COMMUNITY ECOLOGY OF THE GUT MICROBIOME

Andrew Johnson

A DISSERTATION

in

Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2024

Supervisor of Dissertation

Dustin Brisson, Professor of Biology

Graduate Group Chairperson

Dejian Ren, Professor of Biology

Dissertation Committee

Erol Akçay (Chair), Associate Professor of Biology
Paul Schmidt, Patricia M. Williams Term Professor of Biology
Mark Goulian, Charles and William L. Day Distinguished Professor in the Natural Sciences
Corlett Wood, Assistant Professor of Biology

COMMUNITY ECOLOGY OF THE GUT MICROBIOME

For my family.

ACKNOWLEDGEMENT

Over a decade ago, I was working at Charles & Colvard, a company that produced diamond alternatives for the jewelry industry. My job was to wipe clean hundreds of gemstones a day. The stones were heat treated to increase clarity, but any surface contaminants would mar the stone. We were commanded to work silently because talking might slow us down. We were incessantly monitored. I spent countless hours staring through a loupe, wiping spots with a rag. I was depressed.

That job was a blessing. I only understand that now, with hindsight. Sitting in silence for hours a day gave me time to think. I faced the habits that landed me there. I had been immature and squandered all the good opportunities. I lacked a purpose. That much was clear. I accepted that I had to make a fundamental change if I was to recover my life. I needed a goal.

I knew I loved science. I read every day after work and learned that I loved biology more than other fields, with a particular taste for ecology and evolutionary biology. I was promoted at work and given a computer; I used it to watch departmental seminars on the internet. The lofty goal had emerged and it was clear that I had to become a scientist. My goal felt fanciful then, even ridiculous. I decided to try anyway.

These acknowledgements are for all the people that helped me make this dream happen. Each of you were busy with your own lives but took the time to help me nonetheless. I could not have done it alone and I do not know how to adequately thank you. *Thank you.* I will pay it forward, keeping an eye out for those who need help getting where they are going.

There are a few people who should be recognized specifically for their immense impact. Jim Thomas for being a mentor, even though I was not his student (or even enrolled at UNC). Katia Koelle for taking the time to send me in the right direction. Mike Levy for hiring me to work in Peru and giving me my big break. Jennifer Peterson for encouraging me to apply to Penn. Ricardo Castillo-Neyra for years of friendship.

I would like to thank the members of my dissertation committee, Erol Akcay, Paul Schmidt, Mark

Goulian, and Corlett Wood for all their help and kindness. I would like to doubly acknowledge Erol, Paul, and Mark, who interviewed me for admission into the biology program at Penn.

I would like to thank Dustin Brisson for his tremendous role in my life. Dustin, you are a great friend and a fantastic advisor. I will miss our regular talks. I really do hope that we can stay in touch.

Thank you to all the members of the Brisson Lab. Bill Manly for spending so many hours in conversation; Bill, our chats helped me more than you know. Zachary Oppler and Bill for the endless fun. I would also like to acknowledge my graduate cohort. Specifically, Abigail Evans for her years of close friendship. Abby, going through the program with such a close friend meant a lot to me.

I would like to thank my family. Generations of hard work and prudence have given me this chance. My grandparents worked in cotton mills, drove trucks, and mixed paint to raise my parents. I watched my parents work tirelessly to give my sister and me a stable, happy childhood. I grew up surrounded by so much love. Mom and Dad, especially now that I am a parent, I am blown away by your grace. The fact that I am able to accomplish something like this is a testament to the overwhelming support and warmth I receive from my family. I am sad to say that many family members have passed since I moved to Philadelphia. I miss them dearly.

Nicole, how can I say what you have meant to me through all this? You were with me at Charles & Colvard when I had nothing. You were there in Peru; I remember that wishful conversation in December 2016 when we imagined what it would be like if I was actually accepted. You were with me when I interviewed, and when I *really was* accepted. There have been tough times since. You kept me standing after every disappointment. I always want to talk to you first, whether I am lost or elated. And to our daughter, Naomi, you have added something to my life that I cannot articulate. I well up thinking about it. Being your father is the most meaningful experience of my life. *I love you both.* I want to write it a hundred times.

ABSTRACT

COMMUNITY ECOLOGY OF THE GUT MICROBIOME

Andrew Johnson

Dustin Brisson

The gut microbiome is a complex microbial community that plays an important role in shaping host biology. Despite immense academic interest surrounding the microbiome and its relation to the host, the ecological processes governing the gut community remain poorly understood. This dissertation investigates the ecological processes influencing gut microbiome assembly and its effects on host behavior using a simplified *Drosophila melanogaster* model. Chapter 1 examines niche-based and neutral processes driving microbial coexistence, focusing on the interactions between closely related *Acetobacter* species. The results highlight how niche overlap influences competition and colonization success. Chapter 2 explores the role of variation in habitat quality in shaping co-abundances of colonizing microbiota. This study reveals that even subtle differences in habitat can significantly impact species abundance and community composition, especially among taxa with overlapping niches. Chapter 3 shifts the focus from the ecology of the gut microbiome to the consequences of microbiome composition on host behavior, specifically female mating behavior in *D. melanogaster*. We demonstrate that distinct microbial communities can alter mating preferences and latency, suggesting a potential influence of the microbiome on host evolutionary trajectories. Together, these chapters provide insights into the factors driving gut microbiome assembly and the broader implications for host biology, highlighting the importance of ecological context in understanding host-microbe interactions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1

INTRODUCTION

The gut microbiome is a community of trillions of microbes that interact with each other and their host, profoundly influencing host physiology and behavior (Sommer and Bäckhed, 2013; Vuong et al., 2017). For example, microbiome compositions have been linked to host immune function, metabolism, mood, and even behavior (Yoo et al., 2020; Visconti et al., 2019; Rogers et al., 2016; Buffington et al., 2016). The gut microbiome's impact may extend beyond immediate host phenotype and even play a role in host evolution (Shapira, 2016). The wide-reaching impacts of the gut microbiome have garnered significant academic attention, with hundreds of scientific articles focused on the microbiome's impact on host phenotype published in 2023 alone.

Microbiome research has seen exponential growth over the past decades. Advances in sequencing technologies led to an explosion of data, revealing the diverse and complex nature of microbial communities in various environments, especially within the human gut (Navas-Molina et al., 2017). This expanding field has focused heavily on medical research, uncovering a range of health consequences associated with microbiome dysbiosis. For example, studies have linked microbiome community dynamics to chronic metabolic disorders such as diabetes, heart disease, and cancer (Vallianou et al., 2018; Yoshida et al., 2018; Pouncey et al., 2018; Schwabe and Jobin, 2013). Additionally, it is now known that a wide range of medical treatments can dramatically affect the host microbiome, leading to serious medical conditions like *C. difficile* infections (Maier et al., 2018; Theriot et al., 2014). While these studies have identified numerous microbiome-host associations, the sheer complexity of the gut community limits our ability to understand the processes that govern it. A broader theoretical framework is needed to better understand the gut microbiome.

The trajectory of microbiome research mirrors the early development of ecology in the late 19th century. Initially, microbiome studies were largely descriptive, focusing on cataloging the vast diversity of microbial species without a cohesive theoretical framework. This parallels early ecological research, which began with descriptive natural history before developing into a more structured and

hypothesis-driven discipline (Duvallet et al., 2017; Koren et al., 2013). Early ecologists moved from cataloging species to developing theories that provided an organizing framework for understanding ecological communities (Kingsland, 2012). The current state of microbiome research is ripe for a guiding theoretical framework to navigate the complexities of microbial communities. Hard won theoretical frameworks in ecology may offer insight into the gut microbiome. After all, the gut microbiome is an ecological community.

Community ecology is a theoretical framework used to study species interactions within a community, focusing on community structure, species distributions, interactions, and abundances (Putman, 1994). This framework offers powerful perspectives for understanding the species-species interactions driving community assembly and stability. Applying community ecology to microbiome research is particularly useful because the magnitude and proportions of these interactions are key determinants of stable states within ecosystems (May, 1972; Coyte et al., 2015; Qian and Akçay, 2020). Integrating principles from community ecology could offer researchers deeper insight into the dynamics of microbiome communities and better anticipate the consequences of various disturbances.

Niche theory and neutral theory are two foundational concepts in community ecology that offer valuable insights into the coexistence of species. Niche theory is predicated on deterministic species relationships, like mutualisms or commensalisms, while neutral theory assumes equivalency among similar species and offers a null model for interactions. These theories are not mutually exclusive and both have successfully predicted species richness and dynamics in natural communities.

Niche theory provides a framework for understanding how species coexist within ecosystems by examining their interactions, resource use, and environmental tolerances (Grinnell, 1917; Elton, 1927; Hutchinson, 1957). Central to this theory is the idea that species survive within the boundaries of their niche—a combination of their resource requirements and the range of conditions they can tolerate. For example, the Venus flytrap (*Dionaea muscipula*) requires low-nitrogen soil, ample sun exposure, and a sufficient source of prey to survive; together, these factors define the *D. muscipula* niche (Kologiski, 1977). When the niches of distinct species overlap, direct competition can occur, leading to the exclusion of one species unless the competition is mitigated (Gause, 1932; Hardin,

1960). Niche theory explains how coexistence is possible through mechanisms like niche partitioning, where species divide resources or occupy different ecological roles Mittelbach and McGill (2019). Connell's study of barnacle species in the intertidal zone exemplifies this concept, showing how different species occupy distinct vertical zones on the shoreline, each adapted to varying levels of exposure to air and water (Connell, 1961). This stratification reduces direct competition, as each barnacle species has a competitive advantage in its specific niche.

In contrast, neutral theory suggests that the diversity and composition of ecological communities are driven primarily by stochastic processes rather than deterministic interactions (Hubbell, 2001). This theory posits that similar species within the same trophic level are functionally equivalent, with differences in community composition arising mainly from random events such as birth, death, immigration, and emigration (Hubbell, 2001; HilleRisLambers et al., 2012). In other words, these similar species occupy a single niche under neutral theory (Behrenfeld and Bisson, 2024). Neutral theory has been been used to successfully predict patterns of biodiversity across a range of ecological communities, such as tropical forests and coral reefs (Norris, 2003).

While both niche and neutral theories offer valuable insights, they do not fully account for the influence of environmental factors on community structure. Habitat quality, which encompasses resource availability, habitat suitability, and environmental stability, plays a significant role in determining community dynamics (Chesson, 2000b; Pekkonen et al., 2013; van Beest et al., 2014). High-quality habitats, characterized by ample resources and favorable conditions, support greater biodiversity by providing niches that reduce competition (Wang et al., 2023; Ye et al., 2013). Conversely, poor habitat quality can lead to reduced species richness and unstable communities (Poniatowski et al., 2018; Silva et al., 2022). For instance, the diversity and abundance of fish species in coral reefs have been shown to correlate strongly with habitat complexity and resource availability (Jones et al., 2004). Similarly, studies of tropical rainforests have demonstrated that areas with richer soil nutrients and greater canopy cover support a higher diversity of plant species (Phillips et al., 1994). Understanding the impact of habitat quality is essential for a comprehensive view of community assembly, as it interacts with both deterministic and stochastic processes to shape species interac-

tions.

Niche-based and neutral processes, along with environmental factors, collectively shape ecological communities. While significant progress has been made in broader ecological contexts, their roles within microbiome communities remain poorly understood. Much of the existing research has focused on mammalian systems, where highly complex gut microbiomes with hundreds of interacting taxa make disentangling species interactions, stochastic effects, and environmental influences intractable (Douglas, 2018). To gain clearer insights into these processes, simplified and controllable microbiome models are essential (Douglas, 2018).

To address this need, we use a *Drosophila melanogaster* microbiome model, providing a simple, controlled system for studying gut microbiome interactions. Our model utilizes genetically identical flies from an isofemale line, ensuring uniform genetic backgrounds across all experiments. The flies are maintained in a consistent environment to minimize variability. We employ microbiota that naturally evolved within the *D. melanogaster* gut and are capable of stable colonization, including three genera and five species from a single genus collected by Pais et al. (2018). This approach creates synthetic microbiomes that are both realistic yet simplified, enabling precise analysis of the roles of deterministic, stochastic, and environmental processes in community assembly.

In Chapter 1, we explore how niche-based and neutral processes contribute to coexistence within the gut microbiome, particularly within microbiomes composed of closely related *Acetobacter* species. Species that share overlapping niches, such as closely related *Acetobacter* strains, are expected to experience greater competition due to similar resource requirements (Gause, 1932; Hardin, 1960). Niche overlap in this context is estimated by phylogenetic distance, with closer genetic relatedness often correlating with higher ecological similarity and, consequently, more intense competition (Darwin, 1859; Hardin, 1960; Webb et al., 2002; Wiens and Graham, 2005; Cahill Jr et al., 2008). Our study examines the colonization success and population abundance of a focal strain, *A. cibinogensis* (strain 28/18), when co-colonizing with other *Acetobacter* species that vary in their degree of relatedness. By analyzing these interactions, we investigate the balance between niche-based deterministic processes and neutral processes that may facilitate species coexistence within the gut

microbiome, providing insight into how these dynamics may vary with the degree of niche overlap.

In Chapter 2, we investigate the role of habitat variability on the gut microbiome, focusing on how minor variations in habitat quality—such as those arising from individual fly foraging behavior and gastrointestinal lumenal diameter—affect microbial community assembly. Habitat quality is a key determinant in shaping ecological communities, with high-quality habitats providing the resources needed to support greater overall abundance, while low-quality habitats can inhibit community assembly Chesson (2000a,b); van Beest et al. (2014); Liu et al. (2021). In this study, we leveraged these minor, natural variations in habitat quality within our controlled *Drosophila melanogaster* microbiome model. We examined the impact of these variations on the co-abundance of microbial species within the gut, focusing on interactions both within the genus *Acetobacter* and across genera. Our findings emphasize the role of niche overlap in predicting species' responses to habitat quality variations. We show that species with a high degree of niche overlap tend to exhibit stronger positive associations in abundance due to their similar responses to habitat quality.

In Chapter 3, we shift focus from the community ecological factors driving microbiome assembly to the broader impacts of microbiota on host behavior. Using our controlled *Drosophila melanogaster* model, we examine how the gut microbiome influences female mating behavior, including mate preference and mating latency. Understanding these behaviors is important because they can directly affect the evolutionary trajectory of the host (Hamilton and Zuk, 1982; Price, 2006; Eraly et al., 2009; Price et al., 2003). Our study focuses on specific gut microbial communities, including combinations of *Lactobacillus brevis* and *Acetobacter* species, to assess how variations in microbiome composition impact mating outcomes. The experiments reveal that distinct microbial communities lead to significant differences in mating behaviors. We find that specific microbiome communities can lead to a strong in-group mate preference, while other microbiomes can drive female mating latency. These findings contribute to our understanding of the complex interactions between host organisms and their microbiomes, with potential implications for evolutionary biology.

Together, these three chapters shed light on the factors that shape microbiome community assembly and their effects on host biology. Chapter 1 examines how niche-based and neutral processes

contribute to microbial coexistence, revealing the complexity of species interactions within the gut microbiome. Chapter 2 explores the impact of habitat variability, showing that even minor differences in habitat quality can significantly influence microbial community structure, especially when niche overlap is present. Chapter 3 shifts the focus to host behavior, demonstrating that the gut microbiome can influence mating behaviors, with potential implications for host evolution.

By applying community ecology principles to microbiome research, this dissertation provides new insights into the factors driving microbial community assembly. These findings underscore the value of ecological theory in understanding gut microbiomes and highlight the importance of continued research in this area to further unravel the complexities of host-microbe interactions.

CHAPTER 2

NICHE AND NEUTRAL PROCESSES SHAPING THE GUT MICROBIOME

2.1. Abstract

The gut microbiome is a diverse ecosystem that plays an important role in host biology. However, the ecological mechanisms that allow for such high microbial diversity remain poorly understood. This study examines the role of niche-based and neutral processes acting on the assembly of communities of closely related species. We utilized a controlled *Drosophila melanogaster* model to create synthetic microbiomes, varying the relatedness of co-colonizing species to a focal strain, *Acetobacter cibinogensis* (strain 28/18). Our results reveal that closely related species, which exhibit greater niche overlap, experience greater competition, leading to a marked decrease in both colonization success (Logit regression, coefficient = 0.7464, $p < 0.01$) and population abundance (OLS regression, coefficient = 0.3177, $p < 0.01$) of the focal strain. The resulting community proportions of closely related co-colonizing species was statistically neutral, while the community proportions of distantly related co-colonizers were not predicted by neutral theory. This is a slight deviation from expectations because the focal strain and *A. thailandicus* are of similar trophic level, and yet neutral interactions were not detected in this system. These findings demonstrate that the gut microbiome is shaped by a combination of niche and neutral processes, with the degree of species relatedness playing a key role in determining these dynamics.

2.2. Introduction

Species coexistence within communities is essential for the maintenance of biodiversity. Direct and indirect competition between species poses a challenge to coexistence, particularly among related species that occupy similar ecological niches and utilize similar limited resources (Darwin, 1859; HilleRisLambers et al., 2012). Competitive exclusion, where only one species persists, is a common outcome in conditions when resource requirements are too similar (Gause, 1932; Hardin, 1960). However, many natural communities exhibit high levels of biodiversity, including species with similar niche requirements, despite this expected competition (Hutchinson, 1961; Mayfield and Levine,

2010). The mitigation of competition between similar species that permits coexistence depends on a range of ecological processes including deterministic niche-based mechanisms and neutral mechanisms (Hutchinson, 1959; HilleRisLambers et al., 2012; Mittelbach and McGill, 2019). The microbiomes associated with multicellular hosts feature immense biodiversity despite species requiring similar ecological resources (Cuellar-Gempeler, 2021; Chen et al., 2024). Thus, many ecological processes mitigating interpecies competition must be operating in these communities.

Gut microbiomes play crucial roles in shaping host phenotypes (Huang et al., 2022; Buffington et al., 2016). For example, microbiome richness is correlated to aspects of both host metabolism and host behaviors (Murga-Garrido et al., 2021; Lozupone et al., 2012; Alvarez et al., 2020; Cox et al., 2022). Despite the importance of gut microbiome biodiversity to multicellular hosts, the ecological processes maintaining or even permitting microbial species coexistence remain unclear. Concepts from the field of community ecology, specifically niche theory and neutral theory, explain the processes allowing coexistence of multicellular species communities and could explain the processes resulting in the coexistence of gut microbiome species. After all, the gut microbiome is an ecological community of interacting microbiota and their host.

Ecological niche theory is a deterministic model describing how species can coexist within shared environments by considering their interactions and resource use (Hutchinson, 1957, 1959). This theory assumes that species have deterministic relationships, such as mutualistic or competitive interactions, which result in the predictable allocation of resources (Pocheville, 2015). Niche partitioning is a key deterministic niche-based process explaining how similar species avoid competitive exclusion and coexist (Mittelbach and McGill, 2019). Thus, coexistence among species with largely overlapping niches is challenging as they have few ways to avoid competition due to resource requirement similarities. MacArthur's seminal study on wood warblers demonstrated that similar species can coexist by utilizing different subsets of resources within their fundamental niche, a reduced state space of resources known as the realized niche (MacArthur, 1958). The ability to partition resources sufficiently for coexistence relies on marginal differences in the ecology of competing species. These marginal differences allow each species to acquire resources from a subset of the niche where they

have a competitive advantage and avoid areas of the niche space where they are less competitive. For instance, the Cape May warbler, skilled at vertical flight, nests in tree tops to avoid direct competition for nesting sites with other warbler species.

Community ecological processes that support coexistence are not only comprised of deterministic mechanisms like niche partitioning. They also include neutral processes, driven by stochastic events like birth-death dynamics, random dispersal, and competitive equivalency (Hubbell, 2001). Neutral theory in ecology offers a null hypothesis for competition by assuming ecological equivalence between species within the same trophic level (Alonso et al., 2006). Importantly, neutral theory does not dismiss direct resource competition between similar species; it only assumes there will be no deterministic 'winner' of the competition (Hubbell, 2001; HilleRisLambers et al., 2012). Neutral theory has predictive power across diverse ecological communities, predicting patterns of biodiversity in ecosystems such as tropical forests and coral reefs (Norris, 2003). Hubbell's Unified Neutral Theory of Biodiversity, for instance, has been instrumental in explaining species abundance distributions in tropical forests (Hubbell, 2001).

Importantly, niche-based and neutral theories are not mutually exclusive and both successfully predict richness and species dynamics in natural communities, indicating that both niche and neutral mechanisms influence community dynamics (Morrow, 2024). The relative influence of each mechanism varies depending on the specific community context.

Here, we use the controllable microbiome system within the *Drosophila melanogaster* intestinal tract to assess the effects of niche overlap, estimated by phylogenetic distance, on neutral and niche based processes that impact coexistence. Phylogenetic distance is a sensible estimate of niche overlap because genetic similarity often correlates with functional and ecological similarity (Darwin, 1859; Hardin, 1960; Webb et al., 2002; Wiens and Graham, 2005; Cahill Jr et al., 2008). If two species are similar enough, neither should have an overwhelming competitive advantage and they will be closer to ecological equivalency. If the difference between species is greater, the effect of competitive advantage should be stronger than neutral forces. In this study, microbiomes are engineered with a combination of a focal bacterial species, *A. cibinogensis* (strain 28/18), and one of four other species

that vary in genetic relatedness, and thus vary in the degree of niche overlap. The influence of niche and neutral processes facilitating coexistence in the microbiome can be inferred from differences in the colonization rate and population abundance of *A. cibinogensis* (strain 28/18) when co-colonizing with each other species.

2.3. Methods

2.3.1. Germ-free *Drosophila* production

We used the protocol outlined in Pais et al. (2018) to generate germ-free and gnotobiotic flies. Eggs were dechorionated with 2% sodium hypochlorite for 10 minutes, washed in 70% ethanol for 7 minutes, and then rinsed in sterile, DI water. Sterile eggs were then placed on autoclaved food and incubated at 25°C until adulthood.

2.3.2. Bacterial culture

All *Acetobacter* species were cultured in Mannitol salt media, and *Lactobacillus brevis* in MRS media, for 24 hours at 30°C before being resuspended in PBS. We then generated a 50/50 mix of 5% sucrose and bacterial solution, with a final bacterial concentration of $4x10^7$ cell/mL of each species, to feed to the germ-free flies.

2.3.3. Gnotobiotic *Drosophila* production

We generated several treatment groups of gnotobiotic flies for these experiments. Each experimental group consisted of a young, germ-free female *D. melanogaster* fly colonized by a focal commensal gut bacterial species, *Acetobacter cibinogensis*, and second commensal gut bacterial species. Flies were placed in autoclaved agar vials with filter paper inoculated with the appropriate microbial combination and allowed to feed for four hours. Flies were then placed individually in autoclaved chambers for 24 hours, transferred to new autoclaved chambers for another 24 hours, and transferred once more for 24 hours to allow the gut community to stabilize and prevent additional fecal-oral bacterial immigration into the gut.

2.3.4. Recording gut community abundances

Female flies from each treatment group were anesthetized using CO2 and placed in microcentrifuge tubes. Flies were then washed in 70% ethanol twice before being rinsed with sterile, DI water. The surface-sterilized flies were individually homogenized in 500µL of PBS. The homogenate was then further diluted and plated on MRS and Mannitol agar plates, and evenly distributed using glass beads. Plated *Acetobacter* species were distinguished from *A. cibinogensis* (strain 28/18) by replicate plating the original homogenate plate onto a tetracycline plate with the appropriate concentration; 3 µg/mL for *A. malorum*, 5 µg/mL for *A. thailandicus*, or 8 µg/mL for *A. cibinogensis* (strain 114/12). Colonies were counted on the original and replicate plates.

A. cibinogensis (strain 28/18) was differentiated from *Lactobacillus brevis* by plating each homogenate on a set of Mannitol and MRS plates. Visual differential growth on each media type was used to identify each species. *L. brevis* formed opaque white colonies that were larger on MRS plates whereas *A. cibinogensis* (strain 28/18) formed pale yellow colonies with defined borders that were larger on Mannitol plates.

2.4. Results

The presence and abundance of *A. cibinogensis* (strain 28/18) is negatively impacted to the greatest degree when co-colonizing flies with microbiota with the greatest niche overlap. The abundance of the focal strain, *A. cibinogensis* (strain 28/18), is significantly lower in the presence of the most closely related co-colonizing strain. By contrast, the abundance of the focal strain is similar in flies in which it co-colonizes with distantly related microbial species and in flies in which it is the only colonizing species. The lower abundance of the focal strain in flies with closely related co-colonizing microbial species is likely driven primarily by a competition-mediated reduction in colonization rate of the focal strain as opposed to smaller population sizes in successfully colonized flies. Further, colonization success rates of both microbial species are significantly lower when co-colonizers have the greatest niche overlap. That is, the rate at which both co-colonizers failed to establish a population was greatest when co-colonizers shared the most niche space, suggesting the degree of competition is highest among closely related co-colonizers. Importantly, although competition is

most pronounced among closely related species, the outcomes of competition appear stochastic, suggesting neutral ecological processes.

There is a negative correlation between the abundance of *A. cibinogensis* (strain 28/18) and the expected niche overlap with its co-colonizer (Figure 2.1; OLS regression, coefficient = 0.3177, $p < 0.01$). That is, as relatedness between *A. cibinogensis* (28/18) and its co-colonizer decreases, the focal strain abundance trends closer to levels found when colonizing alone. The abundance of the focal *A. cibinogensis* strain is significantly lower when it co-colonizes with a conspecific *A. cibinogensis* strain (1114/12) (Figure 2.1; Mann-Whitney, $p < 0.01$). The focal *A. cibinogensis* abundance is also significantly lower, albeit less so, when it co-colonizes with a closely related species, *A. malorum* (Mann-Whitney, $p < 0.01$). The abundance of the focal *A. cibinogensis* strain is lower than baseline when co-colonizing with *A. thailandicus* and *L. brevis*, although the abundance of the focal strain is not significantly depressed by these more distantly related species (Mann-Whitney, $p = 0.06$ and $p = 0.09$, respectively, after Bonferroni correction).

The overall abundance measure of focal *A. cibinogensis* (28/18) (Figure 2.1A) is a combined metric including the colonization rate and population size given colonization. Similar to the trend identified in overall abundance, there is a negative correlation between the colonization success rate of the focal *A. cibinogensis* (28/18) and the expected niche overlap with its co-colonizer (Figure 2.1B; Logit regression, coefficient = 0.7464, $p < 0.01$). Co-colonization with species whose niches overlap less with the focal strain permits the focal strain to establish a populations at rates similar to those when *A. cibinogensis* colonizes alone. By contrast, the focal *A. cibinogensis* colonization rate is significantly diminished when it co-colonizes with *A. cibinogensis* strain 1114/12 (Binomial, $p < 0.01$) and by the presence of *A. malorum* (Binomial, $p < 0.01$). The effect of *A. thailandicus* on *A. cibinogensis* colonization rate is substantial, but not statistically significant after Bonferroni correction (Binomial, $p = 0.02$). The presence of *L. brevis* had a minimal impact on the colonization rate of the focal *A. cibinogensis* (Binomial, $p = 0.26$). The reduced colonization rate of the focal strain when co-colonizing with species that have the greatest niche overlap explains the majority of the overall effect of co-colonizer niche overlap on focal strain abundance. This suggests that the

colonization success is a primary driver of overall abundance patterns.

The proportion of cases where both species failed to colonize is also correlated with the degree of niche overlap (Table 2.1). The highest rate of colonization failure by both species occurs when flies were challenged with the focal strain and the conspecific *A. cibinogensis* strain (1114/12), with 51 of 71 trials resulting in failure to colonize by either species. Double colonization failure is lower with less niche overlap between co-colonizers: only 11 of 45 trials resulted in double colonization failure when the focal strain was introduced with *A. malorum*, 5 of 39 trials when introduced with *A. thailandicus*, and 3 of 57 trials when introduced with *L. brevis*.

The proportion of the community comprising the focal strain and each co-colonizing strain within flies deviates slightly from the expectations of strictly neutral processes, excluding cases where both species failed to colonize. (Figure 2.2). For example, the proportion of the community made up of the focal strain and the conspecific strain was not significantly different from neutral (50:50) after Bonferroni correction (Wilcoxon signed-rank, Bonferroni-adjusted $p = 0.0769$) (Table 2.2). These results indicate that the proportion of the focal strain in the microbiome significantly deviates from neutrality when co-colonizing with the more distantly related species (*A. thailandicus* and *L. brevis*), but not the more closely related species (*A. cibinogensis* (1114/12) and *A. malorum*). This is a slight deviation from expectations because the focal strain and *A. thailandicus* are of similar trophic level, and yet neutral interactions were not detected in this system.

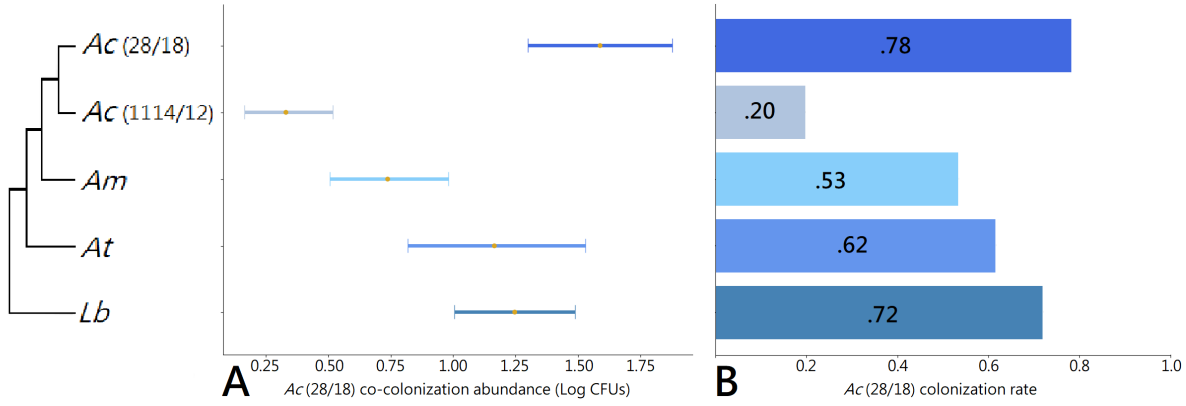

Figure 2.1: **The niche overlap between the focal strain and its co-colonizer predicts depression of focal strain abundance. (A)** The abundance of the focal *A. cibinogensis* (28/18) strain in flies (log CFU) is negatively correlated with the degree of niche overlap with the co-colonizing strain (OLS regression, coefficient = 0.3177, $p < 0.01$). For example, the abundance of *A. cibinogensis* strain 28/18 is significantly lower when co-colonizing flies with the most closely related strain (*A. cibinogensis* strain 1114/12; Mann-Whitney, $p < 0.01$). Similarly, the focal strain abundance is significantly lower when co-colonizing with the next closest species, *A. malorum* (Mann-Whitney, $p < 0.01$), but to a lesser degree than observed when co-colonizing with the most closely related species. While the next closest related species - *A. thailandicus* and *L. brevis* - suppress focal strain abundance, their effects are not statistically significant (Mann-Whitney, $p = 0.06$ and $p = 0.09$, respectively). **(B)** The niche overlap between co-colonizers is predictive of colonization success rates. The bar plot shows the proportion of successful colonization events (CFU > 0) for the focal strain alone and when co-colonizing with species along a phylogenetic gradient. There is a significant negative correlation between the colonization success rate of the focal strain and its niche overlap with the co-colonizer (Logit regression, coefficient = 0.7464, $p < 0.01$). As the niche overlap decreases, the focal strain's colonization success approaches the rate observed when it colonizes alone. The colonization rate of the focal strain is significantly decreased when co-colonizing with *A. cibinogensis* (1114/12) (Binomial, $p < 0.01$) and *A. malorum* (Binomial, $p < 0.01$). While the presence of *A. thailandicus* also reduces the colonization rate of the focal strain, this effect is not statistically significant after Bonferroni correction (Binomial, $p = 0.02$). The presence of *L. brevis* does not significantly affect the colonization rate of the focal strain (Binomial, $p = 0.26$).

Focal strain co-colonization	**Double Zero/Total Experiments**
A. cibinogensis (1114/12)	51/71
A. malorum	11/45
A. thailandicus	5/39
L. brevis	3/57

Table 2.1: The number of trials and the corresponding number of double colonization failures are shown for each co-colonizer.

Focal strain co-colonization	**Neutral**	**p-value**	**Mean proportion, 95% CI**
A. cibinogensis (1114/12)	Yes	0.0769	0.3168 (0.2004 to 0.4432)
A. malorum	Yes	0.1188	0.3791 (0.2792 to 0.4798)
A. thailandicus	No	0.0016	0.1983 (0.0969 to 0.3214)
L. brevis	No	<0.001	0.2208 (0.1521 to 0.2974)

Table 2.2: Wilcoxon signed-rank test results for the deviation of the final species proportion from neutral expectations. *Bootstrapping was used to estimate the means and 95% confidence intervals for each group, excluding cases where both co-colonizers failed to colonize. P-values are corrected using Bonferroni correction.

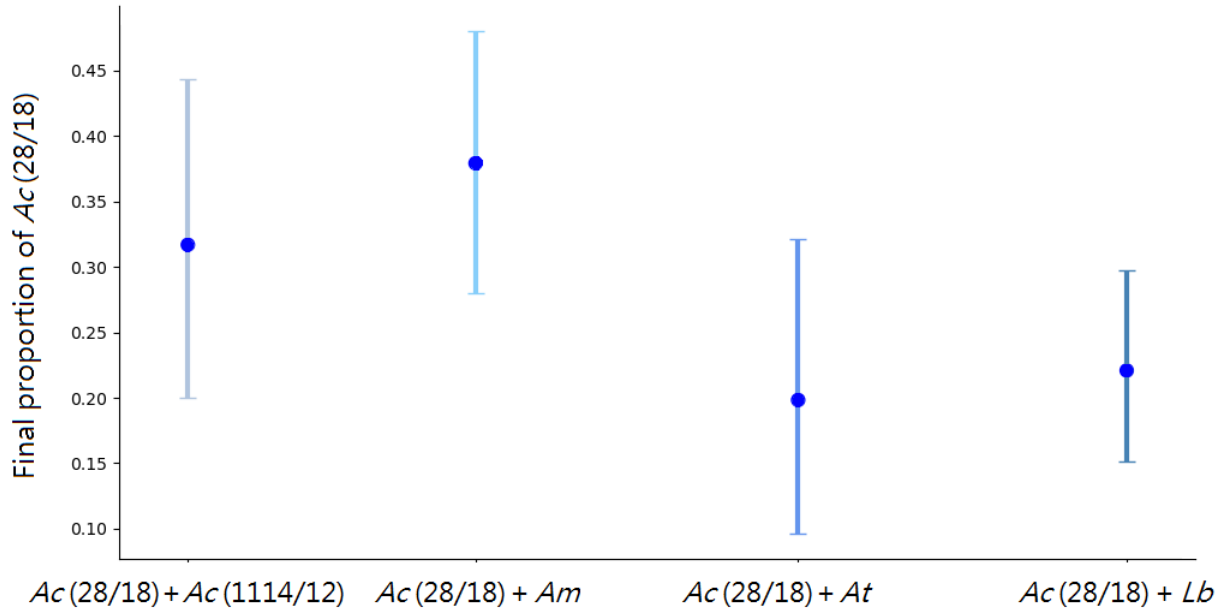

Figure 2.2: **Outcomes are neutral between closely related species but deterministic among more distantly related co-colonizers.** The mean proportions and 95% confidence intervals of the focal strain *A. cibinogensis* (28/18) when co-colonizing with different species are displayed. The proportions of the focal strain when introduced with *A. cibinogensis* (1114/12) and *A. malorum* were not significantly different from neutral expectations ($p = 0.0769$ and 0.1188, respectively), while the proportions of the focal strain and *A. thailandicus* or *L. brevis* were significantly different from neutral expectations ($p = 0.0016$ and < 0.001, respectively). Bootstrapping was used to estimate the means and confidence intervals, excluding cases where both species failed to colonize. The proportions were tested against a neutral expectation of 0.5 using Wilcoxon signed-rank tests with Bonferroni correction.

2.5. Discussion

Direct competition for limited resources poses a challenge to ecological coexistence, particularly among closely related species exhibiting a high degree of niche overlap. When similar species compete directly, only one species is likely to persist unless other ecological processes mitigate the competition (Gause, 1932; Hardin, 1960). Coexistence of microbiota within the gut microbiome is particularly important for host biology. The gut microbiome is an ecosystem that influences an incredible range of host phenotypes (Huang et al., 2022; Buffington et al., 2016). In particular, microbiome community richness and stability play an important role in host health (Murga-Garrido et al., 2021; Lozupone et al., 2012; Alvarez et al., 2020; Cox et al., 2022). Despite the importance of gut biodiversity, little is known about the processes that facilitate microbiota coexistence. Community ecology framework offers many useful perspectives to investigate the mechanisms underlying the extreme biodiversity in host-associated microbiomes. Here, we investigate the role of niche overlap on competition among closely related microbiota.

The negative association between focal strain abundance and co-colonizer relatedness (Figure 2.1A & 2.1B) aligns with niche theory, highlighting the role of niche overlap in competition among microbiota. The trend in overall abundance (Figure 2.1A) is strongly influenced by the negative impact of competition on colonization rate (Figure 2.1B). The relationship between competition severity and co-colonizer relatedness is emphasized by the rate of total colonization failure (Table 2.1). This phylogenetic relationship suggests that niche-based interactions play an important role in shaping microbiome communities. As niche theory predicts, species with the greatest similarities face the most intense competition, likely due to substantial niche overlap that severely reduces the realized niche of both species.

These results also support a role for neutral processes in gut microbiome community diversity. Neutral theory assumes that species within a trophic level are ecologically equivalent and that closely related species are ecologically indistinguishable. This equivalency does not imply that there is no competition between similar species but that neither species has a consistent competitive advantage. Neutral theory predicts the proportion of the community comprising each co-colonizing

species will be stochastic such that average co-colonizer proportions across flies should be equal to the proportions inoculating each fly (50:50). Closely related co-colonizing strains aligns with predictions from neutral theory (Figure 2.2). That is, interactions between the focal strain and its conspecific strain were statistically neutral, supporting the prediction that neither strain has a consistent competitive advantage. Similarly, interactions with the next closest relative, *A. malorum*, were also neutral. However, the interaction between the focal strain and *A. thailandicus* did not align with neutral expectations despite being in the same trophic level, showing competitive dynamics similar to those observed with the most distantly related species (*L. brevis*). Generally, closely related species seem to have the most consequential interactions, both niche-based and neutral, while interactions with more distantly related species have little impact on either population.

While the data support the impact of neutral processes in maintaining microbiome diversity, they also highlight its limitations. The interactions between the focal strain and its conspecific strain and with *A. malorum* were suggestive of niche processes, although they did not reach the accepted threshold of statistical significance. The weak statistical evidence for neutrality suggests that even closely related species exhibit dynamics consistent with niche theory. This marginally-supported neutrality calls into question the extent to which neutral theory alone can explain microbiome diversity. These findings emphasize the need for a more nuanced approach that incorporates both niche and neutral processes to fully capture the complexities of microbial community assembly in the gut.

The gut microbiome is an ecosystem that significantly impacts host biology. Although the importance of the gut community has been recognized, the complexity of species interactions has limited our understanding of the underlying mechanisms. Our results highlight the value of community ecological theory as a framework for understanding the processes governing the gut community. The extensive literature in community ecology offers further insights. For example, future research could use the classical Lotka-Volterra framework to quantify competition between species in this experimental system. This approach would allow for the estimation of competition coefficients (α), providing a quantitative measure of species relationships. Such analysis would be useful for study-

ing multi-generational niche partitioning and understanding how these dynamics contribute to the long-term stability of microbial communities.

Our study highlights the complexity of microbial community assembly in the gut microbiome through the lens of both niche and neutral processes. We show that species relatedness can be a useful predictor of microbiota interactions, as has been shown in a range of eukaryotic ecological communities Cahill Jr et al. (2008); Godoy et al. (2014). Closely related species face intensified competition due to higher degrees of niche overlap, reducing colonization success and abundance, and thus evolutionary fitness. Neutral theory explains marginally neutral interactions but has limitations in fully capturing these dynamics. Thus, neither niche nor neutral theory alone is sufficient to predict community assembly. There are many future research directions including quantification of the impacts of species interactions, exploring other microbiome systems, and refining theoretical models to better capture the observed complexities. This integrative approach will enhance our understanding of the gut ecosystem and the broader principles governing microbial coexistence.

CHAPTER 3

THE ROLE OF HABITAT VARIATION IN MICROBIOME COMMUNITY ASSEMBLY

3.1. Abstract

Ecological coexistence depends on a range of biotic and abiotic factors. Habitat quality, a critical abiotic factor, influences species coexistence by modulating ecological relationships and competition. Despite the gut microbiome's known impact on host biology, the role of habitat quality in microbiota coexistence remains poorly understood. Using a controlled *Drosophila melanogaster* model, we generated synthetic microbiomes with bacteria of varying relatedness to a focal *Acetobacter* species, *A. cibinogensis* (strain 28/18). Our results show that closely related co-colonizers exhibit strong positive correlations in abundance, while more distantly related species show weaker correlations. Across genera, slight positive correlations in abundance were also observed. Importantly, we show these inter-genera correlations are likely driven by habitat quality rather than mutualistic relationships. These findings highlight the crucial role of habitat quality in shaping microbiome community structure and coexistence, especially among closely related species.

3.2. Introduction

Coexistence among species is dependent on both biotic and abiotic factors that affect ecological processes Chesson (2000b). Habitat quality in particular can be an important abiotic factor influencing among-species coexistence Chesson (2000a); van Beest et al. (2014); Liu et al. (2021). Resource availability and species-specific habitat suitability can affect the ecological relationships between species with similar niche requirements, influencing coexistence Chesson (2000b); Pekkonen et al. (2013); Dunham (1980). High quality habitats contain a diverse range and ample supply of resources are correlate positively with biodiversity Lomolino et al. (2010); Wang et al. (2023); Ye et al. (2013); Poniatowski et al. (2018); Silva et al. (2022). Despite the important role of the gut microbiome on host health, the impact of habitat quality on its community ecology remains poorly understood. Here, we examine how variation in habitat quality influences microbiota abundance and coexistence by analyzing the covariance between microbial species that range in degrees of relatedness.

Higher quality habitats, with ample resources, support larger populations of a species. By definition, the carrying capacity of an environment is linked to its resource abundance (Hadwen and Palmer, 1922; O'M, 1971). Higher quality habitats can affect the degree of competition between trophically similar species and facilitate coexistence (Dunham, 1980; Mas-Carrió et al., 2024). For example, survivorship of ornate tree lizards (*Urosaurus ornatus*) is suppressed by interspecific competition with Canyon lizards (*Sceloporus merriami*) only in low quality habitats with limited food availability (Dunham, 1980, 1983).

Richer habitats, characterized by a diverse range of resources and favorable habitat conditions, can support ecologically diverse communities. These rich habitats can also vary in resource abundance. Higher-quality rich habitats are capable of sustaining biodiverse populations of higher abundance (Lomolino et al., 2010; Brown, 2014; Vitousek and Sanford, 1986). For example, studies of the Barro Colorado Island (BCI) forest in Panama show that diverse resources in high abundance allow for the coexistence of both dominant and subordinate tree species, leading to high levels of biodiversity and abundance (Wright et al., 2010).

The role of habitat variation on the gut microbiome remains poorly understood despite the many recognized impacts of microbiota on the biology and health of the host. The habitat quality for gut microbial species varies between, as well as temporally within, hosts (De Filippo et al., 2010; Yatsunenko et al., 2012; Thaiss et al., 2014). Host behavior plays an important role in gut habitat quality. For example, the gut community has access to abundant resources as hosts consume nutrients, which become scarce during fasting periods (Thaiss et al., 2014). These fluctuations in nutrient availability cause dramatic shifts in microbiome community composition (Thaiss et al., 2014) and likely impact inter-species interactions. Thus, host-associated habitat quality could play a crucial role in maintaining the microbiota coexistence, similar to the effects on eukaryotic ecological communities.

Here, we investigate the role of habitat quality in microbiome community assembly using a controlled *Drosophila melanogaster* microbiome model. We examine how variations in habitat quality affect microbial species abundance within the gut by analyzing communities of closely related *Acetobacter*

species and broader interactions across microbial genera. Despite controlling the *D. melanogaster* microbiome system by using an isofemale fly line and constant environmental conditions, habitat quality varies among individual flies. For example, variation in individual fly foraging behavior or in gastrointestinal lumenal diameter impacts habitat quality for host-associated microbes. Since a species' carrying capacity is driven by habitat quality (Hadwen and Palmer, 1922; O'M, 1971), evidence of variation in habitat quality can be inferred by variation in microbiota population size. By leveraging the natural variation in habitat quality, we investigate how habitat quality variation affects gut microbiome community assembly. This approach provides a deeper understanding of the ecological processes governing microbial coexistence within metazoan guts.

3.3. Methods

3.3.1. Generation of Germ-free *Drosophila*

We followed the method described by Pais et al. (2018) to produce germ-free and gnotobiotic flies. We dechorionated the eggs using a 2% sodium hypochlorite solution for 10 minutes, followed by a 7-minute wash in 70% ethanol, and subsequent rinsing in sterile deionized water. The sterilized eggs were then transferred onto autoclaved food and incubated at 25°C until they reached adulthood.

3.3.2. Culturing Bacteria

All *Acetobacter* species were grown in Mannitol salt media, while *Lactobacillus brevis* was cultured in MRS media. Cultures were incubated for 24 hours at 30°C and then resuspended in PBS. A 50/50 mix of 5% sucrose and bacterial solution was prepared, resulting in a final concentration of $4x10^7$ cells/mL for each species. This mixture was then used to feed the germ-free flies.

3.3.3. Production of Gnotobiotic *Drosophila*

For the within-*Acetobacter* experiments, we created several treatment groups of gnotobiotic flies. Each group consisted of young, germ-free female *D. melanogaster* colonized by a focal commensal gut bacterial species, *Acetobacter cibinogensis* (strain 28/18), along with a second commensal gut bacterial species. The flies were placed in autoclaved agar vials containing filter paper inoculated with the respective microbial combinations and allowed to feed for four hours. Subsequently, the flies were housed individually in autoclaved chambers for 24 hours, then transferred to new autoclaved

chambers for another 24 hours, and transferred once more for a final 24-hour period. This protocol allowed the gut community to stabilize and minimized additional fecal-oral bacterial immigration into the gut.

For the between-genera experiments, we followed the same procedure described above to generate germ-free and gnotobiotic flies. Each experiment consisted of three treatment groups. In one treatment group, female *D. melanogaster* would be colonized with a single *Acetobacter* species, either *A. cibinogensis* (strain 28/18) or *A. thailandicus*. Flies in the second treatment group would be colonized with *L. brevis*. In the third treatment group, flies were colonized with a 50/50 mixture of the corresponding *Acetobacter* species and *L. brevis*.

3.3.4. Recording Gut Community Abundances

Female flies from each treatment group were anesthetized using CO_2 and placed in microcentrifuge tubes. The flies were washed twice in 70% ethanol and then rinsed with sterile deionized water. Each surface-sterilized fly was individually homogenized in 500 µL of PBS. The homogenate was further diluted and plated on MRS and Mannitol agar plates, with even distribution achieved using glass beads. *Acetobacter* species were differentiated from *A. cibinogensis* (strain 28/18) by replicate plating the original homogenate onto tetracycline plates with species-specific concentrations: 3 µg/mL for *A. malorum*, 5 µg/mL for *A. thailandicus*, and 8 µg/mL for *A. cibinogensis* (strain 114/12). Colony counts were recorded from both the original and replicate plates.

A. cibinogensis (strain 28/18) was differentiated from *L. brevis* by plating each homogenate on both Mannitol and MRS plates. Visual differential growth on each media type was used for species identification. *L. brevis* formed opaque white colonies that were larger on MRS plates, whereas *A. cibinogensis* (strain 28/18) formed pale yellow colonies with defined borders that were larger on Mannitol plates.

3.4. Results

We co-colonized a single line of isofemale *D. melanogaster* with various bacterial communities to investigate the role of habitat variation in the community ecology of the gut microbiome. Broadly,

co-colonizers with the highest degree of niche overlap exhibited the strongest positive association in population sizes. This positive association in abundance between co-colonizers waned with decreasing niche overlap, indicating that favorable habitat conditions are niche-specific, but can extend across genera. Due to the high degree of niche overlap, co-colonizers in the same genus responded similarly to variation in habitat quality. *Acetobacter* species with the highest degree of niche overlap had the strongest positive correlation in population sizes. Even within-genera, this positive association waned with increasing evolutionary distance among co-colonizing *Acetobacter* species. Co-colonizers from different genera, by contrast, showed only a faint positive association despite no detectable ecological relationships, e.g. facilitation, mutualism, etc. between species of different genera.

The association between the abundance of the focal strain and the abundance of its co-colonizer trended toward positivity in all pairwise conditions. However, the positive association is greatest when the co-colonizers have the greatest niche overlap (closest evolutionary relatedness) and decreased with decreasing niche overlap (Figure 3.1). The covariance in population sizes of the most closely related species (*A. cibinogensis* strain 28/18 and *A. cibinogensis* strain 1114/12) is the most positively correlated (OLS regression, coefficient = 0.92, $p = 0.002$). The covariance in population sizes of *A. cibinogensis* (28/18) and *A. malorum*, the next most closely related species, is also significantly positive (OLS regression, coefficient = 0.46, $p = 0.013$). The covariance of *A. thailandicus* with the focal *A. cibinogensis* strain was not significantly positive, however it matched the expected degree of positivity following the pattern of decreasing association with phylogenetic distance (OLS regression, *A. thailandicus* coefficient = 0.41, $p = 0.076$).

The positive correlation in population sizes observed between distantly related species within the same genus is also present between species from different genera (Figure 3.2). The correlation between *L. brevis* and *A. thailandicus* abundances is positive (OLS regression, coefficient = 0.53, standard error = 0.146, $p = 0.001$). The correlation between *L. brevis* and *A. cibinogensis* population sizes is not statistically significant (OLS regression, coefficient = 0.48, standard error = 0.239, $p = 0.062$). The slight positive relationship between *L. brevis* and these two *Acetobacter* species

could be due to habitat variation, but it could also result from a mutualistic relationship between these genera.

Co-colonization revealed no significant mutualistic or antagonistic relationships between *Acetobacter* and *Lactobacillus* species (Figure 3.3). That is, the population size of *L. brevis* was not significantly different when co-colonizing with *A. cibinogensis* (KS statistic = 0.1104, $p = 0.6117$) or *A. thailandicus* (KS statistic = 0.2561, $p = 0.0595$) compared to when it was the only species colonizing the fly. Similarly, the abundance of *A. cibinogensis* nor *A. thailandicus* were affected by the presence of *L. brevis* (KS statistic = 0.0782, $p = 0.9530$ and KS statistic = 0.1364, $p = 0.8479$, respectively). These results indicate that the microbial population sizes in these co-colonization scenarios are likely influenced by environmental quality rather than direct interactions between microbial species.

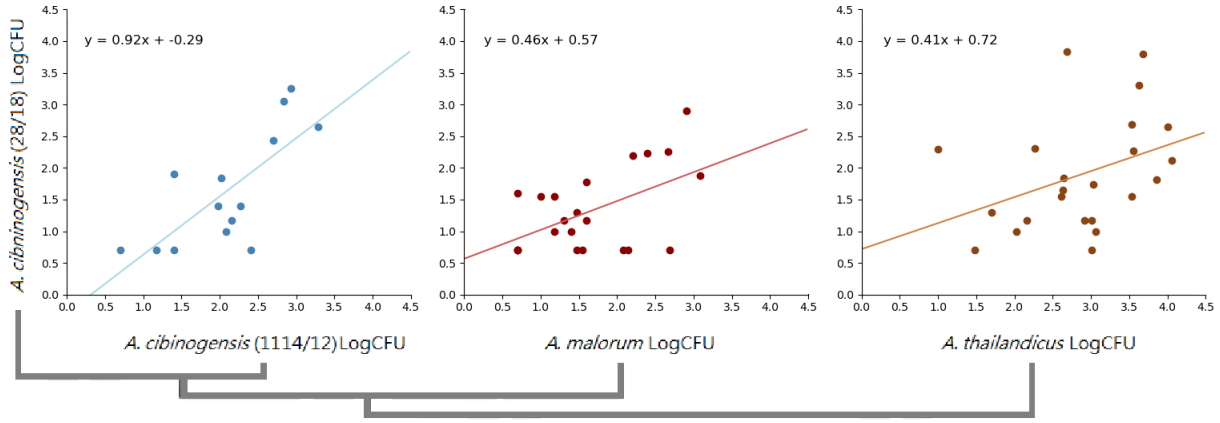

Figure 3.1: **Positive associations in the population sizes of the focal strain and its co-colonizers wanes with decreasing niche overlap**. As niche overlap decreases, the positive association in abundance between *A. cibinogensis* (strain 28/18) and its co-colonizer decreases. Each panel shows the relationship between the log-transformed abundance of the focal strain and one of its co-colonizing species. The abundance of *A. cibinogensis* (strain 28/18) is most positively correlated with *A. cibinogensis* (strain 1114/12; $p = 0.002$), followed by the significant positive correlation with *A. malorum* ($p = 0.013$), and the non-significant positive trend in population sizes with the most distantly related species in the genus, *A. thailandicus* ($p = 0.076$). These results support the hypothesis that diminishing niche overlap results in dissimilar responses to habitat variation (OLS regression, *A. thailandicus* coefficient = 0.41, $p = 0.076$).

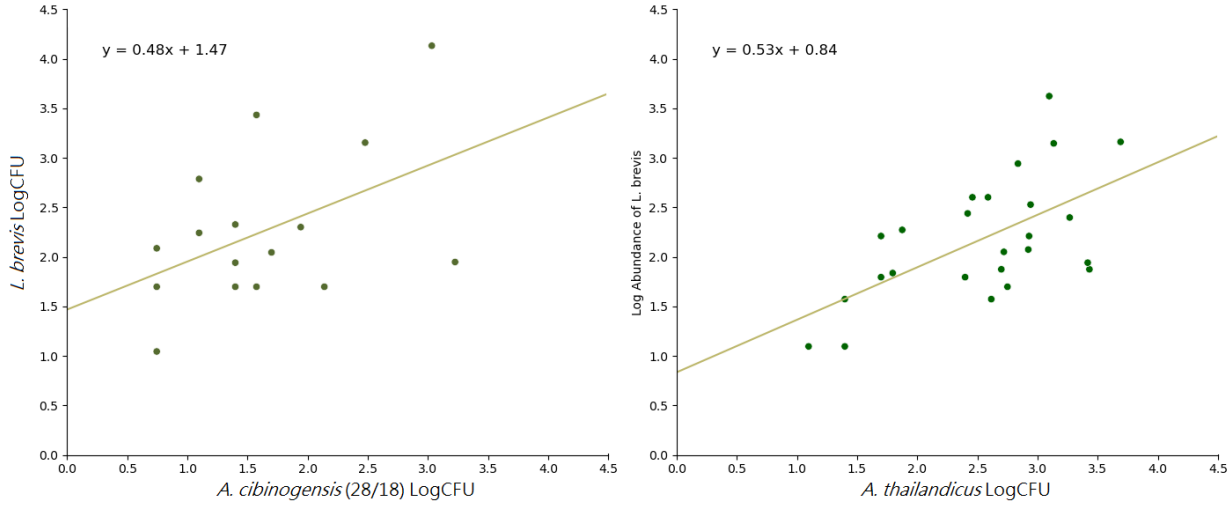

Figure 3.2: **The population sizes of *Lactobacillus brevis* and two *Acetobacter* species are weakly correlated.**. The correlation between *L. brevis* and *A. thailandicus* was statistically significant (OLS regression, coefficient = 0.53, standard error = 0.146, $p = 0.001$) while the correlation between *L. brevis* and *A. cibinogensis* was not statistically significant (OLS regression, coefficient = 0.48, standard error = 0.239, $p = 0.062$).

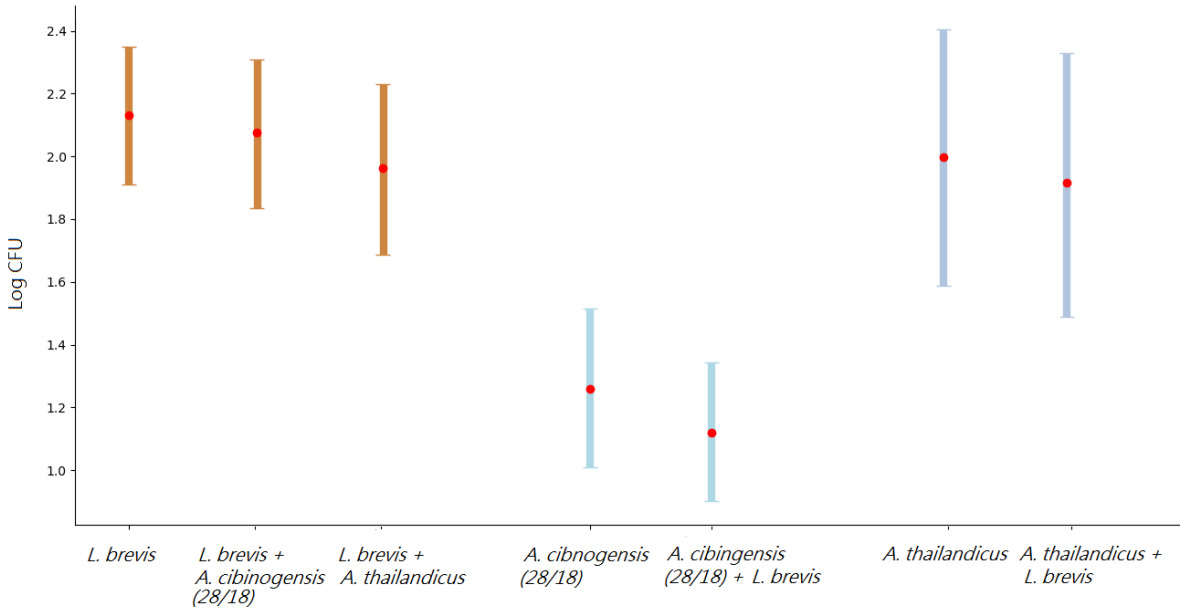

Figure 3.3: **Mutualistic interactions do not drive the observed positive correlations between *Acetobacter* and *Lactobacillus* species.** The abundance of *L. brevis* was not significantly different when co-colonizing with *A. thailandicus* (KS statistic = 0.2561, $p = 0.0595$) or *A. cibinogensis* (KS statistic = 0.1104, $p = 0.6117$) compared to when it was alone. Similarly, no significant changes in the abundance of either *A. thailandicus* or *A. cibinogensis* were detected co-colonizing with *L. brevis* (KS statistic = 0.1364, $p = 0.8479$ and KS statistic = 0.0782, $p = 0.9530$, respectively). These results indicate that the microbial abundances in these co-colonization scenarios are likely influenced by environmental quality rather than direct inter-species interactions.

3.5. Discussion

We investigated the role of habitat quality in microbiota coexistence among similar species within a controlled *Drosophila melanogaster* microbiome model. We use the variation in microbiota population size as a proxy for variation in species-specific habitat quality, since a species' carrying capacity is driven by habitat quality (Hadwen and Palmer, 1922; O'M, 1971). The data demonstrate that closely related strains with similar ecological niches exhibit strong positive associations in population sizes, suggesting they benefit similarly from favorable habitat conditions. This positive association diminishes with increasing phylogenetic distance between co-colonizers, indicating that variation in habitat quality has the strongest impact on the maintenance of coexistence among species with similar niches. Weaker positive correlations between *Lactobacillus* and *Acetobacter* population sizes were also observed, supporting the idea that broader community abundance and coexistence can be affected by beneficial habitat conditions. The lack of detectable species-species interactions between populations from different genera (Figure 3.3) supports the idea that the positive inter-genera correlations observed (Figure 3.2) are likely driven by variation in habitat quality rather than a mutualistic interactions between *Acetobacter* and *Lactobacillus*.

Closely related strains with similar ecological niches benefit similarly from variation in habitat quality, likely due to overlapping resource requirements. This hypothesis is supported by the strong positive association in abundance between *A. cibinogensis* strain 28/18 and conspecific strain 1114/12 (Figure 3.1). The pattern of diminishing positive associations between the focal strain with *A. malorum* and with *A. thailandicus* further highlights the role of niche overlap in shaping the impact of habitat quality on coexistence.

The positive association in abundance between closely related species is counter-intuitive given the expected increase in competition for mutually required resources. That is, species with similar niche requirements are expected to compete for resources which predicts negative correlations in population sizes. In fact, prior research indicates that competition is most pronounced between closely related species (Chapter 1 as well as (Darwin, 1859; Hardin, 1960; Webb et al., 2002; Wiens and Graham, 2005; Cahill Jr et al., 2008)). However, competition among microbial species

in the fly microbiome primarily affected colonization rates with only limited effects on population sizes post-colonization. The present study exclusively analyzed cases where both co-colonizers established populations within the fly, thus excluding impacts of competition on colonization rates. The combination of these results suggests that both niche-based mechanisms and variation in habitat quality impact the coexistence of similar microbiota within the gut.

Variation in habitat quality can broadly affect microbiome communities. The slight positive association between *Lactobacillus* and *Acetobacter* abundances (Figure 3.2) suggests that, despite relatively low niche overlap between genera, these microbial species both react to variation in habitat quality. The lack of significant interactions between genera (Figure 3.3) indicates that these correlations arise from variations in habitat quality rather than direct ecological relationships, like commensalisms or mutualisms. Importantly, these species were introduced into the gut environment simultaneously, thus excluding the potential impacts of priority effects or ecosystem engineering. These mechanisms could affect the ecological relationship within the gut. For example, some *Lactobacillus* species produce glycans that form an extracellular matrix that increases suitable habitat for some *Acetobacter* species Dodge et al. (2023). Thus, *Lactobacillus* ecosystem engineering could result in a facilitative relationship with *Acetobacter* bacteria in natural cases with regular introduction of new bacteria into the gut.

The positive correlation between the focal strain and most distantly related *Acetobacter* co-colonizer, *A. thailandicus* (Figure 3.1), is similar to the correlation between *L. brevis* and the two *Acetobacter* strains (Figure 3.2). This weakly positive correlation indicates that these species share minimal niche overlap and thus do not respond to variations in habitat quality in the same ways. This lack of ecological relation between the focal strain and distantly related co-colonizers is further emphasized by the absence of detectable species interactions identified previously in this system (Chapter 1). That is, species-species interactions among distantly related co-colonizers are not detectable and they respond differently to habitat quality variation. The similarity in relationships between the focal strain and *A. thailandicus* and the focal strain and *L. brevis* highlights the limitation of historically popular genera-level ecological analyses of microbiome communities.

This study highlights the role of habitat quality in shaping microbial community assembly within a controlled *Drosophila melanogaster* microbiome model. The observed positive correlations in abundance among closely related strains underscore the importance of niche overlap in mediating species' responses to habitat variations. These correlations diminish with decreasing niche overlap, resulting in only a slightly positive correlation in abundance between the focal *Acetobacter* strain and the distantly related *A. thailandicus*. Moreover, the weak positive associations in abundance observed between genera, despite the lack of significant direct interactions, emphasize the role of beneficial habitat conditions extending across broader taxonomic groups. Future research should aim to disentangle these factors, incorporate a wider range of microbial taxa, and refine theoretical models to investigate ecological dynamics within the gut microbiome. This approach will advance our knowledge of microbial coexistence and the broader principles governing ecological community structure.

CHAPTER 4

THE GUT MICROBIOME CAN INFLUENCE FEMALE MATING BEHAVIOR IN *D. MELANOGASTER*

4.1. Abstract

Environmentally-determined, sexually-relevant phenotypes can act as false indicators of *good genes*, thus affecting the rate of a species' evolution. The gut microbiome is an environmental factor that has been associated with a range of phenotypic outcomes. However, isolating the role of the gut microbiome in shaping false indicators of heritable fitness has been a challenge. Here, we evaluate the direct role of the gut microbiome on female mate preference and latency using an isofemale line of *D. melanogaster* with tightly controlled microbiome communities. Singly colonized mating trials were conducted with one female fly, colonized with either *Lactobacillus brevis* or *Acetobacter thailandicus*, and two males; one colonized with *L. brevis* and the other male with *A. thailandicus*. Co-colonized mating trials were conducted with female flies colonized with both *L. brevis* and *A. thailandicus* simultaneously. We found that distinct gut communities result in significantly different female mate preferences (Fisher's Exact, $p = 0.019$). Moreover, female flies displayed a bias for males with matching microbiome communities. Females colonized with *L. brevis* strongly preferred males colonized with *L. brevis* (Binomial, $p = 0.007$), while females colonized with *A. thailandicus* had a bias for males colonized with *A. thailandicus* (Binomial, $p = 0.240$). Co-colonized females did not exhibit a significant bias, yet mated substantially faster than singly inoculated females (Kolmogorov–Smirnov, $p = 0.022$). These results suggest that the gut microbiome can play an important role in the development of false indicators of good genes.

4.2. Introduction

The phenotypes preferred by females in potential mates are expected to represent honest indicators of *good genes* that will increase survival or reproductive success in their offspring (Hamilton and Zuk, 1982; Price, 2006). However, phenotypes are the result of both genetic and environmental influences (Simpson et al., 2011). Thus, the heritable alleles partially underlying attractive phenotypes can

be obscured by environmental factors that influence phenotypic development (Fisher, 1930; Greene, 1989; Brakefield et al., 2009). These environmentally-dominated phenotypes are false indicators of heritable fitness that impact the rate of allele frequency changes, and thus the rate of biological evolution by natural selection (Eraly et al., 2009; Langerhans and DeWitt, 2002; Ghalambor et al., 2007; Gienapp et al., 2013; Price et al., 2003).

Environmentally-influenced phenotypes have been shown to impact mate choice (Espeset and Forister, 2022; Montague et al., 2013). For example, great tits (*Parus major*) are attracted to the yellow coloration of males – a trait heavily influenced by the preponderance of caterpillar-sourced carotenoids (Hegyi et al., 2007, 2015; Pagani-Núñez et al., 2014; Slagsvold and Lifjeld, 1985). Similarly, guppies (*Poecilia reticulata*) prefer males with vibrant, diet-mediated orange markings (Grether, 2000). As another example, Male bower birds (*Chlamydera maculata*) with a greater numbers of bower decorations, mostly consisting of glass and bones, are more preferred mates for females (Borgia, 1995). Each of these phenotypes are dependent on environmental context and can, therefore, act as false indicators of heritable fitness.

The gut microbiome is a recently recognized environmental factor that impacts many phenotypes of ecological and medical importance (Gilbert et al., 2018; Barko et al., 2018; McFall-Ngai, 2015; Metchnikoff, 1908; Turnbaugh et al., 2007; Lynch and Hsiao, 2019). Multiple microbiome-influenced phenotypes have been identified including adiposity in mice (*Mus musculus*), camouflage in bobtail squid (*Euprymna scolopes*), and dietary span in wood-feeding termites (*Nasutitermes takasagoensis*) (Jones and Nishiguchi, 2004; Tokuda et al., 2018). Even complex social phenotypes, like aggression and in-group recognition, can be influenced by gut microbiota (Teseo et al., 2019). These microbiome-mediated phenotypes can indirectly affect survival or mating success.

Host-associated microbes have been shown to directly influence some phenotypes that impact mating success. For example, microbiome differences can alter the duration of copulation as well as fecundity (Morimoto et al., 2017). Female mate preference has even been shown to be impacted by dietary differences that may have been mediated through changes in microbial communities (Sharon et al., 2010; Dodd, 1989). The populations maintained on distinct diets over many generations had notably

divergent microbiome communities, although the impact of dietary differences could affect mate preference in many ways (Schultzhaus et al., 2017; Bakker et al., 1999; Hunt et al., 2005), making it difficult to understand the isolated role of the gut microbiome on this phenotype.

Here, we use an isofemale line of *D. melanogaster* with tightly controlled microbiomes to isolate the effect of the gut microbiome on mate preference and clarify its role as a false indicator of *good genes* (Hamilton and Zuk, 1982). All flies were reared in identical environments, with identical food, aside from distinct, experimentally controlled gut microbial communities. The fact that all flies originate from one isogenic line ensures that any phenotype that could affect female mate preferences are derived from microbiome differences, not differences in genetic content. The mating trials demonstrate a direct impact of gut microbial communities on female mate preference and average time to mating. The direct influence of the non-heritable microbes on sexually-relevant phenotypes demonstrates that gut microbiomes can play an important role in the efficacy of evolution by natural selection.

4.3. Methods

4.3.1. Overview

For these experiments, we generated several treatment groups of gnotobiotic flies. Each experimental group was colonized by a single cultured species of commensal gut bacteria or simultaneously colonized by two microbial species (co-colonized flies). We performed a mating assay in which a female was allowed to choose between two males to assess if the identity of the microbial species in the gut microbiome influences female mate selection. All mating trials contained one female, one male colonized by *Lactobacillus brevis* and one male colonized by *Acetobacter thailandicus*.

4.3.2. Bacteria culture

L. brevis and *A. thailandicus* were cultured in MRS and Mannitol salt media, respectively, for 24-48 hours at 30° C before being resuspended in PBS. They were then diluted with a 200ul of 5% sucrose solution and 200ul of PBS, with a bacterial concentration of $0.4x10^8$ cell/mL of each species.

4.3.3. Generation of gnotobiotic flies

To generate germ-free flies, we used the protocol outlined in (Pais et al., 2018). Collected eggs were dechorionated with 2% sodium hypochlorite for 10 minutes, sterilized with 70% ethanol for 7 minutes, and then washed with sterile DI water. Embryos were then placed on sterilized food vials and were inoculated with 100ul of the prepared bacteria solution. The vials were then sealed and placed into incubator to mature.

4.3.4. Microbiota confirmation assay

10 female flies from each treatment group were anesthetized and placed into microcentrifuge tubes. They were washed with 70% ethanol for 15 seconds to sterilize the surface of the flies, then rinsed with sterile deionized water. This process was repeated once more. The washed flies were then individually homogenized in 500 ul of PBS by a Mini-BeadBeater (BioSpec Products, Inc., Bartlesville, OK, USA). The homogenate was then further diluted in PBS, plated onto MRS and Mannitol agar plates, and evenly distributed using glass beads. The plates were placed into the incubator at 30°C for 48-72 hours. Colony phenotypes were observed to confirm infection with desired bacteria.

4.3.5. Mating trials

Mating chambers were 60mm petri dishes filled partially with plain agar. Alternating male flies from separate treatment groups had their wing tips clipped to distinguish between groups during the test. Wing clipping has been demonstrated to not affect *D. melanogaster* mating behavior (Van den Berg et al., 1984). The two males were introduced to the chamber first. The female was then introduced to choose its mate. Female matings with males with the same microbiome treatment were considered homophily, and female matings with males of a different microbiome treatment were considered to be heterophily. Eight assays were run simultaneously and video recorded via an overhead camera for 45-60 minutes. The time and nature of each mating event were determined from recorded footage.

4.4. Results

The impact of microbiome differences on female mate choice was evaluated using an isofemale fly line originally isolated from a wild population in Pennsylvania. The isofemale line was inbred for at least

10 generations to eliminate phenotypic variation associated with genetic differences. Germ-free *D. melanogaster* eggs were inoculated with one or two cultured bacterial species to create gnotobiotic experimental groups. Each tested fly contained only the experimentally-inoculated microbes; no contaminating microbial species was detected on or in any fly.

The mating preferences of female *D. melanogaster* colonized by *L. brevis* is significantly different than females colonized with *A. thailandicus* (Figure 4.1; Fisher's Exact, $p = 0.019$). Importantly, *L. brevis*-colonized females strongly preferred mating with male flies colonized with *L. brevis* over males colonized with *A. thailandicus* (67% of matings, Binomial, $p = 0.007$). Similarly, *A. thailandicus*-colonized females had a slight preference for males colonized with *A. thailandicus* over males colonized with *L. brevis* (56% of matings, Binomial, $p = 0.240$). That is, females tended to prefer mating with males colonized by a microbiome matching their own.

The trend toward in-group mating preferences was magnified among choosier female flies that took longer to mate. Choosier *L. brevis*-colonized females (those mating ten minutes after the trial began) have an even stronger bias for males colonized with *L. brevis* (70% of matings, Binomial, $p = 0.01$). Late mating *A. thailandicus*-colonized females, too, had a greater bias toward *A. thailandicus*-colonized males (60% of matings, Binomial, $p = 0.16$).

Female flies colonized simultaneously with *L. brevis* and *A. thailandicus* do not demonstrate a mating preference (Figure 4.2), with 48% of females mating with *L. brevis*-colonized males (n=56; Binomial, $p = 0.66$). The mating preferences of co-colonized females, *L. brevis*-colonized females, and *A. thailandicus*-colonized females differed significantly ($X^2 = 6.63$, $p = 0.036$), driven primarily by strong preference biases in *L. brevis*-colonized females (48% vs 67%, n=111; post-hoc Fisher's Exact, $p = 0.055$). In contrast, mating preferences of co-colonized females were very similar to those of females colonized with only *A. thailandicus* (52% vs 56%, n=106; Fisher's Exact, $p = 0.70$).

The time to copulation for co-colonized females is significantly shorter than the time to copulation for females colonized by only one microbial species (Kolmogorov–Smirnov, $p = 0.022$; Figure 4.3). Of 56 co-colonized female trials, 27 copulated within the first 10 minutes while only 4 did not mate

within the first 20 minutes. By contrast, only 18 of 55 *L. brevis*-colonized females and 15 of 50 *A. thailandicus*-colonized females mated within the first 10 minutes. Further 14 *L. brevis*-colonized females and 11 *A. thailandicus*-colonized copulated more than 20 minutes after being introduced to males. Time to copulation was similar between the *L. brevis*- and *A. thailandicus*-colonized females (Kolmogorov–Smirnov, $p = 0.843$).

Mate preferences in females colonized with both *L. brevis* and *A. thailandicus* were similar to the mate preferences of females co-colonized with *L. brevis* and a sibling *Acetobacter* species, *A. cibinogensis* (Figure 4.4; Fisher's Exact, $p = 0.86$). In all mate preference trials, males were colonized with *L. brevis* and the *Acetobacter* species in the co-colonized females. Similar to the lack of observed preference in females co-colonized with *L. brevis* and *A. thailandicus*, females co-colonized with *L. brevis* and *A. cibinogensis* have no discernible bias for males harboring different microbial species (Binomial, $p = 0.76$).

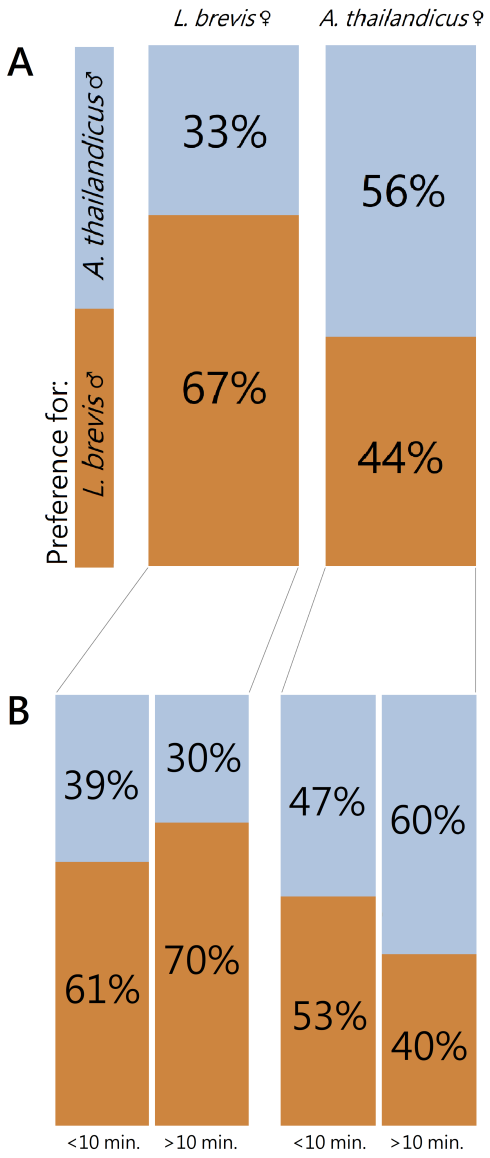

Figure 4.1: **Female *D. melanogaster* prefer to mate with males colonized with the same microbiome species.** **A**. The mating preferences of *L. brevis*-colonized female flies (n = 55) differed significantly from the preferences of *A. thailandicus*-colonized females (n = 50) (Fisher's Exact, $p = 0.019$). Female flies preferred males colonized by the same microbial species, with 37 of 55 *L. brevis*-colonized females mating with *L. brevis*-colonized males (Binomial, $p = 0.007$) and 28 of 50 *A. thailandicus*-colonized females choosing males with a *A. thailandicus* microbiome (Binomial, $p = 0.240$). **B**. The preference for mating with males colonized by the same microbial species increases in later mating female flies (>10 minutes), with 26 of 37 *L. brevis*-colonized females mating with *L. brevis*-colonized males (Binomial, $p = 0.01$) and 21 of 35 *A. thailandicus*-colonized females choosing males with a *A. thailandicus* microbiome (Binomial, $p = 0.16$). The proportion of matings with *L. brevis*-colonized males is shown above in brown while matings with *A. thailandicus*-colonized males are represented in blue.

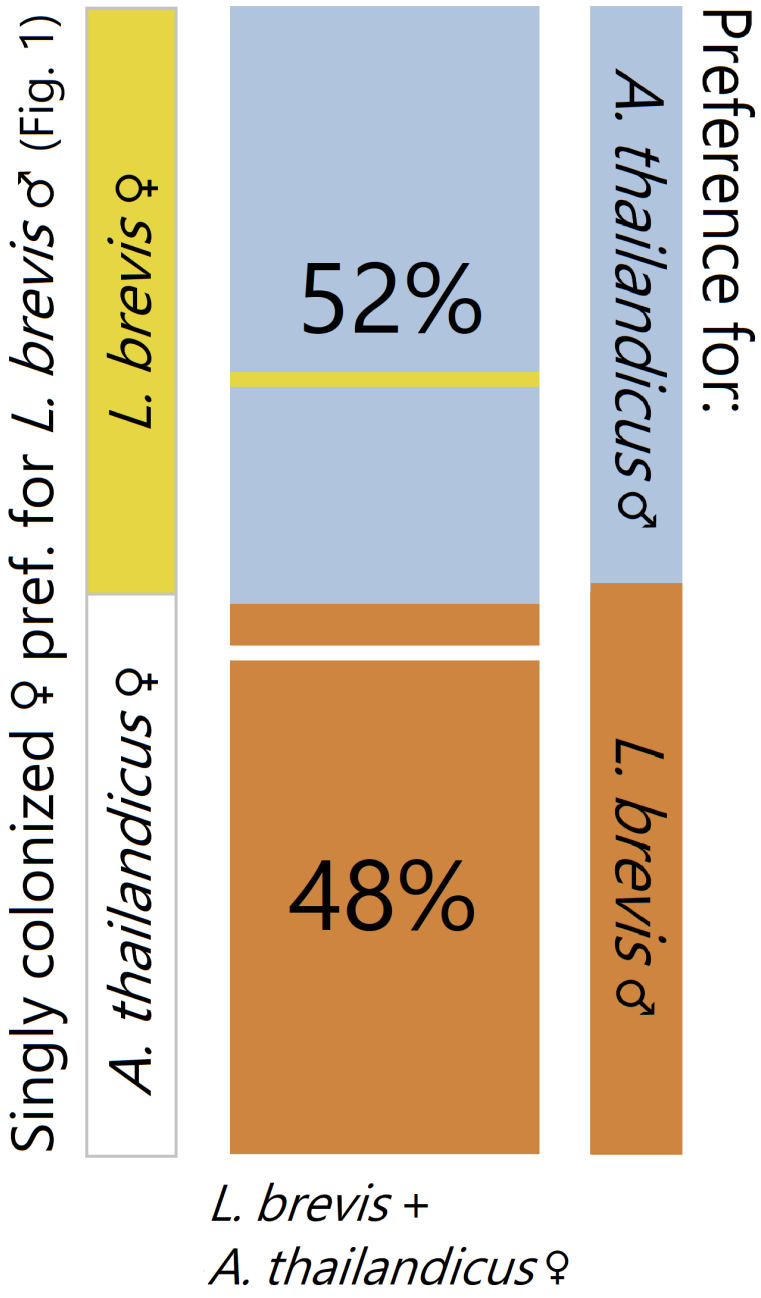

Figure 4.2: **Co-colonized female *D. melanogaster* mate with *L. brevis*- and *A. thailandicus*-colonized males with near-equal frequencies.** Of 56 co-colonized females, 27 mated with *L. brevis*-colonized males (48%, Binomial, $p = 0.66$). The mating preference of co-colonized females differed substantially from the preference of *L. brevis*-colonized females (yellow line; 48% vs 67%, n=111; Fisher's Exact, $p = 0.055$). In contrast, the mating preference of co-colonized females are very similar to the preferences of *A. thailandicus*-colonized females (white line; 52% vs 56%, n=106; Fisher's Exact, $p = 0.\ 70$).

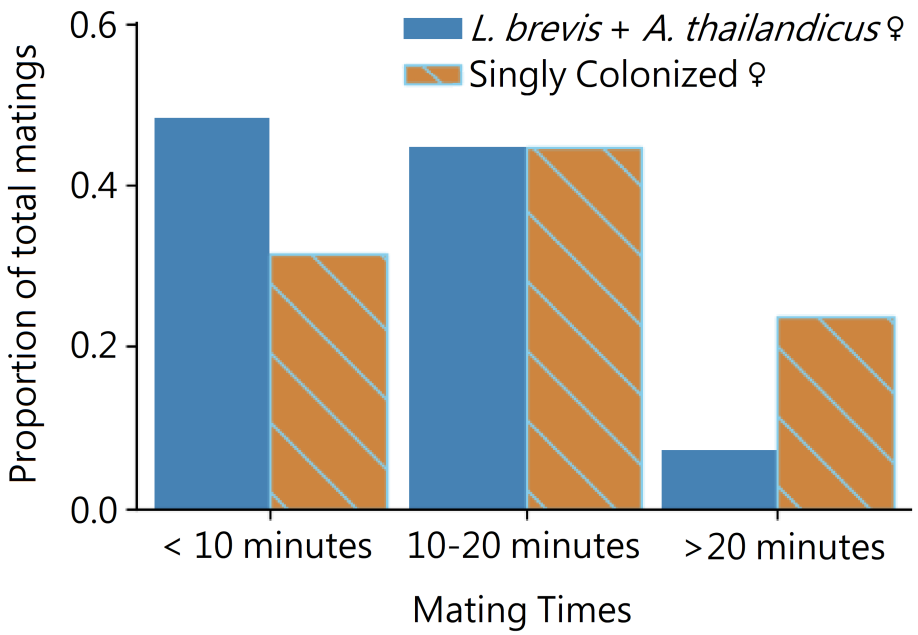

Figure 4.3: **The time to first mating was significantly faster for co-colonized female *D. melanogaster* than for females colonized by *L. brevis* or *A. thailandicus*.** The time to first mating of the 56 co-colonized female flies (blue) is significantly shorter than time to first mating of females colonized with *L. brevis* or *A. thailandicus* alone (brown with blue stripe) (Kolmogorov–Smirnov, $p = 0.022$). Female flies colonized with only *L. brevis* or *A. thailandicus* have similar distributions of the time to first mating (Kolmogorov–Smirnov, $p = 0.843$).

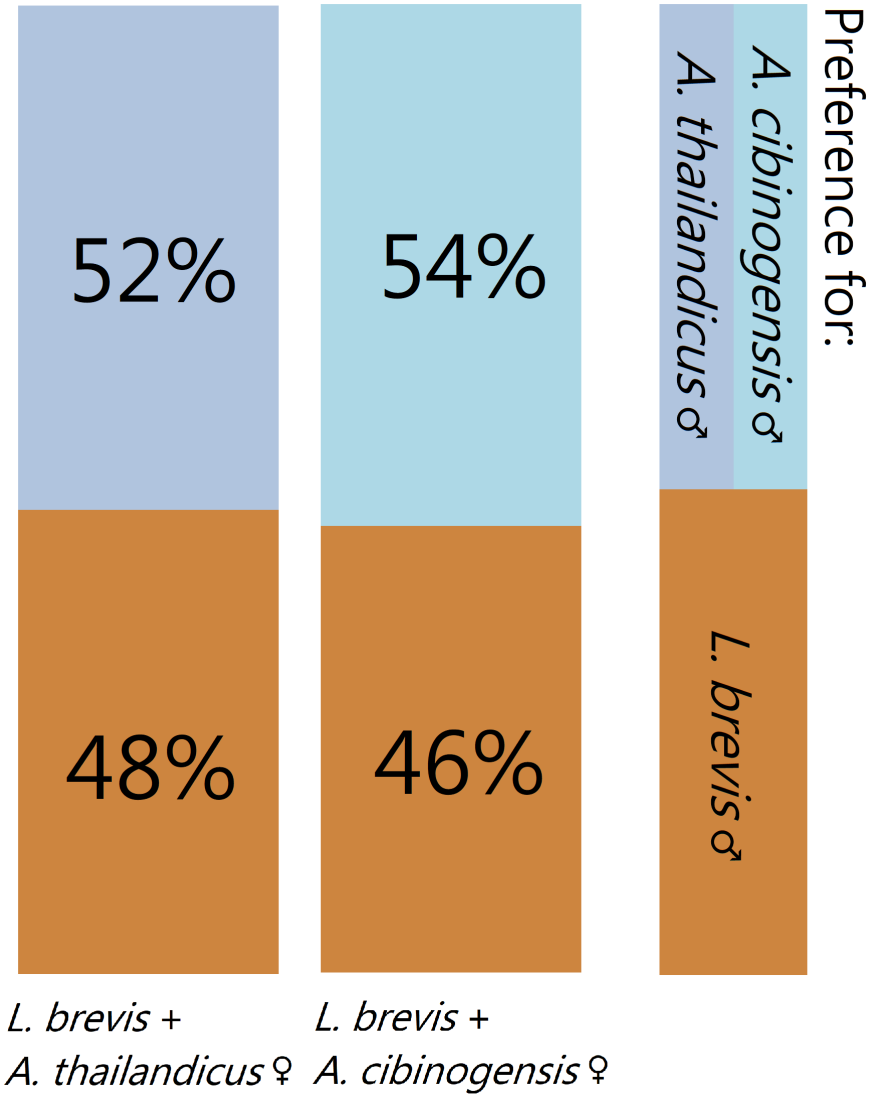

Figure 4.4: **Females co-colonized with *L. brevis* and a different *Acetobacter* species, *A. cibinogensis* have no discernible mate preferences.** Of the 71 females co-colonized with *L. brevis* and *A. cibinogensis* (light blue), 33 mated with *L. brevis*-colonized males (Binomial, $p = 0.76$). This mate preference bias is indistinguishable from that of females co-colonized with *L. brevis* and *A. thailandicus* (46% vs 48%, Fisher's Exact, $p = 0.86$).

4.5. Discussion

Populations evolve via natural selection when phenotypes that increase mating success or survival are derived from heritable genetic variants (Wade and Kalisz, 1990; Saccheri and Hanski, 2006). The efficacy of evolution by natural selection can be diminished by environmental factors that impact favored phenotypes, thus decoupling phenotypes from genotypes. Gut microbiomes have recently been recognized as a crucial environmental factor that influences multiple host phenotypes of evolutionary and medical importance (Kinross et al., 2011; Singh et al., 2017; Macke et al., 2017; Himler et al., 2011). Therefore, the gut microbiome may play an important role in modulating the efficacy of evolution by natural selection.

The gut microbiome serves as a potent environmental feature impacting female mate preference (Figure 4.1A). Experimental mating trials demonstrated that female *D. melanogaster* colonized by *L. brevis* significantly preferred copulating with males also colonized by *L. brevis* while female *D. melanogaster* colonized by *A. thailandicus* showed a bias toward copulating with males colonized by *A. thailandicus*. Homophilic mate preferences were even more evident among more selective females that took longer to choose a mating partner (Figure 4.1B). These results show that differences in gut microbiota affect the interaction between the female mate preference and male attractiveness phenotypes in *D. melanogaster*. The impact of the microbiota on mate preference was isolated from any genetic determinants of mate preferences or other environmental influences, like dietary variation, as all flies were isogenic and were raised in otherwise identical environments.

Females flies co-colonized with *L. brevis* and *A. thailandicus* had no mating bias and were quicker to copulate than singly-colonized females (Figure 4.2, 4.3). This willingness to mate with either male could be caused by microbiome-mediated phenotypic familiarity with both males. That is, co-colonized females see males as equally similar, as both males have microbiomes that overlap with the female's microbial profile. Further, the time to copulation may be impacted by the perceived in-group membership of both males; there is no need for females to spend time choosing among equivalent males. However, it remains unclear how the degree of gut community overlap impacts the female's perception of phenotypic familiarity. This question is relevant in natural populations

where richer microbiome communities are likely to have variable degrees of community overlap. This hypotheses can be addressed with mating trials in which flies have richer gut microbial communities with differing degrees of microbial species overlap.

The relative impact of the gut microbiota and host genotype, or interactions between these two factors, on mate preferences is unknown. In the current trials, isogenic *D. melanogaster* were used to control for the influence of genotype on mate preference. While this experimental design removed the potential for flies to choose *good genes* (Hamilton and Zuk, 1982), controlling for genetic variation removed the possibility to detect gene-by-environment (GxE) interactions. GxE interactions are widespread and commonly have nonlinear effects on phenotype (Monaghan, 2008; Via and Lande, 1985). Thus, the observed effect of gut microbiomes on mate preference could be exaggerated by GxE interactions. Additional mate choice trials including another isogenic line and a hybrid line could address the impacts of GxE interactions on the strength of selection pressures on microbiome-mediated phenotypes.

Assortative mating that consistently reduces gene flow among subpopulations is a primary driver of speciation in sexually reproducing organisms (Dieckmann and Doebeli, 1999; Merrill et al., 2019). Thus, microbiome differences that result in assortative mating have the potential to reduce gene flow among subpopulations and promote speciation in some cases. Although not specifically heritable, gut microbiome communities are strongly influenced by maternal microbiomes, especially in species where the oviparous and excretory systems share common structures (Bakula, 1969; Habayeb et al., 2009). Thus, microbiome-mediated mate preferences could result in the inbreeding of subpopulations of related lineages with similar, semi-heritable microbial communities, possibly reducing gene flow and fostering speciation events. As an example, it is possible that microbiome differences contributed to the assortative mating leading the textbook example of sympatric speciation in the apple maggot fly, *Rhagoletis pomonella* (Bush, 1969). The apple maggot fly speciation event was driven primarily by fruit-specific habitat preferences that could have led to differing microbiome communities (Pinto-Tomás et al., 2011; Miyake et al., 2015; David et al., 2014; Ringø et al., 2016) that contributed to assortative mating.

The gut microbiome is a complex environmental factor that impacts many host phenotypes that are targets of natural selection. Although an incredible range of microbiome-mediated phenotypes have been identified, the complex ecology of the gut community complicates identification of direct effects of microbial species as well as mechanistic effects. Controllable, tractable microbiome-host models like the *D. melanogaster* system presented here can be used to gain a mechanistic understanding of the role of the gut microbiome in host biology. This system employs naturally-cultivated gut microbes and retains all of the inherent advantages of *D. melanogaster* as a model system. This system could also be used to study the impact of the gut microbiome on higher-level biological interactions like host ecology and evolution.

BIBLIOGRAPHY

David Alonso, Rampal S Etienne, and Alan J McKane. The merits of neutral theory. *Trends in ecology & evolution*, 21(8):451–457, 2006.

Yelina Alvarez, Lila G Glotfelty, Niklas Blank, Lenka Dohnalová, and Christoph A Thaiss. The microbiome as a circadian coordinator of metabolism. *Endocrinology*, 161(6):bqaa059, 2020.

Theo CM Bakker, Reto Künzler, and Dominique Mazzi. Condition-related mate choice in sticklebacks. *Nature*, 401(6750):234–234, 1999.

Marion Bakula. The persistence of a microbial flora during postembryogenesis of drosophila melanogaster. *Journal of invertebrate pathology*, 14(3):365–374, 1969.

PC Barko, MA McMichael, Kelly S Swanson, and David A Williams. The gastrointestinal microbiome: a review. *Journal of veterinary internal medicine*, 32(1):9–25, 2018.

Michael J Behrenfeld and Kelsey M Bisson. Neutral theory and plankton biodiversity. *Annual Review of Marine Science*, 16(1):283–305, 2024.

Gerald Borgia. Complex male display and female choice in the spotted bowerbird: specialized functions for different bower decorations. *Animal Behaviour*, 49(5):1291–1301, 1995.

Paul M Brakefield, W Anthony Frankino, et al. Polyphenisms in lepidoptera: Multidisciplinary approaches to studies of evolution and development. *Phenotypic plasticity of insects: mechanisms and consequences*, pages 337–368, 2009.

James H Brown. Why are there so many species in the tropics? *Journal of biogeography*, 41(1): 8–22, 2014.

Shelly A Buffington, Gonzalo Viana Di Prisco, Thomas A Auchtung, Nadim J Ajami, Joseph F Petrosino, and Mauro Costa-Mattioli. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell*, 165(7):1762–1775, 2016.

Guy L Bush. Sympatric host race formation and speciation in frugivorous flies of the genus rhagoletis (diptera, tephritidae). *Evolution*, pages 237–251, 1969.

James F Cahill Jr, Steven W Kembel, Eric G Lamb, and Paul A Keddy. Does phylogenetic relatedness influence the strength of competition among vascular plants? *Perspectives in Plant Ecology, Evolution and Systematics*, 10(1):41–50, 2008.

Jason Z Chen, Zeeyong Kwong, Nicole M Gerardo, and Nic M Vega. Ecological drift during colonization drives within-host and between-host heterogeneity in an animal-associated symbiont. *Plos Biology*, 22(4):e3002304, 2024.

Peter Chesson. General theory of competitive coexistence in spatially-varying environments. *Theoretical population biology*, 58(3):211–237, 2000a.

Peter Chesson. Mechanisms of maintenance of species diversity. *Annual review of Ecology and Systematics*, 31(1):343–366, 2000b.

Joseph H Connell. The influence of interspecific competition and other factors on the distribution of the barnacle chthamalus stellatus. *Ecology*, pages 710–723, 1961.

Timothy O Cox, Patrick Lundgren, Kirti Nath, and Christoph A Thaiss. Metabolic control by the microbiome. *Genome Medicine*, 14(1):80, 2022.

Katharine Z Coyte, Jonas Schluter, and Kevin R Foster. The ecology of the microbiome: networks, competition, and stability. *Science*, 350(6261):663–666, 2015.

Catalina Cuellar-Gempeler. Diversity–function relationships and the underlying ecological mechanisms in host-associated microbial communities. *Microbes: The Foundation Stone of the Biosphere*, pages 297–326, 2021.

Charles Darwin. On the origin of species, 1859.

Lawrence A David, Corinne F Maurice, Rachel N Carmody, David B Gootenberg, Julie E Button, Benjamin E Wolfe, Alisha V Ling, A Sloan Devlin, Yug Varma, Michael A Fischbach, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505(7484):559–563, 2014.

Carlotta De Filippo, Duccio Cavalieri, Monica Di Paola, Matteo Ramazzotti, Jean Baptiste Poullet, Sebastien Massart, Silvia Collini, Giuseppe Pieraccini, and Paolo Lionetti. Impact of diet in shaping gut microbiota revealed by a comparative study in children from europe and rural africa. *Proceedings of the National Academy of Sciences*, 107(33):14691–14696, 2010.

Ulf Dieckmann and Michael Doebeli. On the origin of species by sympatric speciation. *Nature*, 400 (6742):354–357, 1999.

Diane MB Dodd. Reproductive isolation as a consequence of adaptive divergence in drosophila pseudoobscura. *Evolution*, pages 1308–1311, 1989.

Ren Dodge, Eric W Jones, Haolong Zhu, Benjamin Obadia, Daniel J Martinez, Chenhui Wang, Andrés Aranda-Díaz, Kevin Aumiller, Zhexian Liu, Marco Voltolini, et al. A symbiotic physical niche in drosophila melanogaster regulates stable association of a multi-species gut microbiota. *Nature communications*, 14(1):1557, 2023.

Angela E Douglas. The drosophila model for microbiome research. *Lab animal*, 47(6):157–164, 2018.

Arthur E Dunham. An experimental study of interspecific competition between the iguanid lizards sceloporus merriami and urosaurus ornatus. *Ecological Monographs*, 50(3):309–330, 1980.

Arthur E Dunham. Realized niche overlap, resource abundance, and intensity of interspecific competition. In *Lizard ecology: studies of a model organism*, pages 261–280. Harvard University Press, 1983.

Claire Duvallet, Sean M Gibbons, Thomas Gurry, Rafael A Irizarry, and Eric J Alm. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. *Nature communications*, 8(1):1784, 2017.

Charles S Elton. *Animal ecology*. Sidgwick Jackson, 1927.

Debbie Eraly, Frederik Hendrickx, and Luc Lens. Condition-dependent mate choice and its implications for population differentiation in the wolf spider pirata piraticus. *Behavioral Ecology*, 20 (4):856–863, 2009.

Anne E Espeset and Matthew L Forister. In search of an honest butterfly: sexually selected wing coloration and reproductive traits from wild populations of the cabbage white butterfly. *Annals of the Entomological Society of America*, 115(2):156–162, 2022.

Ronald Aylmer Fisher. *The genetical theory of natural selection: a complete variorum edition*. Oxford University Press, 1930.

Georgy Frantsevich Gause. Experimental studies on the struggle for existence. *J Exp Biol*, 9: 389–402, 1932.

Cameron K Ghalambor, John K McKay, Scott P Carroll, and David N Reznick. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional ecology*, 21(3):394–407, 2007.

Phillip Gienapp, Marjolein Lof, Thomas E Reed, John McNamara, Simon Verhulst, and Marcel E Visser. Predicting demographically sustainable rates of adaptation: can great tit breeding time keep pace with climate change? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1610):20120289, 2013.

Jack A Gilbert, Martin J Blaser, J Gregory Caporaso, Janet K Jansson, Susan V Lynch, and Rob Knight. Current understanding of the human microbiome. *Nature medicine*, 24(4):392–400, 2018.

Oscar Godoy, Nathan JB Kraft, and Jonathan M Levine. Phylogenetic relatedness and the determinants of competitive outcomes. *Ecology letters*, 17(7):836–844, 2014.

Erick Greene. A diet-induced developmental polymorphism in a caterpillar. *Science*, 243(4891): 643–646, 1989.

Gregory F Grether. Carotenoid limitation and mate preference evolution: a test of the indicator hypothesis in guppies (poecilia reticulata). *Evolution*, 54(5):1712–1724, 2000.

Joseph Grinnell. The niche-relationships of the california thrasher. *The Auk*, 34(4):427–433, 1917.

Mazen S Habayeb, Rafael Cantera, Gabriela Casanova, Jens-Ola Ekström, Shannon Albright, and Dan Hultmark. The drosophila nora virus is an enteric virus, transmitted via feces. *Journal of invertebrate pathology*, 101(1):29–33, 2009.

S Hadwen and LJ Palmer. Reindeer in alaska (no. 1089). *US Department of Agriculture*, page 56, 1922.

William D Hamilton and Marlene Zuk. Heritable true fitness and bright birds: a role for parasites? *Science*, 218(4570):384–387, 1982.

Garrett Hardin. The competitive exclusion principle: an idea that took a century to be born has implications in ecology, economics, and genetics. *science*, 131(3409):1292–1297, 1960.

Gergely Hegyi, Beáta Szigeti, János Török, and Marcel Eens. Melanin, carotenoid and structural plumage ornaments: information content and role in great tits parus major. *Journal of Avian Biology*, 38(6):698–708, 2007.

Gergely Hegyi, Miklós Laczi, Gergely Nagy, Eszter Szász, Dóra Kötél, and János Török. Stable correlation structure among multiple plumage colour traits: can they work as a single signal? *Biological Journal of the Linnean Society*, 114(1):92–108, 2015.

Janneke HilleRisLambers, Peter B Adler, W Stanley Harpole, Jonathan M Levine, and Margaret M Mayfield. Rethinking community assembly through the lens of coexistence theory. *Annual review of ecology, evolution, and systematics*, 43(1):227–248, 2012.

Anna G Himler, Tetsuya Adachi-Hagimori, Jacqueline E Bergen, Amaranta Kozuch, Suzanne E Kelly, Bruce E Tabashnik, Elad Chiel, Victoria E Duckworth, Timothy J Dennehy, Einat Zchori-Fein, et al. Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *science*, 332(6026):254–256, 2011.

Zhiqiang Huang, Kun Liu, Wenwen Ma, Dezhi Li, Tianlu Mo, and Qing Liu. The gut microbiome in human health and disease—where are we and where are we going? a bibliometric analysis. *Frontiers in Microbiology*, 13:1018594, 2022.

Stephen P Hubbell. *The unified neutral theory of biodiversity and biogeography (MPB-32)*. Princeton University Press, 2001.

John Hunt, Robert Brooks, and Michael D Jennions. Female mate choice as a condition-dependent life-history trait. *The American Naturalist*, 166(1):79–92, 2005.

G Evelyn Hutchinson. Homage to santa rosalia or why are there so many kinds of animals? *The American Naturalist*, 93(870):145–159, 1959.

G Evelyn Hutchinson. The paradox of the plankton. *The American Naturalist*, 95(882):137–145, 1961.

GE Hutchinson. Population studies: animal ecology and demography: concluding remarks. *Cold Spring Harbor Symposia on Quantitative Biology*, 1957.

BW Jones and MK Nishiguchi. Counterillumination in the hawaiian bobtail squid, euprymna scolopes berry (mollusca: Cephalopoda). *Marine Biology*, 144(6):1151–1155, 2004.

Geoffrey P Jones, Mark I McCormick, Maya Srinivasan, and Janelle V Eagle. Coral decline threatens fish biodiversity in marine reserves. *Proceedings of the National Academy of Sciences*, 101(21): 8251–8253, 2004.

Sharon E Kingsland. Foundational papers. *Foundations of Ecology:\Classic Papers with Commentaries*, page 1, 2012.

James M Kinross, Ara W Darzi, and Jeremy K Nicholson. Gut microbiome-host interactions in health and disease. *Genome medicine*, 3:1–12, 2011.

Russell L Kologiski. The phytosociology of the green swamp, north carolina. *North Carolina Agricultural Experiment Station Technical Bulletin*, (250):17, 1977.

Omry Koren, Dan Knights, Antonio Gonzalez, Levi Waldron, Nicola Segata, Rob Knight, Curtis Huttenhower, and Ruth E Ley. A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. *PLoS computational biology*, 9 (1):e1002863, 2013.

R Brian Langerhans and Thomas J DeWitt. Plasticity constrained: over-generalized induction cues cause maladaptive phenotypes. *Evolutionary Ecology Research*, 4(6):857–870, 2002.

Jia-Jia Liu, Yong Xu, You-Xia Shan, Kevin S Burgess, and Xue-Jun Ge. Biotic and abiotic factors determine species diversity–productivity relationships in mountain meadows. *Journal of Plant Ecology*, 14(6):1175–1188, 2021.

MV Lomolino, BR Riddle, RJ Whittaker, and JH Brown. Biogeography, 4th edn sunderland. *MA: Sinauer Associates [Google Scholar]*, 2010.

Catherine A Lozupone, Jesse I Stombaugh, Jeffrey I Gordon, Janet K Jansson, and Rob Knight. Diversity, stability and resilience of the human gut microbiota. *Nature*, 489(7415):220–230, 2012.

JB Lynch and EY Hsiao. Microbiomes as sources of emergent host phenotypes. *Science*, 365(6460): 1405–1409, 2019.

Robert H MacArthur. Population ecology of some warblers of northeastern coniferous forests. *Ecology*, 39(4):599–619, 1958.

Emilie Macke, Aurélie Tasiemski, François Massol, Martijn Callens, and Ellen Decaestecker. Life history and eco-evolutionary dynamics in light of the gut microbiota. *Oikos*, 126(4):508–531, 2017.

Lisa Maier, Mihaela Pruteanu, Michael Kuhn, Georg Zeller, Anja Telzerow, Exene Erin Anderson, Ana Rita Brochado, Keith Conrad Fernandez, Hitomi Dose, Hirotada Mori, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature*, 555(7698):623, 2018.

Eduard Mas-Carrió, Marcin Churski, Dries Kuijper, and Luca Fumagalli. Niche overlap across landscape variability in summer between two large herbivores using edna metabarcoding. *Plos one*, 19(2):e0279672, 2024.

Robert M May. Will a large complex system be stable? *Nature*, 238(5364):413, 1972.

Margaret M Mayfield and Jonathan M Levine. Opposing effects of competitive exclusion on the phylogenetic structure of communities. *Ecology letters*, 13(9):1085–1093, 2010.

Margaret J McFall-Ngai. Giving microbes their due–animal life in a microbially dominant world. *The Journal of experimental biology*, 218(12):1968–1973, 2015.

Richard M Merrill, Pasi Rastas, Simon H Martin, Maria C Melo, Sarah Barker, John Davey, W Owen McMillan, and Chris D Jiggins. Genetic dissection of assortative mating behavior. *PLoS biology*, 17(2):e2005902, 2019.

Elie Metchnikoff. *The prolongation of life*. Putnam, 1908.

Gary G Mittelbach and Brian J McGill. *Community ecology*. Oxford University Press, 2019.

Sou Miyake, David Kamanda Ngugi, and Ulrich Stingl. Diet strongly influences the gut microbiota of surgeonfishes. *Molecular ecology*, 24(3):656–672, 2015.

Pat Monaghan. Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1497):1635–1645, 2008.

Mary J Montague, Marine Danek-Gontard, and Hansjoerg P Kunc. Phenotypic plasticity affects the response of a sexually selected trait to anthropogenic noise. *Behavioral Ecology*, 24(2):343–348, 2013.

Juliano Morimoto, Stephen J Simpson, and Fleur Ponton. Direct and trans-generational effects of male and female gut microbiota in drosophila melanogaster. *Biology letters*, 13(7):20160966, 2017.

Katie H Morrow. Neutral and niche theory in community ecology: a framework for comparing model realism. *Biology & Philosophy*, 39(1):4, 2024.

Sofia M Murga-Garrido, Qilin Hong, Tzu-Wen L Cross, Evan R Hutchison, Jessica Han, Sydney P Thomas, Eugenio I Vivas, John Denu, Danilo G Ceschin, Zheng-Zheng Tang, et al. Gut microbiome variation modulates the effects of dietary fiber on host metabolism. *Microbiome*, 9(1):117, 2021.

Jose A Navas-Molina, Embriette R Hyde, Jon G Sanders, and Rob Knight. The microbiome and big data. *Current Opinion in Systems Biology*, 4:92–96, 2017.

Scott Norris. Neutral theory: a new, unified model for ecology. *BioScience*, 53(2):124–129, 2003.

S O'M. Fundamentals of ecology., 1971.

Emilio Pagani-Núñez, Francesc Uribe, Sergio Hernández-Gómez, Guillermo Muñoz, and Juan Carlos Senar. Habitat structure and prey composition generate contrasting effects on carotenoid-based coloration of great tit parus major nestlings. *Biological Journal of the Linnean Society*, 113(2): 547–555, 2014.

Inês S Pais, Rita S Valente, Marta Sporniak, and Luis Teixeira. Drosophila melanogaster establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS biology*, 16(7): e2005710, 2018.

Minna Pekkonen, Tarmo Ketola, and Jouni T Laakso. Resource availability and competition shape the evolution of survival and growth ability in a bacterial community. *PLoS One*, 8(9):e76471, 2013.

Oliver L Phillips, Pamela Hall, Alwyn Howard Gentry, SA Sawyer, and R Vasquez. Dynamics and species richness of tropical rain forests. *Proceedings of the National Academy of Sciences*, 91(7): 2805–2809, 1994.

Adrián A Pinto-Tomás, Ana Sittenfeld, Lorena Uribe-Lorío, Felipe Chavarría, Marielos Mora, Daniel H Janzen, Robert M Goodman, and Holly M Simon. Comparison of midgut bacterial diversity in tropical caterpillars (lepidoptera: Saturniidae) fed on different diets. *Environmental entomology*, 40(5):1111–1122, 2011.

Arnaud Pocheville. The ecological niche: history and recent controversies. *Handbook of evolutionary thinking in the sciences*, pages 547–586, 2015.

Dominik Poniatowski, Gregor Stuhldreher, Franz Löffler, and Thomas Fartmann. Patch occupancy of grassland specialists: Habitat quality matters more than habitat connectivity. *Biological Conservation*, 225:237–244, 2018.

Anna Louise Pouncey, Alasdair James Scott, James Leslie Alexander, Julian Marchesi, and James Kinross. Gut microbiota, chemotherapy and the host: the influence of the gut microbiota on cancer treatment. *ecancermedicalscience*, 12, 2018.

Trevor D Price. Phenotypic plasticity, sexual selection and the evolution of colour patterns. *Journal of Experimental Biology*, 209(12):2368–2376, 2006.

Trevor D. Price, Anna Qvarnström, and Darren E. Irwin. The role of phenotypic plasticity in driving genetic evolution. *Proc. R. Soc. Lond. B Biol. Sci.*, 270(1523):1433–1440, July 2003. ISSN 1471-2954. doi: 10.1098/rspb.2003.2372.

Rory Putman. *Community ecology*. Springer Science & Business Media, 1994.

Jimmy J Qian and Erol Akçay. The balance of interaction types determines the assembly and stability of ecological communities. *Nature Ecology & Evolution*, 4(3):356–365, 2020.

EZZV Ringø, Zhigang Zhou, JL Gonzalez Vecino, S Wadsworth, Jaime Romero, Åshild Krogdahl, Rolf Erik Olsen, Arkadios Dimitroglou, Andrew Foey, Simon Davies, et al. Effect of dietary components on the gut microbiota of aquatic animals. a never-ending story? *Aquaculture nutrition*, 22(2):219–282, 2016.

GB Rogers, Damien J Keating, Richard L Young, Ma-Li Wong, Julio Licinio, and Steven Wesselingh. From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Molecular psychiatry*, 21(6):738–748, 2016.

Ilik Saccheri and Ilkka Hanski. Natural selection and population dynamics. *Trends in Ecology & Evolution*, 21(6):341–347, 2006.

Janna N Schultzhaus, Joshua J Nixon, Jose A Duran, and Ginger E Carney. Diet alters drosophila melanogaster mate preference and attractiveness. *Animal behaviour*, 123:317–327, 2017.

Robert F Schwabe and Christian Jobin. The microbiome and cancer. *Nature Reviews Cancer*, 13 (11):800–812, 2013.

Michael Shapira. Gut microbiotas and host evolution: scaling up symbiosis. *Trends in ecology & evolution*, 31(7):539–549, 2016.

Gil Sharon, Daniel Segal, John M Ringo, Abraham Hefetz, Ilana Zilber-Rosenberg, and Eugene Rosenberg. Commensal bacteria play a role in mating preference of drosophila melanogaster. *Proceedings of the National Academy of Sciences*, 107(46):20051–20056, 2010.

Dionei J Silva, Ana Filipa Palmeirim, Manoel Santos-Filho, Tânia Margarete Sanaiotti, and Carlos A Peres. Habitat quality, not patch size, modulates lizard responses to habitat loss and fragmentation in the southwestern amazon. *Journal of Herpetology*, 56(1):75–83, 2022.

Stephen J Simpson, Gregory A Sword, and Nathan Lo. Polyphenism in insects. *Current Biology*, 21(18):R738–R749, 2011.

Rasnik K Singh, Hsin-Wen Chang, DI Yan, Kristina M Lee, Derya Ucmak, Kirsten Wong, Michael

Abrouk, Benjamin Farahnik, Mio Nakamura, Tian Hao Zhu, et al. Influence of diet on the gut microbiome and implications for human health. *Journal of translational medicine*, 15:1–17, 2017.

T Slagsvold and JT Lifjeld. Variation in plumage colour of the great tit parus major in relation to habitat, season and food. *Journal of Zoology*, 206(3):321–328, 1985.

Felix Sommer and Fredrik Bäckhed. The gut microbiota—masters of host development and physiology. *Nature reviews microbiology*, 11(4):227–238, 2013.

Serafino Teseo, Jelle S van Zweden, Luigi Pontieri, Pepijn W Kooij, Søren J Sørensen, Tom Wenseleers, Michael Poulsen, Jacobus J Boomsma, and Panagiotis Sapountzis. The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants. *Animal Behaviour*, 150:239–254, 2019.

Christoph A Thaiss, David Zeevi, Maayan Levy, Gili Zilberman-Schapira, Jotham Suez, Anouk C Tengeler, Lior Abramson, Meirav N Katz, Tal Korem, Niv Zmora, et al. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell*, 159(3):514–529, 2014.

Casey M Theriot, Mark J Koenigsknecht, Paul E Carlson Jr, Gabrielle E Hatton, Adam M Nelson, Bo Li, Gary B Huffnagle, Jun Z Li, and Vincent B Young. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to clostridium difficile infection. *Nature communications*, 5:3114, 2014.

Gaku Tokuda, Aram Mikaelyan, Chiho Fukui, Yu Matsuura, Hirofumi Watanabe, Masahiro Fujishima, and Andreas Brune. Fiber-associated spirochetes are major agents of hemicellulose degradation in the hindgut of wood-feeding higher termites. *Proceedings of the National Academy of Sciences*, 115(51):E11996–E12004, 2018.

Peter J Turnbaugh, Ruth E Ley, Micah Hamady, Claire M Fraser-Liggett, Rob Knight, and Jeffrey I Gordon. The human microbiome project. *Nature*, 449(7164):804–810, 2007.

Natalia G Vallianou, Theodora Stratigou, and Stylianos Tsagarakis. Microbiome and diabetes: where are we now? *Diabetes research and clinical practice*, 146:111–118, 2018.

Floris M van Beest, Philip D McLoughlin, Eric Vander Wal, and Ryan K Brook. Density-dependent habitat selection and partitioning between two sympatric ungulates. *Oecologia*, 175:1155–1165, 2014.

MJ Van den Berg, G Thomas, H Hendriks, and W Van Delden. A reexamination of the negative assortative mating phenomenon and its underlying mechanism in drosophila melanogaster. *Behavior genetics*, 14:45–61, 1984.

Sara Via and Russell Lande. Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, 39(3):505–522, 1985.

Alessia Visconti, Caroline I Le Roy, Fabio Rosa, Niccolò Rossi, Tiphaine C Martin, Robert P Mohney, Weizhong Li, Emanuele de Rinaldis, Jordana T Bell, J Craig Venter, et al. Interplay between the human gut microbiome and host metabolism. *Nature communications*, 10(1):1–10, 2019.

Peter M Vitousek and RL Sanford. Nutrient cycling in moist tropical forest. *Annual review of Ecology and Systematics*, pages 137–167, 1986.

Helen E Vuong, Jessica M Yano, Thomas C Fung, and Elaine Y Hsiao. The microbiome and host behavior. *Annual review of neuroscience*, 40(1):21–49, 2017.

Michael J Wade and Susan Kalisz. The causes of natural selection. *Evolution*, 44(8):1947–1955, 1990.

Zhijie Wang, Shujun Liu, and Yuan Su. Spatiotemporal evolution of habitat quality and its response to landscape patterns in karst mountainous cities: a case study of guiyang city in china. *Environmental Science and Pollution Research*, 30(53):114391–114405, 2023.

Campbell O Webb, David D Ackerly, Mark A McPeek, and Michael J Donoghue. Phylogenies and community ecology. *Annual review of ecology and systematics*, 33(1):475–505, 2002.

John J Wiens and Catherine H Graham. Niche conservatism: integrating evolution, ecology, and conservation biology. *Annu. Rev. Ecol. Evol. Syst.*, 36(1):519–539, 2005.

S Joseph Wright, Kaoru Kitajima, Nathan JB Kraft, Peter B Reich, Ian J Wright, Daniel E Bunker, Richard Condit, James W Dalling, Stuart J Davies, Sandra Díaz, et al. Functional traits and the growth–mortality trade-off in tropical trees. *Ecology*, 91(12):3664–3674, 2010.

Tanya Yatsunenko, Federico E Rey, Mark J Manary, Indi Trehan, Maria Gloria Dominguez-Bello, Monica Contreras, Magda Magris, Glida Hidalgo, Robert N Baldassano, Andrey P Anokhin, et al. Human gut microbiome viewed across age and geography. *nature*, 486(7402):222–227, 2012.

Xinping Ye, Andrew K Skidmore, and Tiejun Wang. Within-patch habitat quality determines the resilience of specialist species in fragmented landscapes. *Landscape ecology*, 28:135–147, 2013.

Ji Youn Yoo, Maureen Groer, Samia Valeria Ozorio Dutra, Anujit Sarkar, and Daniel Ian McSkimming. Gut microbiota and immune system interactions. *Microorganisms*, 8(10):1587, 2020.

Naofumi Yoshida, Tomoya Yamashita, and Ken-ichi Hirata. Gut microbiome and cardiovascular diseases. *Diseases*, 6(3):56, 2018.