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The persistence of host-symbiont mutualisms:
Investigating the roles of genetic variation, coevolution, and transmission mode

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Investigating the roles of genetic variation, coevolution, and transmission mode

By

Kayla S. Stoy

B.S., Indiana University, 2014

Advisor: Nicole Gerardo, PhD

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An abstract of
A dissertation submitted to the Faculty of the
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ABSTRACT

The persistence of host-symbiont mutualisms depends on the alignment of host and symbiont fitness interests. However, many mutualisms persist through processes, such as horizontal transmission, that can readily decouple host and symbiont fitness. How these mutualisms persist despite the potential costs remains unknown. My dissertation examines the eco-evolutionary dynamics that underlie the persistence of horizontally transmitted mutualisms. I evaluated these dynamics using a naturally occurring interaction between the insect host *Anasa tristis* and its horizontally transmitted bacterial symbiont *Caballeronia* spp. I began by testing the hypothesis that coevolution underlies the persistence of horizontally transmitted mutualisms. I tested for evidence of pairwise and diffuse coevolution by measuring patterns of host-symbiont specificity. I observed patterns of specialization consistent with diffuse coevolution, suggesting a potential pathway by which cooperation is maintained within this horizontally transmitted interaction. Specifically, selection from a range of host species may maintain fixed cooperative traits across populations of their shared generalist symbionts. I then directly tested whether symbiont transmission environment, like those experienced under vertical and horizontal transmission, alters the direction of selection for cooperative symbiont traits using experimental evolution. I experimentally passaged a *Paraburkholderia* symbiont of *A. tristis* hosts through four transmission environments, including between *A. tristis* hosts, between *A. tristis* hosts and soil, through soil, and through standard culture media. I found that symbionts passaged through the host environment rapidly evolved deleterious traits affecting host survival. In contrast, when symbiont evolution is decoupled from the host, deleterious symbiont traits evolve a slower rate. This demonstrates that transmission environment can alter the direction of selection for cooperative symbiont traits. Contrary to expectation, this work suggests, in some cases, vertical transmission can facilitate misalignment of host and symbiont fitness, even more rapidly than horizontal transmission. Overall, by combining analysis of natural populations with experimental evolution, this dissertation illuminates a pathway by which horizontally transmitted mutualisms may persist and provides new insights into the role of transmission mode within host-symbiont mutualisms.

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CHAPTER I

INTRODUCTION

Cooperative interactions between species (*i.e.*, mutualisms) have puzzled scientists for generations. In *On the Origin of Species*, Darwin only briefly ponders the underlying impetus for mutualism while describing cooperative interactions between ants and aphids (Darwin 1859). He surmises that such interactions could only result through the selfish interests of each independent species. He then concludes, "…as details on this and other such points are not indispensable, they may be passed over here." Darwin later revisits this bizarre phenomenon of cooperative species interactions while examining the vast phenotypic diversity of orchids. In *Fertilisation of Orchids*, he concludes that the variation exhibited across orchids resulted through cross-pollination, a process, he posits, may result through specialized, cooperative interactions between orchids and insects (Darwin 1862). This hypothesis and the study of mutualism was then largely ignored and remained untested for the following century. It was not until the 1960s that ecologists began to consider the coevolutionary dynamics underlying mutualisms (Janzen 1966). A theoretical basis for the maintenance of mutualistic interactions was then first introduced by Trivers in 1971. This work was succeeded by additional theory over the next two decades that established a foundation for describing the pathways by which species mutualistically interact with one another while avoiding exploitation (Axelrod and Hamilton 1981; Noe and Hammerstein 1994; Doebeli and Knowlton 1998; reviewed in Sachs *et al*. 2004).

Despite its slow and seemingly hesitant embrace, there is now a large body of empirical evidence for these models and new theory continues to refine our understanding of mutualism. Recent

decades have also brought robust efforts to identify and characterize previously unknown mutualistic interactions. Through this work, microbes have been identified as especially pervasive participants in mutualisms. In fact, we now accept mutualism with microbes as integral to the function and development of nearly all organisms. From the evolution of the eukaryotic cell to the development of acquired immunity, the complexity of life on Earth is the result of mutualism with microbes (Margulis 1996; reviewed in Gerardo *et al*. 2020). Mutualism has long surpassed its status as a perplexing anomaly to become accepted as imperative for life.

My dissertation applies fundamental eco-evolutionary techniques to address a process within mutualism that contradicts current evolutionary theory: horizontal symbiont transmission. Horizontal transmission poses challenges for mutualisms by potentially misaligning host and symbiont fitness interests, and by potentially increasing phenotypic and genetic variation within mutualisms (Anderson and May 1982; Ewald 1987; Bull 1994; Frank 1996; Brandvain *et al*. 2011). Below, I discuss the possible deleterious consequences of horizontal transmission for mutualistic interactions. In chapter II, I summarize current mutualism theory and address its limitations for comprehensively characterizing the pathways by which mutualisms persist. I propose evolutionary genetics approaches as an important step forward for developing an understanding of the coevolutionary dynamics that underlie host-symbiont mutualisms. In Chapter III, I test whether coevolution contributes to the persistence of horizontally transmitted mutualisms. I collect insect hosts and their bacterial symbionts from across their native geographic range and perform empirical tests for coevolution by measuring host-symbiont specificity. In Chapter IV, I use experimental evolution, an underutilized tool for the study of mutualism (Hoang *et al*. 2016), to directly test whether transmission environment, like those experienced under vertical and horizontal transmission, alters the direction of selection on

symbionts for cooperation with their hosts. Overall, my dissertation demonstrates a potentially important role for diffuse coevolution, provides new insights into the role of transmission mode, and establishes a path forward for assessing the persistence of horizontally transmitted mutualisms.

The paradox of horizontal transmission

Hosts across all domains of life depend on microbial symbionts to fulfill essential components of their fitness. Symbionts can aid in the digestion of nutrients, absorption of metabolites, defense against pathogens, development, and niche expansion (Baumann et al. 1995*b*; Pais et al. 2008; Kikuchi et al. 2011*a*, 2012*b*; Boucias et al. 2012; Joy 2013; Salem et al. 2013; Masson et al. 2015; Vorburger and Perlman 2018; Gerardo et al. 2020; Kaltenpoth and Flórez 2020). Likewise, microbial symbionts often depend on their hosts for aspects of their fitness, including replication and transmission (Lee & Ruby, 1994; Prell *et al*., 2009; Macdonald *et al*., 2012; Wollenberg & Ruby, 2012). The persistence of these interactions depends on the alignment of host and symbiont fitness interests. Under vertical transmission, parents directly pass their microbial symbionts to their offspring each generation. This transmission mode couples symbiont fitness with host fitness because symbiont transmission depends on host survival and reproduction. Under horizontal transmission, parents do not provide their offspring with microbial symbionts. Instead, offspring must acquire symbionts from the environment. Horizontal transmission then decouples the fitness of host and symbiont because symbionts can survive without their hosts. This presents a number of potential challenges for hosts. First, symbionts that can survive without their hosts may become exploitative by increasing rates of within-host replication and overutilizing nutritional and metabolic host resources (Ewald 1987; Bull 1994; Frank 1996; Porter and Simms 2014). Second, the strength of selection from the environment may supersede

selection from hosts, favoring symbiont traits for survival apart from their hosts (Simms *et al*. 2006). Finally, hosts required to scan their environments each generation may simply fail to find and acquire beneficial symbionts genotypes. Because of the potential costs associated with horizontal transmission, theory suggests that the long-term, stable association of host and symbiont depends on an evolutionary transition to vertical transmission (Ewald 1987; Sachs et al. 2011*a*; Drown et al. 2013). Despite these predictions, ancient horizontally transmitted mutualisms are common in nature (Long 1996; Ruby 1996; Chen et al. 2000; Nussbaumer et al. 2006; Kikuchi et al. 2011*a*; Hartmann et al. 2017*a*; Acevedo et al. 2021*a*).

Most of the predictions set forth by evolutionary theory regarding the persistence of mutualism comes from game theory and epidemiological models. In general, both model types depend solely on the phenotypic outcomes of host-symbiont associations and assume underlying antagonistic tendencies for the partners involved. Using these approaches ignores a critical component of species interactions: variation. In nature, host-symbiont mutualisms are both phenotypically and genetically diverse (Sicard *et al*. 2005; Mikheyev *et al*. 2006; Russell and Moran 2006; Barrett *et al*. 2012; Boutin *et al*. 2014; Chavez-dozal *et al*. 2014; Murfin *et al*. 2015; Harrison *et al*. 2017*b*; Bayliss *et al*. 2019). However, current theory often considers partner quality as a bimodally distributed trait (Heath and Stinchcombe 2014). Partners are either of ‘good’ quality and benefit their hosts or are of ‘bad’ quality and exploit their hosts. A more realistic approach would consider partner quality as a continuously variable trait (Heath and Stinchcombe 2014; Stoy *et al*. 2020). Moreover, the phenotypic outcomes of associations are likely driven by underlying genetic dynamics. Many mutualistic interactions are context-dependent, such that the outcome of association depends on host-symbiont genotype specificity (Lee and Ruby 1994*a*; Heath and Tiffin 2007; Wang *et al*. 2012; Murfin *et al*. 2015; Wood *et al*.

2018). Currently, the field lacks a well-developed theoretical framework that considers the evolutionary genetics underlying mutualistic interactions. My dissertation highlights the need to use broader approaches to understand the pathways by which mutualisms persist despite the potential costs of horizontal transmission.

Coevolution in mutualism is not reserved for vertical transmission

Coevolution is reciprocal evolutionary change between interacting species driven by natural selection (Thompson 2005). A vast array of literature has shown the importance of coevolution for species interactions. Coevolution underlies species survival, ecosystem stability, and biological diversification (Ehrlich and Raven 1964; Janzen 1966; Lively 1999; Thompson 2005; King *et al*. 2009; Yoder and Nuismer 2010; Guimarães *et al*. 2017; Nuismer *et al*. 2018). Coevolution also plays an important role in the maintenance of cooperation between mutualistic hosts and symbionts (Ehrlich and Raven 1964; Janzen 1966; Parker 1999; Moran 2001; Murfin *et al*. 2015; Wilson and Duncan 2015). The maintenance of cooperation within mutualism depends on partners maintaining tight control over one another for the exchange of benefits and inhibition of exploitation (Trivers 1971; Axelrod and Hamilton 1981; Noe and Hammerstein 1994; Sachs *et al*. 2004). This may most effectively be accomplished when species have opportunities for repeat interactions across generations, thus increasing the efficacy of selection for cooperative traits between specific host and symbiont lineages. Vertical transmission increases opportunities for coevolution by preserving interactions between specific host and symbiont lineages across generations. As such, coevolution is attributed a central role in the maintenance of cooperation within vertically transmitted mutualisms.

Coevolution is less often attributed a role in horizontally transmitted mutualisms. In fact, coevolution may not be recognized as generally central to these interactions at all given that their

persistence is considered a paradox for evolutionary theory. The dissonance between the perceived role of coevolution across vertically transmitted compared to horizontally transmitted interactions may result through the conflation of vertical transmission with coevolution. Conflating these processes can result in misconceptions about the prevalence of coevolution across mutualistic interactions. While vertical transmission increases opportunities for coevolution, it does not guarantee it. Vertically transmitted interactions may result through one-sided selection. For example, across many mutualisms, symbionts are sequestered into specialized cells, organs, or structures that allow the host to maintain control over them (Ponsen 1977; Mcfall-ngai 1999; Kikuchi *et al*. 2007; Nakajima *et al*. 2013; Lowe *et al*. 2016; Sørensen *et al*. 2019; Acevedo *et al*. 2021). It is not always clear whether these symbionts exert reciprocal selection on their hosts (Garcia and Gerardo 2014), and recent work suggests some mutualisms may result through host exploitation of sequestered symbionts (Nakajima *et al*. 2013; Lowe *et al*. 2016).

Moreover, horizontal transmission does not preclude coevolution. Spatial structure may promote coevolution in horizontally transmitted mutualisms by conserving interactions between specific host and symbiont lineages across generations (Wilkinson 2001). Horizontal transmission may even increase the efficacy of natural selection on symbiont populations by maintaining sufficient genetic variation on which selection can act (O'Fallon 2008). Repeated interactions between host and symbiont lineages within and across spatially structured host and symbiont populations may then permit adaptive responses between partners, consistent with coevolution, even without vertical transmission.

In general, few studies have tested for evidence of coevolution across horizontally transmitted mutualisms. This may be, in part, because of the perception that horizontal transmission

generally disrupts opportunities for coevolution. Alternatively, the paucity of studies may instead result because coevolution is being assumed given the frequency of genetic specificity, co-cladogenesis, and dependency exhibited within these interactions (Lee and Ruby 1994*b*; Aanen *et al*. 2002; Garcia-Cuetos *et al*. 2005; Brucker and Bordenstein 2012; Wang *et al*. 2012; Murfin *et al*. 2015; Parker *et al*. 2017; Forsman *et al*. 2020). These studies provide an important foundation for demonstrating the potential for coevolution. However, an important aspect of providing evidence for coevolution is demonstrating reciprocal evolutionary change driven by selection (Thompson 1994; Brockhurst and Koskella 2013).

The community context is also an important consideration for mutualistic coevolution (Bronstein *et al*. 2003; Stanton 2003; Guimarães *et al*. 2007, 2017*a*). Non-symbiotic mutualisms are often characterized by complex coevolutionary network interactions, largely shaped by generalists (Bascompte *et al*. 2003; Thompson 2006; Bascompte and Jordano 2007; Bascompte 2009). When community composition is geographically structured, coevolution between generalists can underlie increased trait matching, facilitate the exchange of benefits, and drive diversification of the interaction (Medeiros *et al*. 2018). In general, community dynamics can shape coevolutionary outcomes and have important implications for pairwise interactions (Guimarães *et al*. 2011*a*, 2017*a*; Medeiros *et al*. 2018). Considering the community context within which pairwise interactions occur may yield important insights and illuminate the pathways by which horizontally transmitted mutualisms persist.

Diffuse coevolution results through reciprocal evolutionary change between multiple different species with a shared partner (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997; Inouye and Stinchcombe 2011). Coevolutionary change in these interactions results when pleiotropic genes in the shared partner influence its interactions with multiple mutualistic

species, coupling their evolutionary trajectories (Inouye and Stinchcombe 2011; Ossler and Heath 2018). Under diffuse coevolution, pairwise interactions will not exhibit specialization. Instead, the shared partner will exhibit generalist dynamics with its range of partners (Hougen-Eitzman and Rausher 1994). Diffuse coevolution may play an important role in maintaining horizontally transmitted mutualisms by allowing a diverse assemblage of hosts to maintain constant selection on a shared symbiont. Selection from multiple hosts may maintain selection for cooperative symbiont traits while limiting opportunities for a symbiont to evolve exploitative traits toward a single host. Moreover, diffuse coevolution with multiple hosts may benefit symbionts by increasing opportunities for transmission. In general, few studies have tested for diffuse coevolution across symbiotic mutualisms.

Evaluating the role of coevolution in mutualism is important for determining how these interactions evolve and persist. A focus of mutualism research should be evaluation of the roles of both pairwise and diffuse coevolution within and across these interactions. In general, empirical work must aim to provide evidence for specific coevolutionary pathways and determine the underlying evolutionary genetics mechanisms underlying host-symbiont mutualisms. Developing this mechanistic framework may provide key insights into the pathways by which horizontally transmitted mutualisms persist.

Genetic variation in mutualism: the potential benefits of horizontal transmission

The maintenance of cooperation within mutualisms likely depends on genetic compatibility between host and symbiont. Many mutualisms are characterized by genetic specificity, such that specific combinations of host and symbiont genotypes yield higher fitness outcomes than alternative genotypic combinations (Simms 2002; Mueller *et al*. 2004; Heath and Tiffin 2007; Bever *et al*. 2009*a*; Murfin *et al*. 2015). As a result, theory suggests that mutualism should favor

reductions in genotypic and phenotypic variation as compatible host and symbiont genotypes rise to fixation through positive frequency-dependent selection (Parker 1999; Yoder and Nuismer 2010). However, despite these predictions, mutualisms are often characterized by substantial phenotypic and genetic variation (Simonsen and Stinchcombe 2014; Murfin *et al*. 2015; Chong and Moran 2016; Harrison *et al*. 2017*b*). Horizontal transmission is often responsible for introducing and maintaining variation across mutualisms.

I hypothesize that contrary to classical theoretical predictions, the variation maintained by horizontal transmission may sometimes beneficially contribute to the persistence of mutualistic interactions. First, horizontal transmission may allow hosts and symbionts to escape exploitative interactions by increasing opportunities for partner switching and horizontal gene transfer. Hosts and symbionts that have the ability to abandon exploitative interactions for more favorable ones can maintain higher fitness compared to those dependent on specific partner genotypes (Noe and Hammerstein 1994; Sachs *et al*.). Moreover, opportunities for symbionts to evolve exploitative traits may generally be minimized if symbionts have fewer opportunities to become highly adapted to a single host through repeated interactions with a single host lineage. Horizontal transmission may also increase opportunities for horizontal gene transfer that may facilitate rapid adaptation of symbionts in response to an exploitative host (similar to the role of sexual recombination in host-parasite interactions).

Horizontal transmission also reduces the probability of symbiont population bottlenecks and genome degradation, relative to symbiont populations that are vertically transmitted (Rispe and Moran; Pettersson and Berg 2007; Bennett and Moran 2015). This can have important implications for both host and symbiont fitness. First, population bottlenecks that lead to deleterious mutations through Muller's ratchet can lead to the loss of essential functions

important for both hosts and symbionts (Bennett and Moran 2015). Second, reductions in standing genetic variation within symbiont populations can reduce the efficacy of selection on these populations (O'Fallon 2008). These symbionts may be less effective at responding to selection outside of the mutualisms. Vertical transmission that reduces genetic variation in symbiont populations can then have deleterious consequences for both host and symbiont if environmental conditions shift, and symbionts cannot adapt to provide their hosts environmentally relevant fitness benefits (Wernegreen 2012). In general, horizontal transmission maintains genetic variation in symbiont populations, eliminates the risk of genome degradation, and increases opportunities for selection from the environment to maintain traits relevant for host and symbiont fitness.

Study System

The squash bug *Anasa tristis* is an agricultural pest of cucurbit crops, such as squash and zucchini (Bonjour *et al*. 1990; Pair *et al*. 2004). Their native range includes Central America, the United States, and southern Canada (Beard 1940). The native range of *A. tristis* overlaps with two sister species, *Anasa scorbutica* and *Anasa andresii*, which are also major pests of cucurbit crops (Jones 1916; Brailovsky 2001). *Anasa* nymphs of each species develop through five instar stages before reaching adulthood. Squash bugs of each species harbor *Caballeronia* spp. (formerly called *Burkholderia* spp. and reclassified in 2020, but still a member of the *Burkholderiaceae* family) in a special region of the gut, referred to as the crypt. Research using *A. tristis* nymphs indicates that squash bugs environmentally acquire *Caballeronia* spp. during the second instar development stage (Acevedo *et al*. 2021). The bacteria provide squash bugs with substantial fitness benefits, including increased development rate and survival to adulthood (Acevedo *et al*. 2021).

Empirical evidence demonstrates *Caballeronia* symbionts are not passaged directly from parent to offspring through traditional vertical transmission pathways. Instead, tracking of *Caballeronia* symbionts indicates potential for symbiont transmission between co-localized individuals via the environment (Acevedo et al. 2021*a*). The exact mechanism for symbiont transmission remains unknown but likely involves passaging through a shared environmental source, such as on or through plants. Growth within the crypt benefits *Caballeronia* spp. symbionts by allowing them to grow to high titers relatively free from competition. Symbionts can be cultivated on standard laboratory media and fluorescently labeled using genomic integration. This feature makes this system ideal for laboratory manipulation. The geographic range of the interaction between *A. tristis* with *Caballeronia* spp. makes it ideal to test for evidence of pairwise coevolution using local adaptation assays. The overlapping host species range makes it ideal for testing for evidence of host-species specialization and diffuse coevolution. Throughout this dissertation, I leverage the key features of this system to assess the consequences of horizontal transmission and the pathways by which horizontally transmitted mutualisms persist.

Summary of dissertation chapters

In Chapter II, I review current evolutionary theory for the maintenance of mutualistic interactions. I explain the limitations of current theory for evaluating the causes and consequences of phenotypic and genetic variation within and across mutualistic interactions. I examine the roles of phenotypic and genetic variation across mutualisms and draw inferences from current empirical and theoretical work. I specifically draw attention to two fundamental processes responsible for maintaining variation across mutualistic interactions: horizontal transmission and sexual recombination. I suggest evolutionary genetics approaches capable of considering the variation introduced by these processes as a pathway forwarded for establishing

an underlying mechanistic framework underlying the persistence of host-symbiont mutualisms. This work was published in *Journal of Evolutionary Biology* in 2020 in an article entitled "A need to consider the evolutionary genetics of host-symbiont mutualisms."

In Chapter III, I test for evidence of coevolution between insect hosts and their bacterial symbionts. Specifically, I test for evidence of coevolution within and across host species. To test for evidence of coevolution within host species, I measure local adaptation between the squash bug host *A. tristis* and its bacterial symbiont *Caballeronia* spp.. I collect hosts and their associated bacterial symbionts from across their native geographic range, perform reciprocal inoculations, and analyze the resulting fitness outcomes for each pairwise combination. To test for evidence of coevolution across species, I perform reciprocal inoculations to test for specialization between three closely related species of insect, *A. tristis*, *A. andresii*, and *A. scorbutica*, with their respective *Caballeronia* spp. symbionts. I find no evidence for specialization with or across host species. Instead, these interactions are characterized by generalist interactions between host species with their shared bacterial symbionts, which are likely under selection for fixed phenotypic traits. These results indicate a potentially important role for diffuse coevolution.

In Chapter IV, I directly test whether symbiont transmission environment alters the direction of selection on symbionts for cooperative traits. I experimentally evolve a *Paraburkholderia* symbiont of *A. tristis* hosts through several selection environments. Symbionts are passaged directly through *A. tristis* hosts, between *A. tristis* hosts and soil, through non-sterile soil, or through standard lab media. I find that passaging symbionts through hosts rapidly evolve deleterious traits affecting host survival. In contrast, while bacteria that are passaged through soil decrease in their benefit to hosts, these deleterious traits evolve at a slower rare. The rapid loss of

cooperative traits across host-evolved bacteria likely results through one of two mechanisms. First, selection within the host environment may bottleneck symbiont populations, leading to the loss of important cooperative traits. Alternatively, passage through hosts may select for exploitative symbiont traits by increasing opportunities for symbionts to adapt to their hosts. In general, these results indicate the potential for horizontal transmission to maintain cooperative symbiont traits, contrary to current evolutionary theory.

My dissertation examines the persistence of horizontally transmitted mutualisms. I consider the causes and consequences of genetic and phenotypic variation introduced by horizontal transmission, evaluate the role of coevolution, and directly test the implications of symbiont transmission mode using naturally occurring host-symbiont interactions. My dissertation highlights the need to develop a coevolutionary framework for studying horizontally transmitted mutualisms and indicates a potentially important role for diffuse coevolution. Contrary to expectations, I find that selection within vertical transmission can yield more antagonistic outcomes for an insect-bacterial mutualism than environmental symbiont acquisition. In general, I do not find evidence that the costs of horizontal transmission substantially outweigh those associated with vertical transmission within the mutualism between *Anasa* insect hosts and their horizontally transmitted symbionts. My work suggests that despite theory, horizontally transmitted mutualisms are here to stay.

CHAPTER II

A NEED TO CONSIDER THE EVOLUTIONARY GENETICS OF HOST-SYMBIONT MUTUALISMS

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Abstract

Despite the ubiquity and importance of mutualistic interactions, we know little about the evolutionary genetics underlying their long-term persistence. As in antagonistic interactions, mutualistic symbioses are characterized by substantial levels of phenotypic and genetic diversity. In contrast to antagonistic interactions, however, we, by and large, do not understand how this variation arises, how it is maintained, nor its implications for future evolutionary change. Currently, we rely on phenotypic models to address the persistence of mutualistic symbioses, but the success of an interaction almost certainly depends heavily on genetic interactions. In this review, we argue that evolutionary genetic models could provide a framework for understanding the causes and consequences of diversity and why selection may favor processes that maintain variation in mutualistic interactions.

Introduction

Host-symbiont mutualisms are long-term, intimate associations between two or more species that provide each other reciprocal fitness advantages. These associations often play a critical role in the function and development of both hosts and symbionts (Clay 1988; Sprent and Sprent 1990; Baumann *et al*. 1995*a*; Margulis 1996; Mcfall-Ngai 1999; Dethlefsen *et al*. 2007). Despite the

importance and ubiquity of mutualisms, much remains unknown about their evolutionary genetics (Heath and Stinchcombe 2014). The persistence of mutualistic interactions likely depends strongly on the genetic compatibility of hosts and symbiont lineages. Processes that maintain variation, such as horizontal transmission and sexual recombination, should reduce the probability that compatible interactions are maintained. Despite this, these processes are pervasive, and many mutualistic host and symbiont populations are characterized by substantial phenotypic and genetic variation (Simonsen and Stinchcombe 2014; Murfin *et al*. 2015; Chong and Moran 2016; Harrison *et al*. 2017*b*). How variation arises and its implications for the persistence of mutualism have yet to be elucidated (Heath and Stinchcombe 2014).

Theory has produced several models for the maintenance of mutualisms (Box 1) (Axelrod and Hamilton 1981; Noe and Hammerstein 1994; Nuismer and Doebeli 2004). According to these models, high fitness interactions persist when there is tight control over exploitation, such that species choose cooperative partners (partner choice), harmful partners receive fewer benefits through feedbacks (partner fidelity feedback), or partners withhold benefits after receiving a signal that cheating may occur (host sanctions) (Weyl *et al*. 2010). Maintenance of the interaction depends on the phenotypic outcome, such that only interactions that confer the highest fitness for one for both partners persist. We therefore expect to observe little phenotypic and genetic heterogeneity in a given mutualism: cooperative partner genotypes are favored and increase in frequency, and uncooperative partner genotypes are disfavored and decrease in frequency. Many mutualistic interactions, however, are characterized by substantial phenotypic and genetic variation, and this variation is often maintained by horizontal transmission and sexual recombination (Moran and Dunbar 2006; Kikuchi et al. 2011*b*; Simonsen and

Stinchcombe 2014; Salem et al. 2015; Chong and Moran 2016; Harrison et al. 2017*b*). Addressing the underlying evolutionary genetics of mutualisms may help us to understand the prevalence of these processes that maintain genetic variation and their consequences for the evolution of mutualism.

Box 1: Models for the maintenance of mutualism

Under the field's current theoretical framework, the outcome of mutualistic interactions relies solely on the distribution of fitness benefits and does not consider the underlying genetic factors mediating the interaction. A successful interaction results in increased fitness of each partner relative to their free-living state. An exploitative interaction results in decreased fitness for either partner relative to its free-living state. Individuals may respond to exploitation by withholding benefits from cheaters or choosing new cooperative partners. We outline the three most prominent phenotypic models of mutualism here.

Partner choice

Under this model, individuals will choose to interact with a partner that provides the greatest fitness benefits (Noe and Hammerstein 1994). Empirical work suggests that partner choice mediates the initiation and maintenance of many horizontally transmitted mutualisms (Mueller *et al*. 2004; Nyholm and McFall-Ngai 2004; Sachs *et al*. 2004; Heath and Tiffin 2009; Heath 2010; Murfin *et al*. 2015). For example, the interaction between the Hawaiian bobtail squid and its bacterial symbiont *Vibrio fischeri* is thought to be maintained through partner choice. Each generation, the squid initiates the interaction by only allowing specific strains of *Vibrio fischeri* to colonize its light organ (McFall-Ngai and Ruby 1991). The squid expels most of the *V. fischeri* from its light organ each morning, and the remaining bacteria subsequently repopulate the organ (Lee and Ruby 1994*c*; Ruby 1996) over the course of the day. Empirical evidence suggests hosts select only luminescent bacteria to remain in the light organ (Visick *et al*. 2000).

Partner-fidelity feedback

Under this model, partners interact repeatedly, and feedback from prior interactions ensures that fitness benefits are reciprocal (Sachs *et al*. 2004). An individual will decide whether to cheat or

cooperate based on the actions of its partner in the previous generation (Axelrod and Hamilton 1981; Doebeli and Knowlton 1998; Sachs *et al*. 2004). If the partner cheats, the individual will not reciprocate benefits. Thus, over multiple interactions, the net benefit of cooperation outweighs the net benefit of cheating.

The model of partner fidelity feedback is often applied to the relationship between the pea aphid *Acythrosiphon pisum* and its vertically transmitted bacterial symbiont *Buchnera aphidicola*. Aphids depend on *Buchnera* for the production of essential amino acids absent in their diet. In exchange, aphids provide *Buchnera* with inorganic amino acids. The fitness interests of each partner have become so tightly aligned that neither can survive without the other (Baumann *et al*. 1995*b*; Fisher *et al*. 2017). *Buchnera* that replicate to high titers decrease host reproduction, thus limiting their own transmission and replication (Chong and Moran 2016). Therefore, symbionts that decrease host fitness reduce their own fitness in turn. These dynamics are consistent with partner-fidelity feedback.

Host Sanctions

Under host sanctions, an organism receives a signal that cheating may occur and withholds benefits from its partner (Weyl *et al*. 2010). Mutualisms that utilize host sanctions maintain evolutionary stability by punishing exploitative partners. Host sanctions are thought to stabilize the interaction between yuccas and yucca moths (Pellmyr and Huth 1994). Yuccas and yucca moths rely on one another for reproduction: yucca moths pollinate yuccas and lay their offspring in yucca flowers to consume the developing fruit. Under non-exploitative conditions, yucca moth offspring consume only a small portion of the developing fruit. However, yucca moths sometimes exploit yuccas by increasing egg burden and failing to pollinate flowers. Yuccas prevent exploitation by selectively aborting flowers with high egg burdens and low pollination. In theory, this sanctioning stabilizes the interaction by selecting for high quality non-exploitative partners.

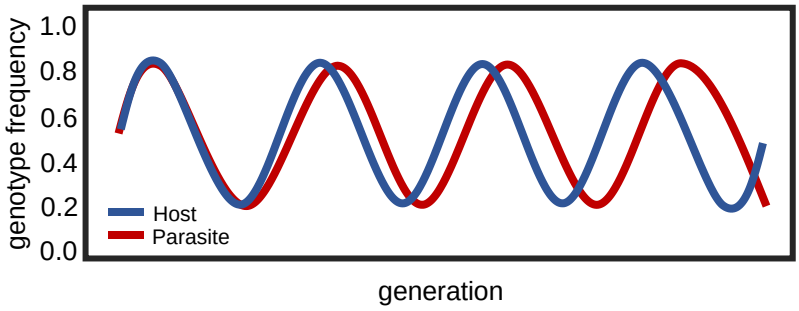
In contrast to host-symbiont mutualisms, a large amount of effort has gone into evaluating the evolutionary genetics of host-parasite interactions. While there are several theoretical approaches for evaluating coevolution (Nuismer 2017), these interactions have largely been studied using population genetics models, particularly matching alleles and gene-for-gene models (Box 2) (Flor 1956; Parker 1994; Carius *et al*. 2001; Agrawal and Lively 2002, 2003; Thrall and Burdon 2002). Using these evolutionary genetic models, we have gained an understanding of the maintenance of genetic variation in host and parasite populations and the implications of this variation for the outcome of antagonistic interactions (Mode 1958; Hamilton *et al*. 1990; Frank 1993; Parker 1994; Otto and Michalakis 1998; Thrall and Burdon 2002; Thrall *et al*. 2012). These models have provided a coherent framework for empirical work that has aimed to assess the evolutionary dynamics of natural host-parasite interactions (Flor 1956; Burdon 1987; Dybdahl and Lively 1998; Burdon *et al*. 1999; Carius *et al*. 2001; Luijckx *et al*. 2013). Furthermore, some studies have begun to demonstrate the value of such models for understanding the coevolutionary dynamics underlying mutualisms (Gomulkiewicz *et al*. 2003; Nuismer *et al*. 2003; Brandvain *et al*. 2011; Drown *et al*. 2013). Evolutionary genetics models like these can serve as a broad framework for future studies evaluating the causes and consequences of genetic variation in mutualism.

Box 2. Evolutionary genetic models for host-parasite and host-symbiont interactions

Coevolutionary dynamics can be evaluated using several theoretical frameworks. Evolutionary genetic models, including matching alleles and gene-for-gene, have been particularly useful to account for the immense phenotypic and genetic variation in populations of hosts and parasites (Thompson and Burdon 1992; Frank 1993; Agrawal and Lively 2002, 2003; Thrall and Burdon 2002). We propose modifying these models to explore the evolutionary genetics of host-symbiont mutualisms.

A brief overview of host-parasite coevolution

Some evolutionary genetic models, such as matching alleles, predict that coevolution between hosts and virulent parasites can result in fluctuating selection that favors the maintenance of genetic variation in host populations. This model is based upon the idea of self-nonself recognition in the host. Hosts are characterized by the alleles they carry at a set of loci. Infection results if a parasite carries alleles that "match" those of the host it encounters: the host fails to recognize the parasite as non-self and does not mount a defense response. Parasites that do not match host alleles are detected and attacked, resulting in a failed infection. Under this model, parasites adapt to infect common host genotypes, and rare host genotypes evade infection, gaining a fitness advantage. As depicted below, this frequency-dependent selection gives rise to oscillations in host genotypes, maintaining genetic variation in host populations at equilibrium (Hamilton *et al*. 1990).

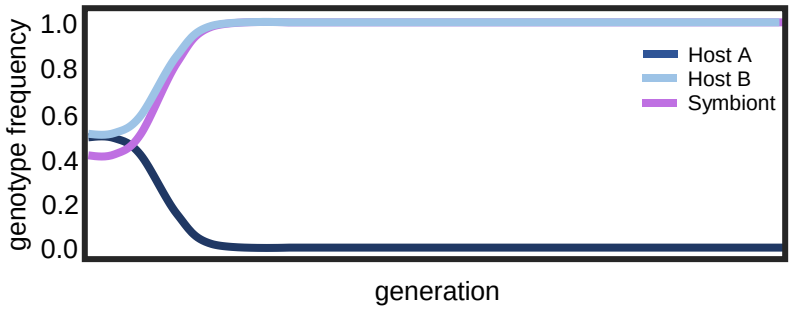


Coevolution with parasite can result in negative frequency-dependent selection on host populations. Parasites adapt to infect common host genotypes, giving rare host genotypes an advantage. These infection genetics lead to oscillations in host and parasite genotype frequencies.

The gene-for-gene model of infection has primarily been used to explain the dynamics of plant-pathogen systems (Flor 1956). This model differs from matching alleles in two ways: patterns of recognition and universal infectivity of parasites. Here, hosts must recognize parasite surface proteins to avoid infection. Hosts with susceptible (r) alleles can be infected by parasites carrying either virulence (V) or avirulence (v) alleles. Hosts with resistant (R) alleles recognize parasites with avirulence alleles and mount an immune response. These hosts fail to recognize virulence alleles and so can be infected by parasites harboring virulence alleles. The recognition patterns of gene-for-gene models allow certain parasite genotypes to be universally infective against all host genotypes, a pattern that does not exist for matching alleles. When hosts incur costs for resistance and parasites experience costs for infectivity, this model predicts negative frequency-dependent selection, similar to matching alleles (Agrawal and Lively 2002). If hosts and parasites do not experience costs, universally infective parasites and resistant hosts are expected to become fixed in the population.

Host-symbiont mutualisms

While negative frequency-dependent selection can maintain variation in antagonistic interactions, it is likely that different dynamics maintain variation in mutualisms. Under many conditions, evolutionary genetic models may predict that host-symbiont mutualism leads to the fixation of cooperative host and symbiont genotypes through positive frequency-dependent selection (Parker 1999).



Evolutionary genetic models may predict that cooperative host and symbiont genotypes gain a fitness advantage and go to fixation through positive frequency-dependent selection. For example, the symbiont "matches" and thus can associate with Host **B**, increasing the fitness of Host **B** relative to that of Host **A.** As a result, Host **B** goes to fixation.

While positive frequency-dependent selection may characterize some mutualistic interactions, positive frequency-dependent selection cannot explain the vast genetic and phenotypic variation observed in mutualisms in nature, suggesting a need to carefully modify evolutionary genetic models to account for the dynamics observed in nature.

Here, we present the need to evaluate the evolutionary genetics underlying the persistence of mutualistic interactions. We begin by discussing the genetic diversity observed in mutualisms, specifically highlighting two ancient and stable symbiotic associations as examples. We then provide an overview of current models of host-symbiont mutualisms and use these models to draw inferences about the implications of variation for the persistence of mutualism. Drawing from these models and empirical work, we discuss conditions under which selection may favor processes, like horizontal transmission and sexual recombination, that increase genetic variation. Finally, we highlight the use of evolutionary genetic models for mutualistic interactions and suggest expanding upon such models as an approach to evaluate the genetic variation and persistence of mutualisms observed in nature (Box 3).

Box 3. Building evolutionary genetic models for mutualism: Questions to consider

Below we outline questions specific to host-symbiont mutualisms that may provide a framework for future evolutionary genetic models.

How do novel host-symbiont mutualisms establish?

- Does the establishment of a mutualism require genetic specificity between host and symbiont?
- Does genetic variation in host and symbiont populations affect the establishment of novel mutualisms?

- How does gene flow between populations of hosts or symbionts affect the probability of establishing novel mutualisms?
- How does sexual recombination or horizontal gene transfer in host or symbiont populations affect the probability of establishing cooperative interactions?

- Does coevolution facilitate the establishment of novel mutualisms?
- Do chance encounters between compatible host and symbiont genotypes underlie the establishment of novel mutualisms?
- Do compatible cooperative genotypes rapidly rise to fixation?

How do host-symbiont mutualisms persist?

- How frequently and under what conditions are mutualisms characterized by positive frequency-dependent selection?
- Why are sexual recombination, horizontal gene transfer, and horizontal transmission common in mutualistic species?
    - What is the role of exploitation?
    - What is the role of spatial or temporal environmental variation?
    - What effect do population bottlenecks have?
- How do gene flow and spatial structure contribute to the persistence of mutualism?
- Does selection from the mutualism always supersede selection from the environment?
- Does coevolution underlie the persistence of mutualism?
    - What genetic dynamics characterize mutualistic coevolution?
    - Do coevolutionary genetic dynamics differ from those in host-parasite interactions?
- How do interactions within an ecological community alter pairwise coevolutionary dynamics?
    - Is coevolution within communities truly ‘diffuse’?
    - What are the genetic dynamics that underlie ‘diffuse’ coevolution?
    - How does indirect selection on pairwise mutualisms affect their long-term persistence?

Important consideration

Host-symbiont mutualisms may rely on interactions at multiple loci underlying infectivity, within host replication, and the exchange of benefits between partners. Antagonistic interactions often do not depend on an exchange of products between partners. As a result, mutualistic interactions may generally rely on interactions at more loci than host-parasite interactions. Basic evolutionary genetic models may not accurately characterize interactions dependent on many loci. More empirical work is necessary to determine the evolutionary genetics that underlie mutualisms. If mutualistic coevolution is dependent on interactions at many loci, then quantitative genetics models may prove a more useful tool.

Genetic variation in mutualistic interactions

Phenotypic and genetic diversity are pervasive across mutualisms, including those that are horizontally and vertically transmitted (Figure 1) (Pellmyr and Huth 1994; Nishiguchi 2002; Sicard *et al*. 2005; Russell and Moran 2006; Mikheyev *et al*. 2007; Poulsen *et al*. 2009; Barrett *et al*. 2012; The Human Microbiome Project 2012; Boutin *et al*. 2014; Chavez-dozal *et al*. 2014; Murfin *et al*. 2015; Bayliss *et al*. 2019). While a substantial number of mutualisms are characterized by genetic variation, we choose to draw attention to two well-studied and ancient interactions. Specifically, we highlight the variation present in the interactions between leguminous plants and their horizontally acquired rhizobial symbionts and between pea aphids and their vertically transmitted bacterial symbionts. By discussing these mutualisms, we hope to demonstrate that variation is maintained even in stable, obligate associations and across multiple transmission modes.

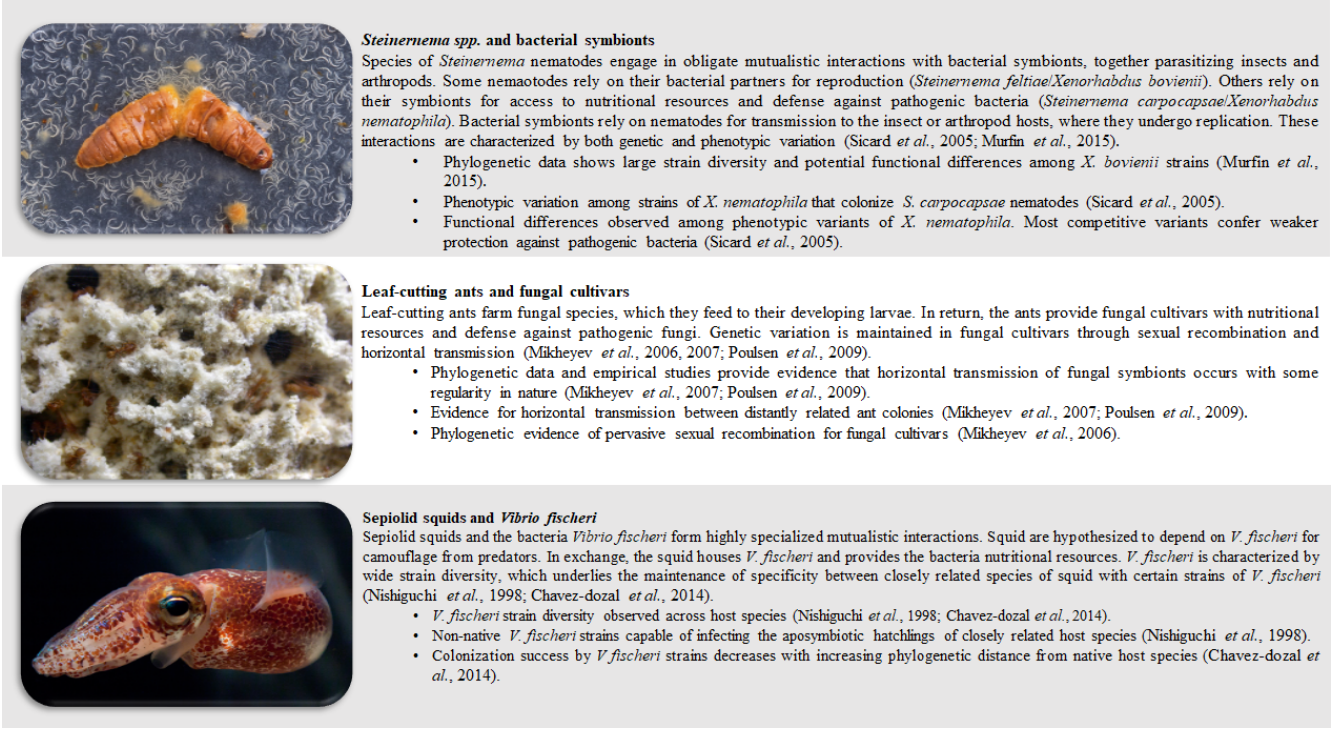


Figure 1. Key systems highlighting the phenotypic and genetic variation present within mutualistic interactions.

The genetic variation present in horizontally transmitted mutualistic interactions is particularly apparent in studies evaluating the initiation of mutualism through partner choice. The initiation of mutualism refers to the association of hosts and horizontally transmitted symbionts at the start of each new generation. Work in extant mutualisms provides evidence for a genetic basis to the ability of hosts to differentiate between beneficial and non-beneficial microbial symbionts during initiation (Mueller *et al*. 2004; Heath and Tiffin 2009; Heath 2010; Murfin *et al*. 2015). For example, rhizobial mutualists provide their leguminous host plants with fixed nitrogen in exchange for carbon compounds. Cross-inoculations between twelve families of *Medicago truncalata* with three strains of its rhizobial mutualist *Sinorhizobium meliloti* indicate plant genotypes express differential preference for specific rhizobium strains, and these specific associations lead to increased fitness benefits for host and symbiont (Heath and Tiffin 2009). The differential fitness outcomes resulting from cross-infections between distinct genetic

backgrounds indicate that genetic variation underlies the phenotypic variation observed in nature and has important implications for the persistence of the interaction.

Genetic and phenotypic diversity has also been observed in obligate mutualisms maintained through vertical transmission. For example, the pea aphid *Acyrthosiphon pisum* requires its intracellular bacterial symbiont *Buchnera aphidocola* for the production of essential amino acids lacking in its diet, and, in exchange, provides *Buchnera* with non-essential amino acids (Wilson *et al*. 2010; Hansen and Moran 2011; Price *et al*. 2011). Despite the obligate nature of the interaction for both partners, phenotypic variation in host control over symbiont replication has been observed. A genetic basis to this variation was revealed by performing crosses between host lineages to produce a range of host-symbiont genotype combinations (Chong and Moran 2016). Host fitness and symbiont titer exhibited significant variation across host-symbiont pairs. Furthermore, increased symbiont titer correlated with decreased host fecundity. Both the conflicting fitness interests of host and symbiont and the variation observed in the interaction suggest the potential for antagonistic coevolution. The interactions between aphids and their microbial symbionts demonstrate that phenotypic and genetic variation are prevalent in vertically transmitted mutualisms, even among the most obligate of these interactions.

The implications of genetic variation: insights from current models of host-symbiont mutualisms

A considerable amount of theoretical work has aimed to understand the dynamics underlying the long-term persistence of mutualistic interactions. Together, these models provide insights into the conditions under which the maintenance of genetic variation may be favored or disfavored.

Here, we highlight current theoretical work in mutualism and draw from this work to make inferences about the implications of horizontal transmission, sexual recombination, and horizontal gene transfer for the persistence of mutualism.

Much of our understanding of mutualism comes from game theory models, including models that predict mutualisms are stabilized through partner choice, partner-fidelity feedback and host sanctions (Box 1) (Axelrod and Hamilton 1981; Noe and Hammerstein 1994; Sachs *et al*. 2004; Weyl *et al*. 2010). These models predict the evolution and persistence of mutualisms by weighing the relative costs and benefits of initiating and sustaining interactions with a given partner. The fitness optimum of a mutualism is achieved by forming and maintaining interactions with partners that contribute the highest fitness benefits, thus favoring the maintenance of specific combinations of host and symbiont. The phenotypic dynamics predicted by these models are consistent with those expected under positive frequency-dependent selection (Parker 1999). Here, genotypic combinations of host and symbiont that achieve the greatest fitness go to fixation, while host or symbiont genotypes that provide few benefits are disfavored by selection and lost. As a result, genotypic diversity should decrease as those host and symbiont genotype combinations conferring high fitness increase in frequency. If host populations are characterized by low levels of genetic diversity, we may expect to observe selective sweeps of beneficial symbiont genotypes and the decline of symbiont genetic diversity (Parker 1999). When mutualisms evolve in genetically diverse host populations, we may expect to observe reductions in host diversity as beneficial symbionts adapt to common hosts, increasing the relative fitness of symbiont-associated host genotypes.

Additional inferences about the role of genetic variation in host-symbiont mutualisms can be made using the game theory model developed by Bergstrom & Lachmann (2003), which considers the effect of each partner's rate of evolution on benefit exchange. This model predicts that the slower evolving partner population obtains the highest benefits in mutualistic interactions. This occurs because the faster evolving partner evolves in response to the "demands" of the slower evolving partner, which less readily changes evolutionary trajectories. Because reductions in genetic diversity can reduce rates of evolution, we may then expect partners from populations with reduced genetic variation to achieve relatively high benefits. However, the persistence of the mutualism may depend on the maintenance of genetic variation in the faster evolving partner population, which would promote adaptation to its genetically homogenous partner. This prediction is consistent with evolutionary genetic models that predict host-symbiont coevolution is stabilized by asymmetric gene flow between host and symbiont populations (Gomulkiewicz *et al*. 2000).

Game theory models have laid a useful foundation for understanding the persistence of mutualisms given the costs and rewards associated with an interaction. In these models, partner quality is bimodally distributed, such that an individual can choose between high- and low-quality partners (Heath and Stinchcombe 2014). This distribution in partner quality underestimates the variation observed in nature (Heath and Stinchcombe 2014; Archetti 2019). Screening models provide some solutions to this problem: they allow for an individual to select from a range of potential partners (Archetti 2011, 2019; Archetti and Scheuring 2011). In these models, hosts are uninformed of potential symbiont quality but force potential symbionts that vary in quality to screen themselves based on a set of costs and rewards offered by the host

(Archetti 2011, 2019; Archetti and Scheuring 2011). Ultimately, higher quality partners receive higher rewards at a higher cost while low-quality partners receive lower rewards at lower costs. Similar to game theory models, early iterations of these models predict that high quality partners attain higher fitness than lower quality partners and go to fixation, thus maintaining little variation in symbiont populations (Archetti 2011; Archetti and Scheuring 2011).

A screening model developed by Archetti (2019), however, assumes hosts offer fewer rewards to high quality symbionts when few low-quality symbionts are present and that hosts occasionally make mistakes by offering low-quality symbionts high rewards. Under these conditions, variation in symbiont quality is maintained through frequency-dependent selection because the presence of partners of low and high quality increases the overall benefits provided to symbionts of both quality types. Screening models like this one provide a useful step forward by demonstrating the conditions under which the distribution of costs and rewards in a mutualism can favor the maintenance of variation.

The distribution of benefits between partners likely depends on several factors, such as the underlying genetics and selection at multiple levels (i.e. on the host, on the symbiont, and on the interaction). Further insights can be gained from studies that consider how additional biologically relevant factors affect the costs and benefits of mutualisms. For example, several studies have used simulations to consider the influence of mutation accumulation on the fitness outcomes for host and symbiont (Rispe and Moran; Pettersson and Berg 2007; O'Fallon 2008). Symbiont populations often undergo bottlenecks, becoming vulnerable to genetic drift that makes selection less effective at removing deleterious mutations from these populations. The

accumulation of deleterious mutations through Muller's ratchet can lead to symbiont genome degradation (Bennett and Moran 2015; Wernegreen 2017), potentially yielding negative consequences for both host and symbiont if symbionts lose genes for essential functions. Genome degradation is especially common in heritable symbioses, where bacterial populations undergo large bottlenecks and opportunities for horizontal gene transfer are severely limited (Moran *et al*. 2009; Wilson *et al*. 2010; Bennett and Moran 2015; Wernegreen 2017). Rispe and Moran (2000) and Petterson and Berg (2007) show that increasing the number of transmitted symbionts within and across hosts decreases the accumulation of deleterious mutations through Muller's ratchet. This occurs as transmission of larger numbers of symbionts increases the probability that symbiont variation is preserved within and across host lineages.

Similarly, O'Fallon (2008) shows that maintaining genetic variation through horizontal transmission stabilizes host-symbiont mutualisms by improving the efficacy of selection on symbiont populations (O'Fallon 2008). Specifically, when symbiont populations maintain intermediate levels of horizontal transmission, symbionts accrue fewer deleterious mutations through Muller's ratchet. Moreover, this model finds that maintaining moderate levels of genetic variation in symbiont populations through horizontal transmission does not reduce host fitness. As a result, host and symbiont populations that preserve pathways for horizontal transmission and maintain genetic variation may gain a selective advantage over those without these pathways.

Finally, some studies have employed evolutionary genetic models, primarily matching alleles, to evaluate the coevolutionary dynamics between hosts and symbionts. These models have

provided valuable insights into a number of processes and are particularly useful to evaluate the maintenance of genetic variation in the light of reciprocal selection between hosts and symbionts. For example, Brandvain *et al.*, (2011) used a matching alleles framework to evaluate the role of transmission mode in the persistence of mutualistic interactions. Long-term mutualistic interactions can give rise to genetic covariation, the nonrandom association of genomes, which is analogous to linkage disequilibrium between alleles in a single genome (Wade and Goodnight 2006). Genetic covariation between interacting loci of host and symbiont genomes is theorized to play an important role in the persistence of mutualism by reducing intergenomic conflict between mutualists (Wade and Goodnight 2006). Brandvain *et al*. (2011) show that genetic covariation depends on consistent associations of host and symbiont genotypes across generations (i.e. vertical transmission). Even infrequent horizontal transmission can completely disrupt genetic covariation. Similar to early models and consistent with models of virulence evolution, this evolutionary genetic model suggests that selection under mutualism results in low levels of genetic variation that maintain specific combinations of host and symbiont genotypes.

The propensity for exploitation in mutualisms could maintain genetic variation through dynamics similar to those observed in antagonistic interactions (Herre *et al*. 1999). Exploitation occurs when “cheater” symbiont genotypes over-utilize host resources, reducing the fitness benefits provided to the host relative to non-cheater genotypes (Jones *et al*. 2015). An important distinction between exploitation and parasitism is that hosts still gain an overall fitness benefit through association with symbionts relative to hosts devoid of symbionts. However, exploited host genotypes experience reduced fitness relative to non-exploited hosts. This may then allow for alternative host genotypes to gain a fitness advantage.

Studies that use a matching alleles framework indicate that the prevalence of exploitation affects the maintenance of genetic variation in host and symbiont populations (Gomulkiewicz *et al*. 2003; Nuismer *et al*. 2003). For example, Gomulkiewicz *et al*. (2003) show that low genetic variation and high trait matching (i.e. compatibility of host and symbiont phenotypes and genotypes) are maintained with gene flow between consistently mutualistic populations (Gomulkiewicz *et al*. 2003). Genetic variation increases, and trait matching decreases, however, when the nature of the interaction varies from antagonistic to mutualistic across connected populations, even with low levels of gene flow from antagonistic to mutualistic populations. A high prevalence of antagonism can even lead to cyclic coevolutionary dynamics between mutualists. Genetic variation may also be favored if the nature of interspecific fitness interactions varies temporally from mutualistic to antagonistic (Nuismer *et al*. 2003).

Taken together we see that: 1) consistently mutualistic interactions are theorized to favor reduced genetic variation and trait matching (Gomulkiewicz *et al*. 2000, 2003); 2) bottlenecks reduce genetic variation and suppress population sizes, which can relax selection and can drive mutation accumulation in symbiont populations (O'Fallon 2008; Wernegreen 2012); and 3) genetic variation in mutualism is theorized to increase when the interaction varies spatially or temporally from mutualistic to antagonistic (Gomulkiewicz *et al*. 2003; Nuismer *et al*. 2003). Given the observed genetic and phenotypic diversity in mutualistic interactions, we might infer that costs of exploitation and mutation accumulation in host and symbiont populations favor the maintenance of genetic variation. Therefore, processes that break up genotypic combinations and increase genetic variation, such as horizontal transmission and sexual recombination, may in fact be

important for the persistence of mutualisms. Below, we explore the implications of maintaining genetic variation through horizontal transmission and sexual recombination.

The maintenance of genetic variation through horizontal transmission

Much of our understanding of the role of transmission mode in symbiotic interactions comes from host-parasite interactions. Epidemiological models predict transmission mode drives virulence evolution. Vertical transmission is predicted to select for reduced parasite virulence due to positive covariance of host and parasite fitness (Ewald 1987; Bull 1994; Frank 1996). Horizontal transmission, however, relaxes the dependence of parasite transmission on host survival. The covariance of host and parasite fitness may then become negative, selecting for increased within-host replication and thus increased virulence of parasites (Anderson and May 1982; Ewald 1987; Bull 1994; Frank 1996).

In addition, for mutualisms, horizontal transmission may limit the maintenance of compatible host and symbiont genotypes. Hosts required to acquire symbionts from the environment each generation are likely to select symbionts from a genetically diverse pool (Stougaard 2000; Knowlton and Rohwer 2003; Radutoiu *et al*. 2003; Blanquer *et al*. 2013). Such hosts may acquire symbiont genotypes different from those of their parents. If beneficial interactions require high levels of genetic specificity between partners, this may result in deleterious fitness consequences and the potential breakdown of the interaction. These observations and theoretical predictions suggest that mutualism should select for limited horizontal transmission in order to maintain beneficial interactions.

Despite these predictions, horizontal transmission is ubiquitous in both plant and animal mutualisms (Simms 2002; Nyholm and McFall-Ngai 2004; Bolker *et al*. 2009; Salem *et al*. 2015; Chrostek *et al*. 2017; Hartmann *et al*. 2017). For example, many species of stinkbugs depend on environmentally acquired *Burkholderia* bacteria for development, survival, and insecticide resistance (Kikuchi *et al*. 2007, 2009, 2012*a*). Phylogenetic analysis indicates the relationship between stinkbugs and *Burkholderia* is "ancient and promiscuous": conspecific stinkbugs may associated with different *Burkholderia* genotypes, while heterospecific stinkbugs may associated with the same bacterial genotypes (Kikuchi *et al*. 2011). Similarly, some plants receive benefits from horizontally acquired mycorrhizal fungi. These fungal mutualists provide plants with many benefits, including improved nutrient uptake and defense against pathogens (Wilkinson 1997). In exchange, plants provide their fungal mutualists with carbon compounds (Wilkinson 1997). The relationship between plants and their mychorrizal fungi has been maintained for millions of years by horizontal symbiont transmission. The persistence of mutualisms, like these, challenge current evolutionary theory.

Given its ubiquity, it is feasible that under certain circumstances horizontal transmission may be favored in order to maintain genetic variation in symbiont populations. Theoretical work highlighted above shows that the maintenance of genetic variation can stabilize mutualisms by increasing the efficacy of selection on symbiont populations and limiting the potential for exploitation between partners (Rispe and Moran; Gomulkiewicz *et al*. 2003; Nuismer *et al*. 2003; Pettersson and Berg 2007; O'Fallon 2008). Therefore, horizontal transmission may present a

long-term benefit for mutualisms, in contrast to the short-term costs predicted by virulence evolution models.

Empirical work in host-pathogen interactions also provides direct support for the potential of horizontal transmission to reduce the accumulation of deleterious mutations in microbial populations (Elena *et al*. 2001). Virulence attenuation in vertically transmitted RNA viruses results from the accumulation of deleterious mutations. In contrast, horizontally transmitted viruses undergoing similar bottlenecks accumulate fewer mutations and experience higher fitness. Empirical work in both facultative and obligate heritable mutualisms have suggested similar benefits (Oliver *et al*. 2010; Mondo *et al*. 2012; Naito and Pawlowska 2016). For example, the facultative, heritable association between an arbuscular mycorrhizal fungus from the Glomeromycota phylum and its bacterial symbiont *Ca.* Glomeribacter gigasporum is an ancient association that seems to have been stabilized through occasional horizontal transmission (Mondo *et al*. 2012; Naito and Pawlowska 2016). The maintenance of horizontal transmission is hypothesized to increase the probability of gene transfer between bacterial symbionts, thus reducing mutation accumulation and genome degradation.

In contrast with predictions of virulence evolution models, horizontal transmission may also minimize exploitation of hosts and symbiont. Exploitation of both hosts and symbionts is often observed in mutualistic interactions (Bronstein 2001; Sørensen *et al*. 2019). Left unchecked, exploitative behaviors may lead to transitions from mutualism toward parasitism (Pellmyr *et al*. 1996; Machado *et al*. 2001; Sachs and Simms 2006). Tightly coupled genetic interactions provide the opportunity for one partner to evolve to better exploit the other (Garamszegi 2006;

Agudelo-Romero and Elena 2008; Leggett *et al*. 2013). Partners that maintain genetic "independence" through horizontal transmission may be relatively protected from exploitation. Moreover, organisms that maintain pathways for horizontal transmission may experience strong selection for partner choice mechanisms that reduce the probability of selecting exploitative partners from the environment (Shapiro and Turner 2014). Therefore, horizontal transmission may allow individuals to take advantage of the genetic variation in their symbiont populations and increase the probability of forming new, less exploitative interactions. For example, the maintenance of horizontal transmission is predicted to allow arbuscular mychorrizal fungal hosts to abandon costly interactions and establish new cooperative interactions with their bacterial symbiont *Ca.* Glomeribacter gigasporum under temporally variable environments (Mondo *et al*. 2012; Naito and Pawlowska 2016).

Spatial structure in host and symbiont populations may also maintain genetic covariance of host and symbiont genotypes under horizontal transmission (Wilkinson 2001). For example, plants typically disperse seeds over short geographic distances, making them likely to interact with the same genetic lineages of mycorrhizae as their parents (Wilkinson 1997). Repeated interactions between specific lineages of hosts and symbionts may facilitate cooperation by giving rise to adaptive responses between partners, similar to those observed under vertical transmission (Wilkinson 1997, 2001). However, spatial structure could also limit the local variation required for cooperation to evolve through partner choice (Akçay 2017), suggesting a need for some mechanism promoting gene flow.

Finally, horizontal transmission may allow symbiont populations to respond to external selection pressures outside of the mutualism. For example, experimental evolution between the nematode

Steinernema carpocapsae and its mutualistic bacteria *Xenhorhabdus nematophila* demonstrates that selection from within the interaction can supersede selection on symbionts from external forces (Morran *et al*. 2016). The inability of symbionts to adapt to external environmental pressures can present substantial costs for both hosts and symbionts. These costs are demonstrated by insects, including stinkbugs, weevils, and aphids, whose responses to thermal stresses are limited by obligate bacterial symbionts whose depleted genomes leave them unable to adapt to heat stress (Wernegreen 2012). When insects are placed in hot environments, bacterial symbionts are lost, leading to a decline in host fitness. Given these costs, horizontal transmission may allow symbiont populations to maintain genetic variation, facilitating the persistence of mutualism by eliminating constraints on symbiont adaptation.

The maintenance of genetic variation through sexual recombination and horizontal gene transfer

The role of sexual recombination in mutualism has received little attention. In contrast, a plethora of theoretical and empirical studies have addressed the role of sexual recombination in host-parasite interactions. According to these studies, sexual recombination in hosts populations increases the probability that host offspring will evade parasitism (Hamilton *et al*. 1990; Lively and Howard 1994; Dybdahl and Lively 1998; Morran *et al*. 2009). This is based upon the matching alleles model, in which parasites must genetically match hosts to form a successful infection. Under this model, parasites are under selection to infect common host genotypes. Sexual recombination can produce offspring with rare genotypes, which parasites are less likely

to match and infect. Empirical evidence supports these predictions (Dybdahl and Lively 1998; Morran *et al*. 2011).

Historically, it has been predicted that sex should disrupt mutualisms by continually generating rare combinations of alleles with which symbionts are poorly adapted to interact. The dynamics observed in host-parasite interactions led Graham Bell to argue that the Red Queen hypothesis predicts mutualists should reproduce asexually (Graham Bell 1982). However, sexual recombination is common across the tree of life, including in organisms that form mutualisms. The potential for exploitation between host and symbiont may favor sexual reproduction in host or symbiont populations (Herre *et al*. 1999). If highly adapted cheater genotypes better exploit their partners, then rare partner genotypes may gain an advantage because cheaters are less effective at exploiting them. As a result, exploitation may confer a fitness advantage to partners that reproduce sexually or maintain horizontal gene transfer, promoting the maintenance of genetic variation.

Sexual recombination may be particularly advantageous in obligate mutualisms. Because obligate mutualists require their partners for fitness, they cannot easily abandon interactions that become exploitative. The dependence of each partner on the other for fitness may increase the severity of exploitation, favoring sexual recombination. However, the positive covariation between host and symbiont fitness in heritable obligate mutualisms is also likely to reduce the prevalence and intensity of exploitation in these interactions (Trivers 1971; Axelrod and Hamilton 1981; Frank 1996; Herre *et al*. 1999).

Hosts may exploit their symbionts by reducing symbiont replication and resource utilization (Nakajima *et al*. 2013; Lowe *et al*. 2016). The establishment of novel mutualisms may even rely on exploitation, with hosts forcing free-living bacteria to cooperate (Law and Dieckmann 1998; Sørensen *et al*. 2019). Furthermore, some hosts maintain control over their symbionts by sequestering them in specialized cells or organs (Funk *et al*. 2001; Kikuchi *et al*. 2009). Sequestration in these specialized structures often bottlenecks symbiont populations, limiting the potential for genetic exchange through horizontal gene transfer. Population bottlenecks and restricted horizontal gene transfer can result in mutation accumulation and genomic decay (Kikuchi *et al*. 2009; Burke and Moran 2011; McCutcheon and Moran 2012). Given these costs, the maintenance of sexual recombination or horizontal gene transfer in symbiont populations may provide an evolutionary defense against exploitative hosts.

Hosts themselves also face exploitation, as demonstrated in the obligate interaction between aphids and *Buchnera* discussed previously (Chong and Moran 2016). Observing antagonistic dynamics in even this most obligate of mutualistic interactions emphasizes the potential significance of selfish and exploitative behaviors for the maintenance of genetic variation in mutualisms. In nature, most aphids alternate between parthenogenic and sexual reproduction (Trionnaire *et al*. 2008). Reproduction is parthenogenic during the spring and summer seasons but switches to sexual reproduction during the fall (Trionnaire *et al*. 2008). It is worth considering whether the maintenance of seasonal sexual reproduction in aphid populations serves to limit exploitation by their obligate bacterial symbiont.

Even in the absence of exploitation, sexual reproduction could confer an advantage in mutualistic interactions. For hosts, sexual reproduction may promote sexual transmission of microbial symbionts. Host offspring that vertically inherit microbial symbionts often acquire their symbionts through maternal transmission. Asexual reproduction then restricts host lineages from acquiring new symbiont strains. In contrast, sexual reproduction may provide opportunities for female lineages to acquire new microbial strains from males during copulation, which may be passed to their offspring. For example, aphids can sexually transmit the heritable, facultative symbionts they rely on for parasite defense and thermal tolerance (Moran and Dunbar 2006). Experimental crosses between males and females harboring different microbial strains demonstrate sexual reproduction can result in the transfer of paternal symbionts to aposymbiotic females, coinfection of paternal and maternal symbionts, and replacement of maternal symbionts by paternal symbionts (Moran and Dunbar 2006). Paternal symbionts can be maternally transmitted to offspring and persist in host lineages for at least ten generations. Sexual symbiont transmission then provides hosts opportunities to acquire symbionts with beneficial traits, such as tolerance to high temperatures and parasitoid defense (Moran and Dunbar 2006; Oliver *et al*. 2008). Furthermore, coinfection of paternal and maternal symbionts allows for horizontal gene transfer, reducing the accumulation of deleterious mutations through Muller's ratchet (Moran and Dunbar 2006; Oliver *et al*. 2008)

Symbionts likely gain analogous advantages from sex and horizontal transmission. The maintenance of recombination or horizontal gene transfer promotes the purging of deleterious mutations from symbiont genomes (Mikheyev *et al*. 2006; O'Fallon 2008; Oliver *et al*. 2010; Mondo *et al*. 2012). For example, phylogenetic evidence suggests that sexual recombination is

maintained in fungal cultivars farmed by leaf-cutting ants (Mikheyev *et al*. 2006). Sex is hypothesized to maintain genetic variation in fungal lineages, allowing selection to purge deleterious mutations (Mikheyev *et al*. 2006; O'Fallon 2008). Given that bottlenecks are a hallmark of many symbiont populations, the maintenance of recombination and horizontal gene transfer may be especially important in their evolution.

Evolutionary genetic models as a tool to assess genetic variation in host-symbiont mutualisms

Theoretical approaches have provided a useful foundation for the evaluation of evolutionary genetics in host-parasite interactions (Box 2). Specifically, evolutionary genetic models have revealed the role of host-parasite coevolution in producing the vast phenotypic and genetic diversity observed in nature. Here, we propose evolutionary genetic models as a valuable theoretical framework to explore the evolutionary genetic dynamics underlying mutualistic interactions.

Evolutionary genetic models have already begun to improve our understanding of the evolutionary trajectories of mutualism given the genetic contributions of each partner, the genetic composition of host and symbiont populations, the effects of gene flow between populations, and the effect of the temporal and spatial variation in fitness interactions (Gomulkiewicz *et al*. 2000, 2003; Nuismer *et al*. 2003; Brandvain *et al*. 2011). These models provide a solid foundation from which future theoretical and empirical work can build to produce general evolutionary genetic principles for both the establishment and persistence of host-symbiont mutualisms. Specifically, this theoretical framework may provide a powerful tool to

address outstanding questions related to the maintenance of genetic variation in host-symbiont mutualisms discussed above, including the roles of horizontal transmission and sexual recombination (Box 3).

While further application of these foundational evolutionary genetics models to the study of beneficial symbioses could provide important insights into the evolution of mutualisms, it is also important to note the possible limitations of these models in characterizing both host-parasite and host-symbiont interactions. These models assume coevolutionary dynamics are mediated by genetic specificity between hosts and symbionts at few genes or loci. However, coevolutionary outcomes are likely mediated by the genetic variation and specificity across multiple loci of host and symbiont genomes (MacPherson *et al*. 2018). Furthermore, evolutionary genetic models often assume pairwise coevolutionary dynamics. We must also consider that interactions between members of an ecological community may alter pairwise coevolution (Guimarães *et al*. 2011*b*, 2017*b*; Nuismer *et al*. 2012; Dáttilo *et al*. 2013; Betts *et al*. 2016; Akçay 2017). Considering community ecology may be important for mutualism because both host and symbiont are likely to interact with species outside of the mutualism. For example, interactions with generalists may stabilize pairwise coevolution in temporally and spatially variable environments (Guimarães *et al*. 2011*b*; Dáttilo *et al*. 2013). Furthermore, pairwise dynamics may be altered if the traits of interacting species evolve in response to the indirect effects of coevolutionary interactions between other members of the ecological community (Guimarães *et al*. 2017). Accordingly, theoretical and experimental approaches should together continue to explore more complex approaches.

Conclusion

As a field, researchers have done extensive work to understand the phenotypic implications of beneficial interactions and to elucidate the genes mediating specific mutualistic interactions. However, unlike for host-parasite interactions, we still lack a basic framework for the evolutionary genetic dynamics underlying the persistence of mutualisms. Future work should focus on improving our understanding of the role of genetics in mediating the success of mutualistic symbioses. Specifically, assessing the genetic dynamics of mutualism may improve our understanding of why the processes of horizontal transmission and sexual recombination are pervasive across mutualisms. Including evolutionary genetic models as a foundational starting point, and coupling this work with empirical approaches, will improve our understanding of the variation observed in mutualisms.

CHAPTER III

EVALUATING COEVOLUTION IN A HORIZONTALLY TRANSMITTED MUTUALISM

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Submitted

Abstract

Many interspecific interactions are shaped by coevolution. Transmission mode is thought to influence opportunities for coevolution within symbiotic interactions. Vertical transmission maintains partner fidelity, increasing opportunities for coevolution, but horizontal transmission may disrupt partner fidelity, potentially reducing opportunities for coevolution. Despite these predictions, the role of coevolution in the maintenance of horizontally transmitted symbioses is unclear. Leveraging a tractable bug-bacteria symbiosis, we tested for signatures of pairwise and diffuse coevolution by assessing patterns of host-symbiont specialization. If pairwise coevolution defines the interaction, we expected to observe evidence of reciprocal specialization between hosts and their local symbionts. We found no evidence for local adaptation between sympatric lineages of *Anasa tristis* squash bugs and *Caballeronia* spp. symbionts across their native geographic range. We also found no evidence for specialization between three co-localized *Anasa* host species and their native *Caballeronia* symbionts. Our results demonstrate generalist dynamics underlie the interaction between *Anasa* insect hosts and their *Caballeronia* symbionts. We predict that selection from multiple host species may favor generalist symbiont traits through diffuse coevolution. Alternatively, selection for generalist traits may be a consequence of selection by hosts for fixed cooperative symbiont traits without coevolution.

Introduction

Hosts across all domains of life form essential mutualistic interactions with microbial symbionts. Mutualistic microbes fulfill a variety of integral functions for host development and survival, including augmenting nutrition, providing defense against pathogens, and facilitating development (Pais *et al*. 2008; Boucias *et al*. 2012; Salem *et al*. 2013; Masson *et al*. 2015; Vorburger and Perlman 2018; Gerardo *et al*. 2020; Kaltenpoth and Flórez 2020). Likewise, symbionts may depend on hosts for nutrition, replication without competition, and transmission (Lee & Ruby, 1994; Prell *et al*.2009; Macdonald *et al*.2012; Wollenberg & Ruby, 2012; but see also Garcia & Gerardo, 2014). The persistence of mutualism requires strong selection for the maintenance of cooperative traits and inhibition of exploitation (Trivers 1971; Axelrod and Hamilton 1981; Noe and Hammerstein 1994; Sachs *et al*. 2004). This may most effectively be accomplished when species have opportunities for coevolution, reciprocal evolutionary change driven by natural selection (Thompson 2005).

Coevolution plays an important role in both antagonistic and mutualistic interspecific interactions (Ehrlich and Raven 1964; Janzen 1966; Thompson 1994, 2005; Dybdahl and Lively 1998; Moran 2001; Currie *et al*. 2003; Decaestecker *et al*. 2007; Wilson and Duncan 2015). Symbiont transmission mode is thought to influence opportunities for pairwise coevolution across symbioses. The direct passage of symbionts from parent to offspring through vertical transmission increases the likelihood for tight, pairwise coevolution because partner fidelity between specific host and symbiont lineages is conserved through time. Across vertically transmitted mutualisms, reciprocal genomic and phenotypic changes underlying transmission and fitness are often observed, demonstrating evidence for pairwise coevolution (Riegler *et al*. 2004; Toft and Andersson 2010; Ilinsky 2013; Wilson and Duncan 2015; Lee *et al*. 2020). In contrast,

the acquisition of microbial symbionts from the environment through horizontal transmission can reduce partner fidelity between host and symbiont lineages, potentially decreasing opportunities for pairwise coevolution. As a result, pairwise coevolution is generally considered to play a larger role in symbioses maintained by vertical transmission than horizontal transmission. However, while generally accepted as a canonical characteristic of symbiosis, little empirical work has demonstrated that rates of pairwise coevolution truly vary across vertically and horizontally transmitted mutualisms.

Whether rates of pairwise coevolution covary with symbiont transmission mode is unknown, in part, due to the paucity of empirical studies directly testing for evidence of pairwise coevolution in horizontally transmitted mutualisms. Horizontally transmitted mutualisms are frequently characterized by obligacy and phylogenetic co-diversification, both of which can be signatures of coevolution (Lee and Ruby 1994*b*; Aanen *et al*. 2002; Garcia-Cuetos *et al*. 2005; Brucker and Bordenstein 2012; Wang *et al*. 2012; Murfin *et al*. 2015; Parker *et al*. 2017; Forsman *et al*. 2020). However, a key feature of pairwise coevolution is reciprocal evolutionary change of both host and symbiont due to reciprocal selection. Few empirical studies have experimentally tested whether hosts and their symbionts are reciprocally adapted to one another. Such tests are necessary to determine whether an association is shaped by pairwise coevolution, as opposed to evolutionary change driven by one-sided selection or correlated change of host and symbiont driven by co-adaptation to a shared environment.

Specialization is a hallmark of coevolution (Janzen 1980; Thompson 1994), and certain patterns of specialization can provide evidence for pairwise coevolution. Specialization consistent with pairwise coevolution may be observed across different levels of the interaction, including within and across species. For example, local adaptation of both host and symbiont requires reciprocal

selection and geographic variation across the range of the interaction (Gandon and Michalakis 2002). As a result, observing local adaptation between host and symbiont lineages within species has generally been considered evidence for reciprocal evolutionary change consistent with pairwise coevolution (Janzen 1980; Kawecki and Ebert 2004; Brockhurst and Koskella 2013). Similarly, evidence for pairwise coevolution across species can be observed by performing reciprocal inoculations to test for specialization between co-localized host species with their 'native' symbiont strains. For mutualistic interactions, specialization consistent with pairwise coevolution most likely results when sympatric or native host-symbiont combinations produce higher fitness interactions for both host and symbiont than those achieved through alternative host-symbiont combinations (Kawecki and Ebert 2004; Hoeksema and Thompson 2007; Barrett *et al*. 2012; Blanquart *et al*. 2013). Although selection for exploitation in symbiont populations could lead to periods of maladaptation. In general, few studies in mutualism have tested for patterns of local specialization of hosts and symbionts (except see Hoeksema and Thompson 2007; Barrett *et al*. 2012; Harrison *et al*. 2017; Caldera *et al*. 2019; Rekret and Maherali 2019). Moreover, studies that have tested for local specialization have primarily taken place within plant-microbe interactions (Hoeksema and Thompson 2007; Barrett *et al*. 2012; Harrison *et al*. 2017*a*; Rekret and Maherali 2019) and often only test for specialization of one partner (except see Hoeksema and Thompson 2007), leaving the question of whether pairwise coevolution generally underlies horizontally transmitted symbiotic mutualisms, especially between animals and microbial symbionts.

Furthermore, there has been a long-recognized need to move beyond pairwise interactions and study mutualistic interactions within a community context (Bronstein *et al*. 2003; Stanton 2003; Thrall *et al*. 2007; Koskella and Bergelson 2020). While recent efforts have improved our

understanding of mutualisms within a community context (Wood *et al*. 2018; Dewald-Wang *et al*. 2022; Meyer *et al*. 2022), generally few empirical studies have considered the implications of community interactions for host-symbiont coevolutionary dynamics. This is despite the fact that there is an extensive body of research examining the coevolutionary community dynamics that shape mutualistic interactions between plants and pollinators.

Research in plant-pollinator interactions has demonstrated that, within communities, mutualistic interactions are stabilized by asymmetric interactions between complex networks of interacting species containing few specialists and a large range of generalists (Bascompte *et al*. 2003, 2013; Nuismer *et al*. 2012). Specialization can increase the rewards partners attain from an interaction (Douglas 1998*a*; Schwartz and Hoeksema 1998) but can also limit the potential partners with which hosts can interact. If environmental change decreases the prevalence of compatible symbiont genotypes, specialized mutualistic interactions may be destabilized leading to their evolutionary breakdown (Futuyma and Moreno 1988; Douglas 1998*b*; Sachs and Simms 2006). Alternatively, when communities are composed of a large number of generalists, which can interact with a range of partners, evolutionary breakdown of mutualistic communities is less likely if a single partner is lost (Bascompte *et al*. 2013). These asymmetric mutualisms driven by generalists are thought to be stabilized by diffuse coevolution, reciprocal selection between a range of partners with a shared, common symbiont (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997), which we hypothesize may also contribute to horizontally transmitted mutualisms.

Under diffuse coevolution, the strength and direction of selection on a common partner is driven by selection from multiple mutualistic partners and/or genetic correlations across these interactions links their evolutionary trajectories. (Hougen-Eitzman and Rausher 1994; Iwao and

Rausher 1997). In mutualism, for example, diffuse coevolution may occur through reciprocal selection for cooperation between a common generalist symbiont with a range of host species. Patterns of specialized versus generalist interactions can provide evidence of the potential for diffuse coevolution within a mutualistic interaction. Under diffuse coevolution, phenotypic variation across pairwise interactions will not be observed (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997; Inouye and Stinchcombe 2011). However, as in plant-pollinator interactions, patterns of asymmetric specialization may be observed.

Here, we tested for evidence of pairwise and diffuse coevolution in a horizontally transmitted mutualism. We leveraged the experimentally tractable interaction between the squash bug *Anasa tristis* and its horizontally transmitted bacterial *Caballeronia* spp. symbionts (Acevedo *et al*. 2021). Squash bugs that harbor *Caballeronia* exhibit increased development and survival rates relative to aposymbiotic squash bugs. *Caballeronia* is harbored in specialized organs, called crypts, where it grows to high titers relatively free from competition. Moreover, *A. tristis* squash bugs overlap in their geographic range with two closely related *Caballeronia*-harboring insects, *A. andresii* and *A. scorbutica*. We assessed these interactions for evidence of pairwise and diffuse coevolution using reciprocal inoculations to test for specialization within and across host species with their respective *Caballeronia* symbionts.

Materials and Methods

Study System

The squash bug *Anasa tristis* is an agricultural pest of cucurbit crops, such as squash and zucchini (Bonjour *et al*. 1990; Pair *et al*. 2004). Their native range includes Central America, the United States, and southern Canada (Beard 1940). Squash bug nymphs develop through five

instar stages before reaching adulthood. During the second instar, squash bugs environmentally acquire the bacterial symbiont *Caballeronia* spp. (formerly called *Burkholderia* spp. and reclassified in 2020, but still a member of the *Burkholderiaceae* family) (Acevedo *et al*. 2021). These bacteria provide squash bugs with substantial fitness benefits, including increased development rate and survival to adulthood. Empirical evidence demonstrates *Caballeronia* symbionts are not passaged directly from parent to offspring through traditional vertical transmission pathways. Instead, tracking of *Caballeronia* symbionts indicates potential for symbiont transmission between co-localized individuals via the environment. The exact mechanism for symbiont transmission remains unknown but likely involves passaging through a shared environmental source, such as on or through plants. Growth within the crypt benefits *Caballeronia* spp. symbionts by allowing them to grow to high titers relatively free from competition. Most often, crypts are colonized by a single cultivable *Caballeronia* spp. strain, but coinfections are occasionally observed (Acevedo *et al*. 2021).

Insect field collections and laboratory preparation

We collected *A. tristis* squash bugs and their associated *Caballeronia* symbionts across three different geographic scales (Figure 1).

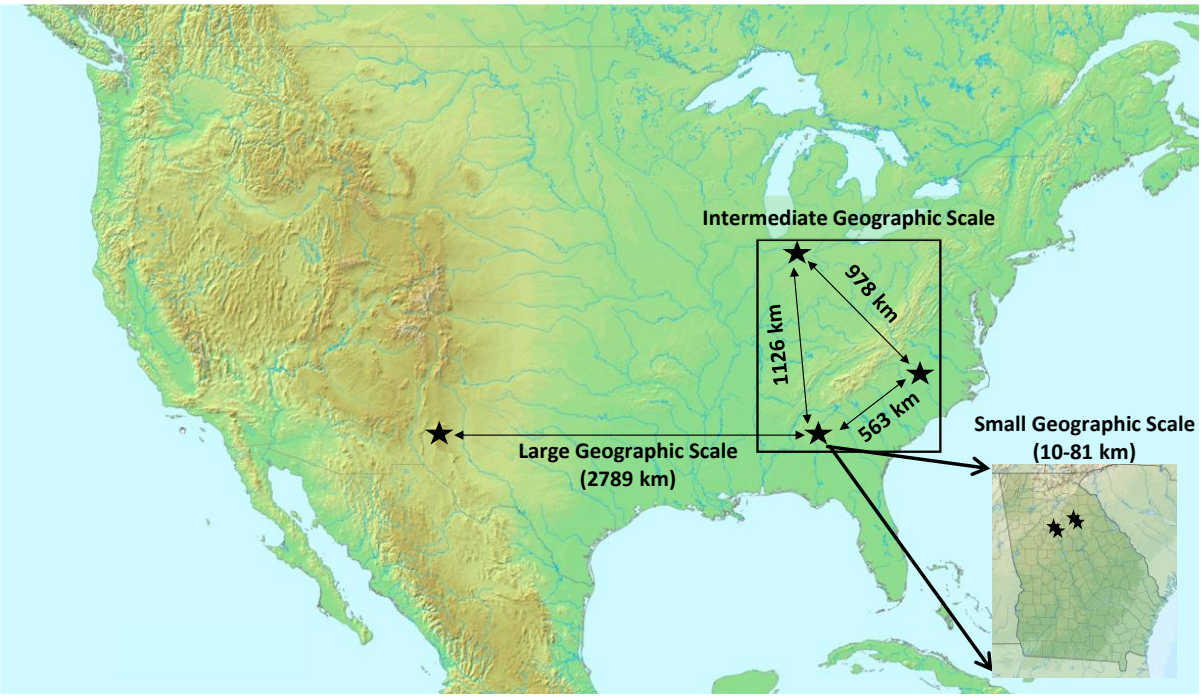


Figure 1. Geographic scales for local adaptation assays. *A. tristis* hosts were collected from sites across three geographic scales: small, medium, and large. At the small geographic scale, bugs were collected from four sites in Georgia, USA. At the intermediate scale, we collected *A. tristis* from three different states: Georgia, Indiana, and North Carolina. At the large spatial scale, Eastern USA *A. tristis* were collected in Georgia sites and Western USA *A. tristis* were collected in Arizona.

At the small geographic scale, we collected *A. tristis* and their associated *Caballeronia* spp. symbionts from four organic farms in Georgia, USA, separated by distances of 10 to 81 km. Field collection sites in Georgia included Woodland Gardens (WG), Front Field Farms (FFF), Crystal Organic Farm (CF), and Oxford Organic Farm (Ox). At the intermediate scale, we collected *A. tristis* and their associated *Caballeronia* spp. symbionts from sites across the United States, including locations in Georgia (GA), North Carolina (NC), and Indiana (IN). Squash bugs were collected from a total of four organic farms and gardens in each state. Distance between states range from 563 km to 1126 km, and distances between collection sites within each state range from 482 m to 97 km. At the large geographic scale, we collected *A. tristis* squash bugs and their associated *Caballeronia* spp. symbionts from the Eastern and Western United States. Western bugs were collected from sites in Arizona, USA, and Eastern bugs were collected from sites in Georgia, USA (2789 km between states). For specialization assays across host species, we collected *A. tristis*, *A. andresii*, and *A. scorubtica* hosts from four sites in Gainesville, Florida separated by a distance of 1.6 to 14.5 km. Following field collections, bugs were returned to the lab, scanned for ectopic parasites, and allocated for use in either the establishment of experimental populations or symbiont isolation. To establish populations, bugs were placed on yellow crookneck squash plants in environmental chambers at 27 °C, 60% humidity, and a 16/8 h day/night cycle. For the local adaptation assays, insects from each collection site were placed separately on two to three plants, each housing two mating pairs each for a total of thirty to fifty bugs per state. For the specialization assays, insects from each species were placed on five plants with conspecifics, each housing three to four mating pairs, for a total of thirty to forty bugs per species.

Genetic analyses of *Anasa tristis*

The population structure of *A. tristis* was assessed using restriction site-associated DNA sequencing (RAD-seq). We extracted DNA from 95 bugs collected from Georgia (n = 50), Indiana (n = 20), and North Carolina (n = 20). DNA was extracted using either Omega Bio-tek E.Z.N.A. Insect DNA kits or Qiagen DNeasy blood and tissue extraction kits. DNA quality and purity was assessed using gel electrophoresis and nanodrop spectrometry. DNA was quantified using a Qubit 2.0 fluorometer. Library construction was performed using an Illumina TruSeq Nano DNA Sample Prep Kit. High throughput sequencing was performed using the Illumina novaseq6000 platform with a read length of 150 bp at each end. Several samples across host populations produced low quality reads that were excluded from analysis. Final bioinformatic analysis included comparisons of 44 GA *A. tristis* bugs and 19 *A. tristis* bugs from both IN and NC. Adapter sequences were trimmed using Cutadapt (Martin 2011). All 82 samples were then mapped against the reference genome using BWA-mem. We used the STACKS pipeline (Catchen *et al*., 2011, 2013) to obtain the vcf file and genome wide Fst (genetic differentiation between populations) and Dxy (absolute genetic divergence) values. We performed principal component analysis (PCA) using the R Bioconductor package 'SNPRelate.' We completed admixture analysis and calculated the cross-validation error rate to determine the best number of populations within the dataset using the R package 'LEA.'

Genetic analyses of *Caballeronia* symbionts

For the *Caballeronia* symbionts, we assessed whether there was genetic variation in the isolates used for experiments and additional isolates from the same populations in three ways. We first used traditional sanger sequencing of 16s rRNA to identify *Caballeronia* symbionts isolated from field collected squash bugs. We then used whole genome sequencing to gain greater insight into the genetic variation present in the symbiont populations. Finally, we used high throughput

16s rRNA sequencing of whole crypt communities to gain greater insight into the distribution of *Caballeronia* variants across individuals and populations.

To isolate individual symbionts for local adaptation assays, we dissected the crypts of one to ten squash bugs per collection site, depending on the availability of field collected bugs from each site, and isolated bacterial symbionts (n = 34 GA bugs + 16 NC bugs + 26 IN bugs = total 76 bug dissections). We also isolated individual symbionts from each host species by dissecting the crypts from bugs that were collected in Gainesville, Florida (n = 44 *A. tristis* bugs + 28 *A. andresii* + 16 *A. scorbutica*). For each crypt, half was placed in 1x phosphate buffered saline (PBS) and homogenized by crushing. The other half was stored in 99% ethanol for later culture-independent assessment of bacterial community composition. Crypts placed in 1x PBS were crushed, dilution plated onto Luria Broth (LB) agar, and grown at 28 °C for 48-72 hours. Resulting colonies that morphologically resembled *Caballeronia* spp. were individually selected from each plate and stored in 15% glycerol at -80 °C. In general, bacteria morphologically similar to *Caballeronia* were the predominant bacteria on the plates.

For sanger sequencing, bacteria stored in glycerol were revived by streaking onto LB agar and grown at 28 °C for 48 hours. DNA from each bacterium was extracted by boiling at 95 °C (Dashti *et al*., 2009). Extracted DNA was amplified by polymerase chain reaction (PCR) using general 16s ribosomal DNA bacterial primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) (Lane 1991). PCR amplifications were performed with an initial four minutes of denaturing at 94 °C, followed by 36 cycles of denaturing for 30 seconds at 94 °C, annealing for 30 seconds at 55 °C, extending for one minute at 72 °C, and a final one-minute extension at 72 °C. PCR products were purified using a Qiagen QIAquick PCR purification kit and protocol. Samples were sanger sequenced, and the resulting sequences were

assembled in DNASTAR SeqMan Pro. Aligned sequences were run through the NCBI NIH BLAST nucleotide database for identification. We identified a total of 26 *Caballeronia* strains from bugs isolated in Georgia, North Carolina, and Indiana (GA = 14; NC = 4; IN = 6). We identified a total of fifteen strains from bugs isolated in Florida (*A. tristis* = 6, *A. scorbutica* = 4, *A. andresii* = 5). Based on results of 16s rRNA sequencing of whole crypt communities (see below), failure to isolate *Caballeronia* strains likely resulted from the limitations of culture-based methods, rather than the absence of *Caballeronia* within squash bug crypts. Sequences identified as *Caballeronia* spp. were aligned using DNASTAR MegAlign Pro. We calculated the percent identity of the sequenced portion of the 16s rRNA gene across strains at each scale. We selected the most genetically dissimilar strains at each geographic scale and from each host species for reciprocal inoculations.

We conducted whole genome sequencing for *Caballeronia* strains isolated from *A. tristis* in Georgia (n = 13 strains), Indiana (n = 6 strains), and North Carolina (n = 4 strains). We also conducted whole genome sequencing for strains isolated from *A. tristis* (n = 6 strains), *A. andresii* (n = 4 strains), and *A. scorbutica* (n = 5 strains) hosts collected in Florida. Bacterial strains were revived from glycerol by streaking onto LB agar and grown for 48 hours at 28 °C. Individual colonies were selected and grown in LB overnight with shaking at 28 °C. DNA was extracted from bacteria in liquid cultures using a Qiagen DNeasy blood and tissue kit. DNA quality was assessed using gel electrophoresis and quantity was measured using a Nanodrop spectrometer. Genomic sequences were obtained using paired-end Illumina Mi-seq whole genome sequencing. Reads were assembled *de novo* using the SPAdes genome assembler, version 3.15.3 (Bankevich *et al*., 2012). Contigs were ordered against a reference genome using Mauve contig mover (Rissman *et al*., 2009). Genome assembly completeness was assessed using

BUSCO (Simão *et al*., 2015). Multiple genome alignment and comparative genome analysis were conducted using anvi'o (Eren *et al*., 2021). We performed pangenome analysis to compare amino acid sequences and gene cluster presence and absence across symbiont strains using the program DIAMOND (Buchfink *et al*., 2014). Evolutionary relationships were estimated using single-copy core gene clusters (homologous single-copy genes present in most bacterial species) for phylgenomic analysis. We assessed genetic variation across genomes by computing the average nucleotide identity across genomes using PyANI (Pritchard *et al*., 2016).

For 16s rRNA-based analyses of entire microbial communities within individuals' crypts, the crypts previously stored in ethanol at -80 °C were thawed and rinsed in 1x PBS. DNA was extracted using the Lucien MasterPure Complete DNA and RNA Purification kit protocol and reagents. DNA was purified using the ZYMO OneStep PCR Inhibitor kit and subsequentially quantified using a Thermo Fisher Scientific NanoDrop One UV Spectrophotometer. Samples for high-throughput sequencing were selected based on successful amplification of the V3-V4 region of 16S rRNA gene via PCR. We used 341f/785r primers, as described in Klindworth *et al*., 2013, for DNA amplification. PCR reagents, protocols, and thermocycling conditions adhered to the New England BioLabs *Taq* PCR kit. PCRs were performed on an Eppendorf Mastercycler. Gel visualizations were run on Agilent's 4200 TapeStation using D1000 Screentape. Samples with clear bands were selected for sequencing. Library preparation and 2 x 300bp paired end Illumnia MiSeq 20K diversity assays were performed by Molecular Research LP (MR DNA) using the Klindworth primers. Raw sequences were downloaded from basespace and demultiplexed into individual fastq files using a FASTQ processor designed by MR DNA. Reads were pre-processed and quality filtered using qiime2 v 2019.7. Computing resources were provided by Cyverse.

To assess variation within and across *A. tristis* crypts, we analyzed the relative abundance of OTUs within the *Burkholderiaceae*. OTU analysis was performed using a sampling depth of 34,993 reads, which included 48% of all reads across 58 *Burkholderiaceae* taxa. We quantified population-specific variation in *Burkholderiaceae* composition using non-metric multidimensional scaling (NMDS). Statistical comparisons of crypt *Burkholderiaceae* communities across host populations were performed using PERMANOVA, and post-hoc analysis was performed using Tukey pairwise analysis. For analysis of the relative abundance of OTUs within the *Burkholderiaceae* across samples, selection of reads was randomized and limited to the 20 most abundant OTUs. Statistical analyses of crypt community composition were performed using the following R packages: 'vegan,' 'phyloseq,' 'pairwiseadonis' and 'qiime2R.'

Experimentally assessing host-symbiont specificity by testing for local adaptation

We established squash bug lineages for local adaptation assays, as described above. Field collected squash bugs reproduced in the environmental chambers, and we performed experiments using the second (F2), third (F3) and fourth (F4) generation progeny to reduce environmental and maternal effects. Reciprocal inoculations at the smallest geographic scale included progeny from the F2 generation, the intermediate scale included a mixture of progeny from the F2 and F3 generations, and the largest scale included a mixture of progeny from the F3 and F4 generations for Eastern *A. tristis* and the F2 progeny for Western *A. tristis*.

We selected a single symbiont strain from each collection site for reciprocal inoculations at the smallest geographic scale (GAWG2-4, GACF4, GAFFF3, and GAOX1). We selected a single strain from each state for reciprocal inoculations at the intermediate scale (GACF4, INML1, and

NCF4) and a single strain for each state at the large geographic scale (GACF4 and AZ1). Strains for the small and intermediate scale were selected as described above, such that reciprocal inoculations were conducted using the most genetically dissimilar strains at each geographic scale.

Prior to inoculations, frozen *Caballeronia* samples were revived as described above. Liquid feeding solutions for symbiont inoculations were prepared as described in Acevedo *et al*. 2021. Specifically, liquid cultures were prepared and incubated overnight at 28 °C with shaking. Overnight cultures were diluted 1:5 in LB and incubated at 28 °C with shaking for two hours. Bacterial feeding solutions (10mL) were prepared by diluting the two-hour liquid cultures with sterile molecular water to ~$2x10^7$ cells/mL. Blue dye (1%) was added to each solution to allow for visual confirmation of the feeding solution in squash bug guts. Feeding solutions were poured into 35mm Petri dishes, and a cotton dental swab was placed in each dish. The dishes were wrapped in parafilm to prevent spilling or squash bug drowning, allowing the bugs to feed from the cotton swab only.

For reciprocal inoculations at the small geographic scale, squash bug eggs from each mating pair for a given collection site were pooled. Eggs were surface sterilized with 70% ethanol and 10% bleach and returned to environmental chambers for about one week, until first instars emerged. Emerging first instar nymphs were fed surface sterilized zucchini or squash wrapped in parafilm. At second instar, nymphs were starved for seven to nine hours. Symbiont inoculations were performed such that squash bugs from each collection site were provided with bacterial feeding solutions from each collection site (*i.e*., one sympatric and three allopatric combinations per host population). After 24 hours, feeding solutions were removed. Thirty nymphs from each treatment were divided into plastic vented containers housing five bugs each and fed pieces zucchini or

squash wrapped in parafilm. Due to limitations in egg production, inoculations using each symbiont strain occurred separately over four consecutive weeks. This procedure was repeated until all symbionts were fed to hosts from each population. In total, hosts from each collection site were inoculated with bacteria from each collection site for a total of four sympatric (n = 30 bugs/treatment x 4 sympatric treatments = 120 bugs receiving sympatric bacteria) and twelve allopatric inoculations (n = 30 bugs/treatment x 12 allopatric treatments = 360 bugs receiving allopatric bacteria). Aposymbiotic controls were established by feeding nymphs water according to the protocol above. Twenty-five hosts from each population were provided water solutions (n = 25 hosts/populations x 4 populations = 100 water control bugs). A small number of bugs died across host populations following water inoculations, and fitness measurements were conducted for 20-25 hosts per population (WG = 20 bugs; FFF = 25 bugs; Ox = 24 bugs; CF = 21 bugs; n = 90 total water control bugs). Developmental stage and survival were assessed every other day until bugs reached adulthood. From these data, we could estimate the number of days each individual spent in each instar and the number of days each individual was alive during each experiment.

Inoculations with each bacterial symbiont were performed asynchronously, which could conflate symbiont effects with replicate effects. To address this limitation, we later tested explicitly for symbiont effects by inoculating nymphs from a single host population with bacteria from all four collection sites on the same day. Tests for symbiont effects were performed for the CF and FFF host populations, which were the only host populations from which enough eggs could be collected. Eggs were sterilized and squash bugs were inoculated according to the procedure described previously. Bacteria from each collection site were fed to 15 to 20 squash bugs from each of the two populations. Host fitness was measured as described above.

Symbiont fitness was measured as the number of colony forming units (CFUs) per adult squash bug crypt. Adult squash bugs were anesthetized using carbon dioxide and surface sterilized in 70% ethanol for five minutes. Crypts were manually dissected, placed in 1x PBS and crushed. The homogenate was then serially diluted. Spread plates were prepared on LB agar and grown at 28 °C for 48 hours. The number of colonies per plate was counted. Observation of multiple colony morphologies was noted, and only colonies with morphological characteristics of *Caballeronia* spp. were counted. Bacteria morphologically typical of *Caballeronia* were the predominant bacteria on the plates.

For reciprocal inoculations at the intermediate geographic scale, eggs from all collection sites for a given state were pooled. Egg sterilization and bacterial inoculations were performed as described above. For each inoculation, second instar nymphs from each state (IN, GA, and NC) were provided bacteria isolated from each state (*i.e.*, one sympatric and two allopatric combinations per inoculation for the intermediate geographic range). Twenty-five nymphs from each treatment were placed in groups of five in plastic vented containers and fed zucchini or squash (total of five containers per treatment). This procedure was repeated three times for full reciprocity for a total of three sympatric combinations (n = 25 bugs/treatment x 3 sympatric treatments = 75 bugs receiving sympatric bacteria) and six allopatric combinations (n = 25 bugs/treatment x 6 allopatric treatments = 150 bugs receiving allopatric bacteria). Aposymbiotic water controls were produced for each host population as described above (n = 15 bugs/populations x 3 populations = 45 water control bugs). Following the establishment of these reciprocal inoculations, the inoculations were replicated a second time so that all host populations were fed bacteria from all collection sites on the same day, allowing distinction between symbiont versus replicate effects. In this case, twenty bugs from each collection site

were inoculated with bacteria from each collection site for a total of three sympatric (n = 20 bugs/treatment x 3 sympatric treatments = 60 bugs receiving sympatric bacteria) and six allopatric inoculations (n = 20 bugs/treatment x 6 allopatric treatments = 120 bugs receiving allopatric bacteria). Across the two replicates, a total of 135 bugs received sympatric bacteria, and 270 bugs received allopatric bacteria. Due to the global COVID-19 pandemic and lab closures, squash bugs from each replicate were removed from environmental chambers and kept at ambient temperature (20 °C) on days 40 and 19 of each replicate experiment, respectively, in order to keep the experiment going. Host fitness was measured over the course of the experiment as described previously. Due to the pandemic and lab closures, we were unable to assess symbiont fitness for reciprocal inoculations at the intermediate geographic scale.

Reciprocal inoculations at the large geographic scale were performed using *A. tristis* collected from Arizona and Georgia. Egg collection, sterilization, and feeding solutions were prepared as described previously. Bugs from each state were provided bacteria from each state for a total of two sympatric (n = 30 bugs/treatment x 2 sympatric treatments = 60 bugs receiving sympatric bacteria) and two allopatric treatments (n = 30 bugs/treatment x 2 sympatric treatments = 60 bugs receiving allopatric bacteria). Inoculated bugs were placed in groups of five in plastic vented containers and fed zucchini or yellow crookneck squash. Host survival and developmental stage were recorded as described previously. Symbiont fitness was measured at this geographic scale as described for the small geographic scale.

Testing for specialization between host species and *Caballeronia* spp. strains

The native geographic range of *A. tristis* overlaps with two closely related *Caballeornia*-harboring sister species, *A. andresii* and *A. scorbutica* (Acevedo *et al*. 2021). In the field, these species are observed inhabiting the same plants, indicating the potential for symbiont sharing

between species. We tested for specialization between these overlapping species with their respective *Caballeronia* spp. strains by performing reciprocal inoculations.

Field collected squash bugs were used to establish laboratory colonies and bacterial strains for reciprocal inoculations, as described previously. We randomly selected a single bacterial strain isolated from each host species for reciprocal inoculations: AAF181, ATF2731, and ASM285. Reciprocal inoculations were performed so that each host species was inoculated with bacteria isolated from each host species for all possible combinations of host and bacteria (*i.e.*, one conspecific-derived symbiont and two heterospecific-derived symbionts for each host species x three host species = three conspecific-derived symbiont inoculations and six heterospecific-derived inoculations). For *A. tristis*, we inoculated a total of 25 bugs per treatment (n = 25 bugs receiving conspecific-derived symbionts + 50 bugs receiving heterospecific-derived symbionts = 75 bugs). For *A. andresii*, we inoculated a total of 25 bugs per treatment (n = 25 bugs receiving conspecific-derived symbionts + 50 bugs receiving heterospecific-derived symbionts = 75 bugs). For *A. scorbutica*, we inoculated a total of 20 bugs per treatment (n = 20 bugs receiving conspecific-derived symbionts + 40 bugs receiving heterospecific-derived symbionts = 60 bugs). We prepared bacterial feeding solutions and measured host and symbiont fitness as described for the local adaptation assays.

Statistical analysis for reciprocal inoculations

For reciprocal inoculations within and across species, we performed cox proportional hazard models using the R package ‘survival’ to test whether host development rate to adult varied in response to the following main effects: host origin, symbiont origin, and an interaction between host and symbiont origin. We chose to perform this analysis for the rate to the adult stage because variation across treatments was greatest at this stage, and bugs reach reproductive

maturity at adult, making the rate to adult the best measure of host fitness. We considered reaching adulthood as an event, and bugs that died before reaching adulthood were censored. We also performed cox proportional hazard models to test whether host survival varied based on the following main effects included in the model: host origin, symbiont origin, and an interaction between host and symbiont origins. Death was considered an event, and bugs were censored once reaching adult. Occasionally bugs were accidentally killed while collecting data, and these bugs were censored. For reciprocal inoculations testing for local adaptation at the intermediate geographic scale, we repeated reciprocal inoculations two times, and the effect of inoculation block was included as a fixed effect for both survival and development rate analyses. If we detected an interaction between host and symbiont origin for survival or development rate to adult, we performed contrasts using the R package 'emmeans' to test for an effect of sympatric versus allopatric combinations of host and symbiont.

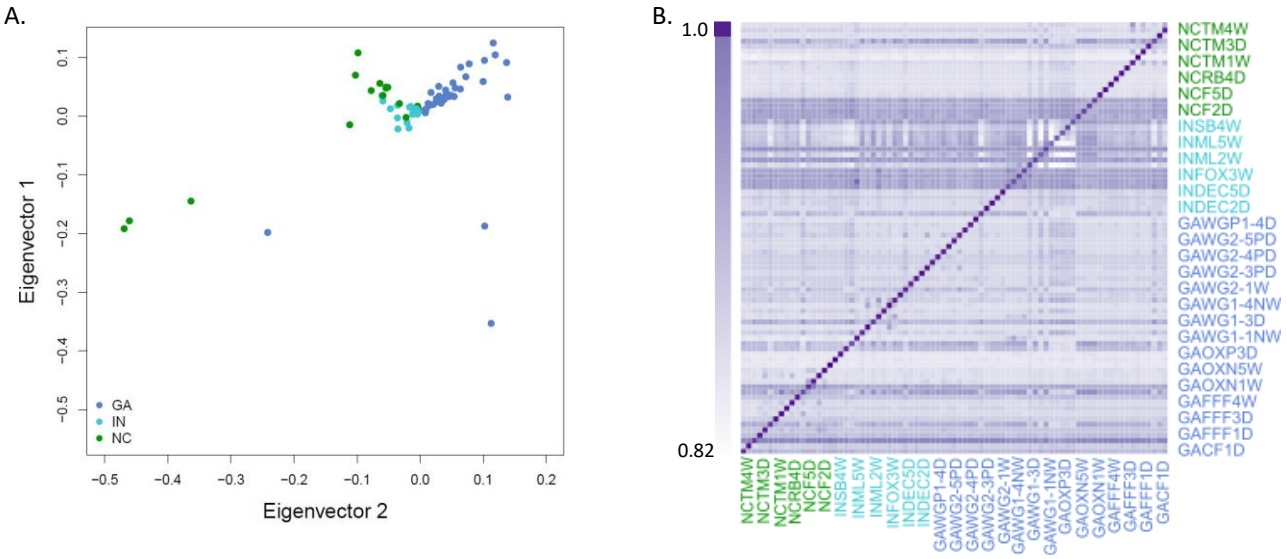
For analysis both within and across species, we further assessed host survival using a Chi-square test to assess whether the proportion of hosts surviving to adulthood varied across sympatric versus allopatric combinations of host and symbiont. We used a quasipoisson distributed generalized linear model to test whether symbiont fitness (logCFU/crypt) varied in response to the following main effects: host origin, symbiont origin, or an interaction between host and symbiont origins. Across all tests, water was excluded from models comparing the effect of host and symbiont origin on partner fitness, and we compared the effect of receiving a symbiont versus water treatment on host development rate and survival using the same statistical tests mentioned above. Models were fit using R, version 4.1.0.

Results

Geographic genetic variation is not observed across *A. tristis* host populations

We performed genetic analysis to test for spatial structure and underlying genetic variation across *A. tristis* host populations at the intermediate geographic scale based on RADseq data. We did not observe genetic spatial structure across *A. tristis* host populations. Pairwise Fst (genetic differentiation between populations) was low across all pairwise combinations (GA-NC = 0.03, GA-IN = 0.03, and IN-NC = 0.05). Likewise, Dxy (absolute genetic divergence) was low across all pairwise combinations (GA-NC = 0.0047, GA-IN = 0.0050, IN-NC = 0.0051). Moreover, we observed no pattern of population-specific clustering in the PCA (Figure 2A), indicating *A. tristis* hosts form a single population.

Figure 2. *A. tristis* host population structure. (A) PCA plot demonstrating genomic variation across the three insect populations at the intermediate geographic scale: GA (purple), IN (blue), NC (green). We do not observe a clear pattern of population-specific clustering, indicating all hosts belong to a single population. (B) Heatmap showing genetic identity across all *A. tristis* samples. Percent genetic identity was high across all samples, ranging from 0.82 to 1.0, and there is no clear pattern of population-specific genetic similarity across these host populations.



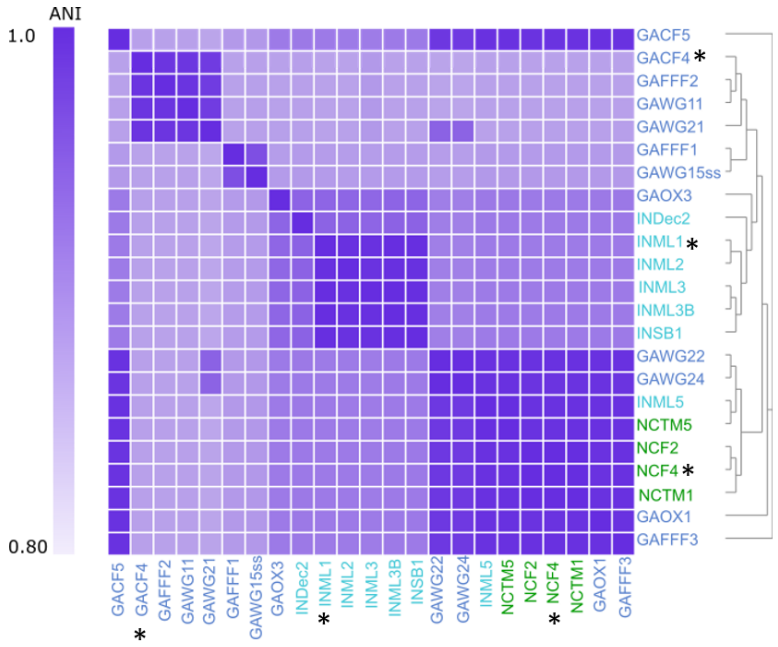
The lack of spatial structure across *A. tristis* populations suggest local adaptation as an unlikely outcome. However, the loci that underlie the symbiotic interaction between *A. tristis* and its *Caballeronia* symbiont are unknown. While we did not observe spatial structure or genetic

divergence across host populations overall, this does not preclude the potential for variation across loci relevant to the mutualism, leaving local adaptation a possible outcome.

Geographic genetic variation is observed across *Caballeronia* symbiont strains

Geographic variation was observed across symbiont strains. Based on analyses of whole genome sequence data, we observed little within-population variation for symbionts isolated from IN and NC (ANI ~0.99; Figure 3). In contrast, we observed higher genetic variation within the GA symbiont population (ANI = 0.82-0.99; Figure 3). In general, symbionts isolated from NC and IN shared high average nucleotide identity with each other (ANI = 0.90 – 0.99; Figure 3, S1). On average, strains from GA shared higher average nucleotide identity with strains from NC than IN, but average nucleotide identity across all pairwise combinations of NC and GA strains varied broadly (ANI = 0.82 – 0.99; Figures 3, S1). This variation was driven by several GA strains that were genetically diverged from all other strains, forming their own monophyletic clade (Figure 3).

Figure 3. Heat map showing the genetic distance across symbiont strains isolated from *A. tristis* from GA (purple), IN (blue), and NC (green). Genetic distance was calculated using average nucleotide identity (ANI), which is a measure of the nucleotide similarity across coding regions. ANI varied from 0.82 to 1.0, with the largest difference resulting when comparing IN and GA symbionts. Evolutionary relationships (far right) between strains were assessed using phylogenomics. Strains GACF4, NCF4, and INML1 (asterisks) were used for experimental reciprocal inoculations.



We further assessed geographic symbiont strain variation using 16s rRNA-based community analysis, which allows for comparison of both cultivable and uncultivable symbiont strains within the host crypts. We observed a similar effect of geographic origin on crypt *Burkholderiaceae* community composition (richness and evenness) ($F_{2,36} = 3.17$, $p = 0.003$; Figures 4, S3) as we did for whole genome-based analyses. Specifically, the *Burkholderiaceae* community composition of crypts isolated from NC and IN bugs were more similar than those isolated from GA bugs ($F_{1,37} = 2.41$, $p = 0.033$; Figures 4, S3). This is the same pattern observed across whole genome analysis of the cultivable symbiont strains across GA, IN, and NC.

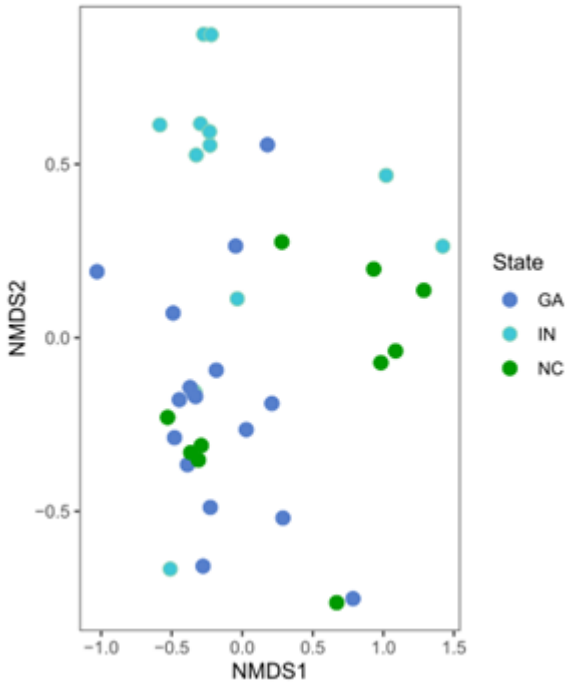


Figure 4. NMDS plot demonstrating variation within the *Burkholderiaceae* crypt communities of squash bugs isolated across the intermediate geographic range: Georgia (GA; purple), Indiana (IN; blue), and North Carolina (NC; green). Each dot represents all OTUs within the *Burkholderiaceae* (bacterial family including *Caballeronia spp.*) for a single crypt isolated from a bug. Crypt community composition varied across the intermediate geographic range ($F_{2, 36} = 3.17$, $p = 0.002$). Differences between strains are driven by differences in GA crypt community composition relative to those of IN and NC ($F_{1,37} = 2.47$, $p = 0.037$).

Genetic variation in symbiont strain is not observed across sympatric host species

Using whole genome sequencing, we observed little nucleotide variation across symbiont strains isolated from sympatric *A. tristis*, *A. scorbutica*, and *A. andresii* hosts. All strains shared high average nucleotide identity (ANI = 0.99; Figure 5, S2), except for one strain isolated from an *A. tristis* host that differed from all other strains (ATM282; ANI = 0.85; Figure 5, S2). Symbiont strains isolated from heterospecific hosts did not vary from one another in their average nucleotide identity, suggesting specialization between strain and host species as an unlikely outcome.

Figure 5. Heat map showing the genetic distance across symbionts strains isolated from *A. tristis* (AT; pink), *A. andresii* (AA; dark purple), and *A. scorbutica* (AS; light purple). All hosts and their associated symbionts were collected in Florida, USA. Genetic distance was calculated using average nucleotide identity (ANI), which varied from 0.85 to 0.99. We observed little variation across strains isolated from heterospecific hosts, with only one strain (ATM282) exhibiting substantial variation from other strains. Evolutionary relationships (far right) between strains were assessed using phylogenomic analysis. Strains ASM285, AAF181, and ATF2731 (asterisks) were used for reciprocal inoculations to test for specialization between host species and symbiont strain.

Local adaptation is not observed between *A. tristis* hosts and their symbionts

We tested for specificity consistent with pairwise coevolution by measuring local adaptation between hosts and symbionts across a small, intermediate, and large geographic scale (Figure 1). We quantified host responses to a sympatric symbiont and a range of allopatric symbionts by measuring host development rate and survival to each life stage. If pairwise coevolution contributes to the maintenance of this interaction, we predicted that we would observe specificity between hosts and their local *Caballeronia* spp. symbiont strains. Specifically, we predicted we would observe faster development rates and higher survival for squash bugs, and higher growth within crypts for symbionts, paired with their sympatric versus allopatric partners. For each reciprocal inoculation, we designated bugs to an aposymbiont water control treatment. Across all analyses both squash bugs receiving water exhibited slower development rate and reduced survival compared to those that received a symbiont, consistent with Acevedo *et al*., 2021. These individuals were not included in subsequent analyses.

Tests for local adaptation at the small geographic scale

At the small geographic scale, we observed a significant effect of the geographic origin of symbiont ($\chi^2 = 41.67$, df = 3, $p < 0.001$) and a significant interaction between host and symbiont geographic origin ($\chi^2 = 18.27$, df = 9, $p = 0.03$) for host development rate to adult (Table 1; Figures 6, S4). Because we observed a significant interaction between host and symbiont, we contrasted the rate of development for hosts paired with sympatric versus allopatric symbionts. Host development rate did not vary in response to sympatric versus allopatric symbionts ($p = 0.23$). Moreover, we detected a significant effect of the geographic origin of host ($\chi^2 = 11.55$, df

= 3, p = 0.009; Table 1) and symbiont (χ^2 = 20.70, df = 3, p = 0.0001; Table 1) on host survival. We did not detect a significant interaction between geographic origin of host and symbiont for host survival (Table 1; Figures 6, S4). There was also no significant difference in the proportion of hosts that survived to adult for sympatric and allopatric combinations of host and symbiont

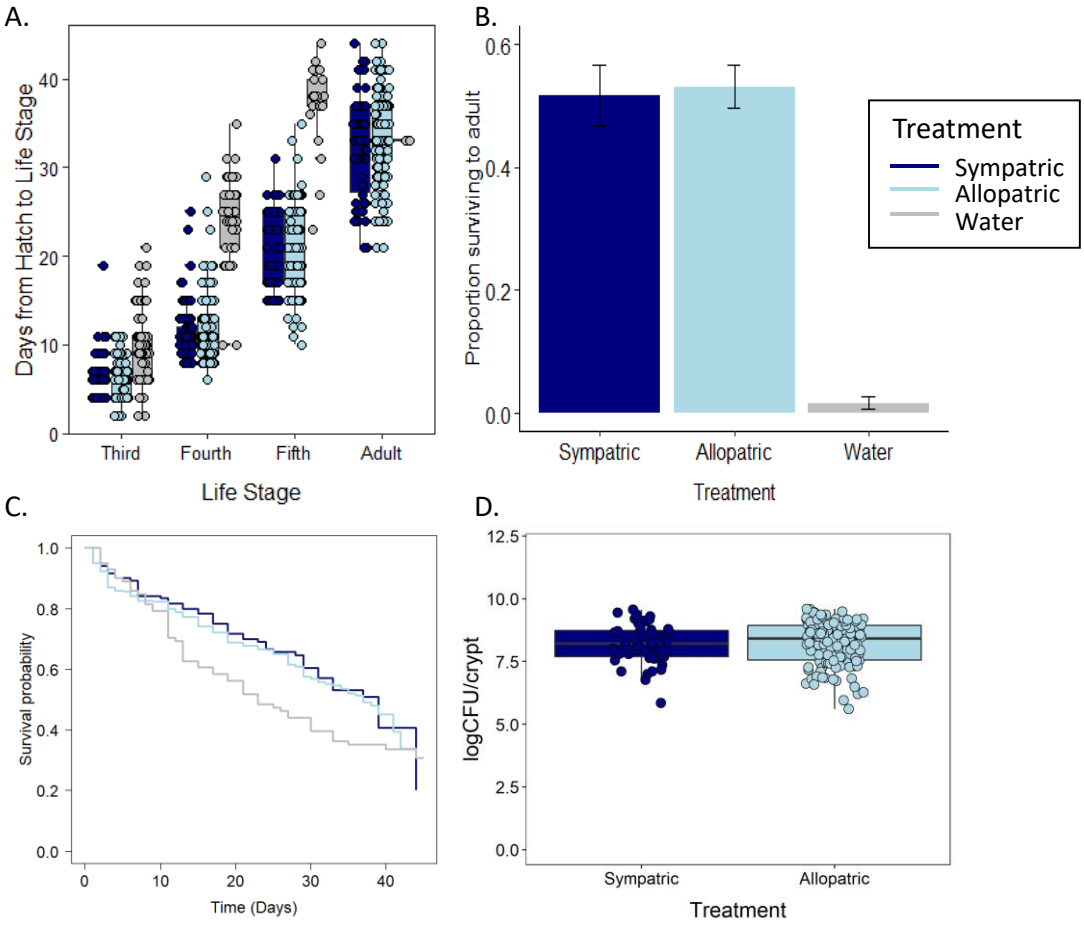


Figure 6. Host and symbiont fitness for reciprocal inoculations to test for local adaptation at the small geographic scale. Figure 5A shows host development rate across all developmental life stages. We observed a significant effect of symbiont (χ^2 = 41.67, p < 0.001) and an interaction between host and symbiont (χ^2 = 18.27, p = 0.03), which was not driven by differences in the effect of sympatric versus allopatric symbionts on host fitness (p = 0.23). Hosts receiving water developed slower than those that received a symbiont across all life stages. See figure S4 for host development rate across all pairwise combinations of host and symbiont. Figure 5B shows the proportion of bugs surviving to adult across experimental treatments. The proportion of bugs surviving to adult did not vary across sympatric and allopatric treatments but was lower for bugs receiving water versus those receiving a symbiont. Figure 5C shows the survival curves across symbiont treatments (see figure S4 for survival curves across all pairwise combinations of host and symbiont). We observed and effect of symbiont (χ^2 = 20.70, p = 0.0001) and host (χ^2 = 11.53, p = 0.009) for host survival. Figure 5D shows the effect of treatment on symbiont fitness (logCFU/crypt) (see figure S6 for each pairwise combination of host and symbiont). We observed no effect of host on symbiont fitness.

(Table 1; Figures 6). For symbiont fitness, we measured the number of CFUs per crypt of surviving adult squash bugs (Figures 6, S5). We detected a significant effect of host ($F_{3,169}$ = 3.0913, p = 0.02878) and symbiont ($F_{3,166}$ = 20.2003, p < 0.001). However, we did not observe a significant interaction between geographic origin of host and symbiont for CFUs per squash bug crypt ($F_{9,157}$ = 10.04, p > 0.11). Taken together, these results indicate that despite phenotypic variation in the effect of the geographic origin of host and symbiont for host fitness, local adaptation has not evolved at this geographic scale.

Because reciprocal inoculations were not performed synchronously, we predicted the observed effects of host and symbiont may reflect variation across replicate rather than variation across strains. To further assess whether geographic origin of host and symbiont affects host development rate and survival, we repeated reciprocal inoculations for two host populations (CF and FFF), such that each symbiont strain was synchronously provided to each host population. When inoculations were performed synchronously, we observed no effect of symbiont geographic origin, host geographic origin, nor an interaction between host and symbiont geographic origin, for rate of development to adult (Table S1; Figure S6). We also did not detect an effect of geographic origin of host nor symbiont for host survival (Table S1; Figure S6). However, we did observe an interaction between host and symbiont geographic origin for host survival (χ^2 = 8.92, df = 3, p = 0.03), and thus we contrasted the survival of hosts paired with sympatric versus allopatric symbionts. We observed no difference in host survival when paired with a sympatric versus an allopatric symbiont (p = 0.90), providing no support for local adaptation between host and symbiont. These results indicate that previous variation in the effect of symbiont for host fitness likely resulted from variation across replicate rather than from variation across sites. These results are consistent with those obtained previously and provide

further support for a lack of local adaptation between host and symbiont at this small geographic scale.

Tests for local adaptation at the intermediate geographic scale

Gene flow can dilute the strength of local selection by decreasing opportunities for conserved interactions and reciprocal selection between host and symbiont lineages across generations. Therefore, we tested for genetic specificity at a larger geographic scale as a means to limit the impacts of gene flow between populations. At the intermediate geographic scale, we observed a significant effect of host geographic origin ($\chi^2 = 12.89$, df = 2, p = 0.002; Table 1, Figures 7, S7) for the rate of *A. tristis* development to adult. However, we observed no effect of geographic origin of symbiont nor an interaction between the geographic origins of host and symbiont on development rate (Table 1; Figures 7, S7). Neither geographic origin of host, symbiont, nor an interaction between host and symbiont origin had a significant effect on host survival (Table 1; Figures 7, S7). We also observed no interaction between geographic origin of host and symbiont for host survival (Table 1; Figures 7, S7). Furthermore, the proportion of hosts surviving to adult did not differ between sympatric and allopatric combinations of host and symbiont (Table 1; Figure 7). Taken together, these results do not provide support for genetic specificity between host and symbiont lineages at the intermediate geographic scale.

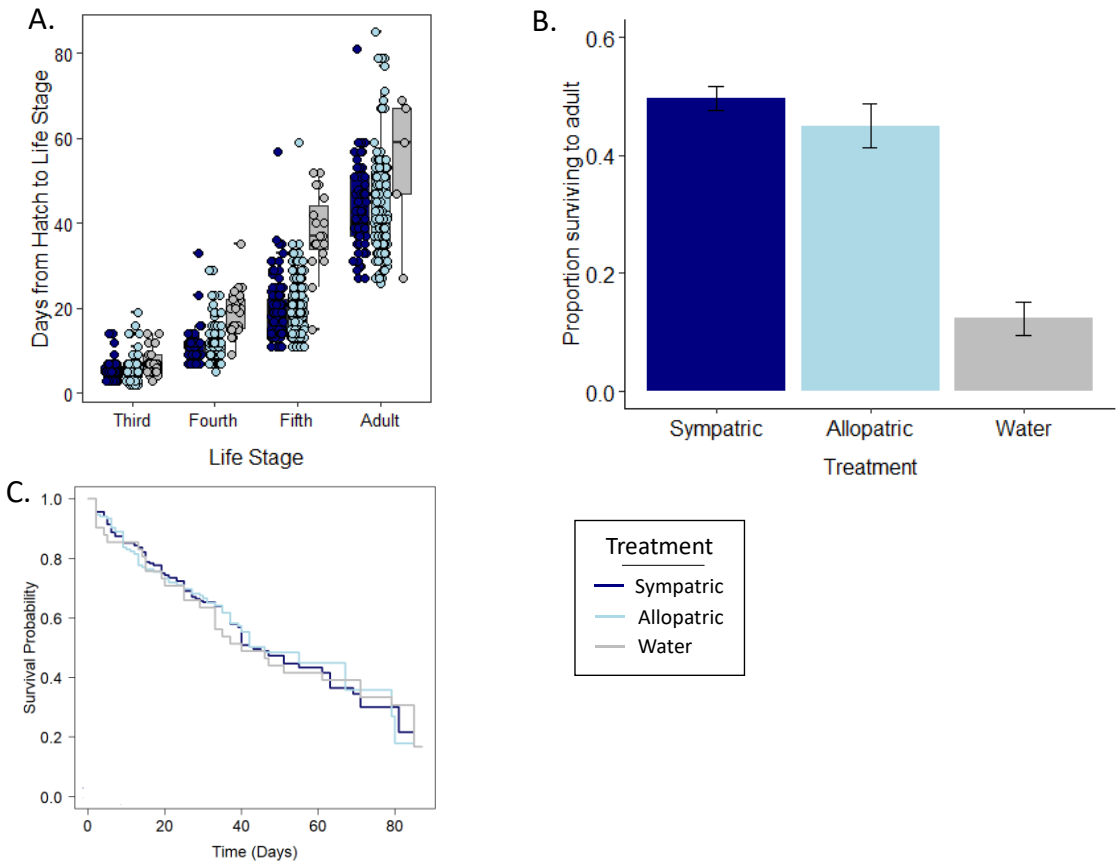


Figure 7. Host fitness at the intermediate geographic scale. Plot A shows overall host development rate when paired with sympatric versus allopatric symbionts (See figure S7 for all host development rate for all pairwise combinations of host and symbiont). We observed a significant effect of host geographic origin for rate of development to adult ($\chi^2 = 12.89$, $p = 0.002$). Plot B shows the proportion of bugs surviving to adult across experimental treatments. Survival to adult for bugs receiving water was significantly reduced compared to those receiving a symbiont ($p < 0.0001$) but did not differ between sympatric and allopatric treatments. Plot C shows survival curves across treatments. We did not observe a significant effect of host origin, symbiont origin, nor an interaction between host and symbiont origin for host survival. Survival over time did not vary across bugs receiving water versus those receiving a symbiont. This trend was driven by bugs that became developmentally "stuck" at a juvenile life stage but took a long time to die. We were unable to collect symbiont fitness data due to lab closures resulting from the COVID-19 pandemic.

Tests for local adaptation at the large geographic scale

Morphological variation exists between *A. tristis* squash bugs located in the Eastern versus the Western United States. Western bugs exhibit increased body size and melanization compared to Eastern squash bugs. The phenotypic variation observed across Eastern and Western U.S. bugs suggests these populations are likely diverged from one another. We predicted phenotypic divergence across Eastern and Western host populations may result from divergent interactions with their microbial symbionts, so we repeated reciprocal inoculations at this largest geographic scale. We observed a significant effect of host origin for development rate to adult ($\chi^2 = 10.49$, df = 1, p = 0.001; Table 1; Figure 8), such that hosts from the Western United States developed slower than those from the Eastern United States. We observed no effect of symbiont geographic origin nor a significant interaction between host and symbiont geographic origin for host development rate (Table 1; Figure 8). We did not observe a significant effect of symbiont origin, host origin, nor an interaction between host and symbiont geographic origin for host survival (Table 1; Figure 8). Furthermore, there was no difference in the proportion of bugs that survived to adulthood between sympatric and allopatric combinations of host and symbiont ($\chi^2 = 0.25013$, df = 1, p = 0.617; Table 1; Figure 8). For symbiont fitness, we measured the number of CFUs per crypt of surviving adult squash bugs. We detected a significant effect of symbiont origin ($F_{1,15} = 8.52$, p = 0.01), but we did not observe a significant effect of host origin nor an interaction between host and symbiont (Figure 8). Overall, using tests for local adaptation, we did not find support for our prediction that pairwise coevolution across spatially structure populations underlies the maintenance of this horizontally transmitted mutualisms.

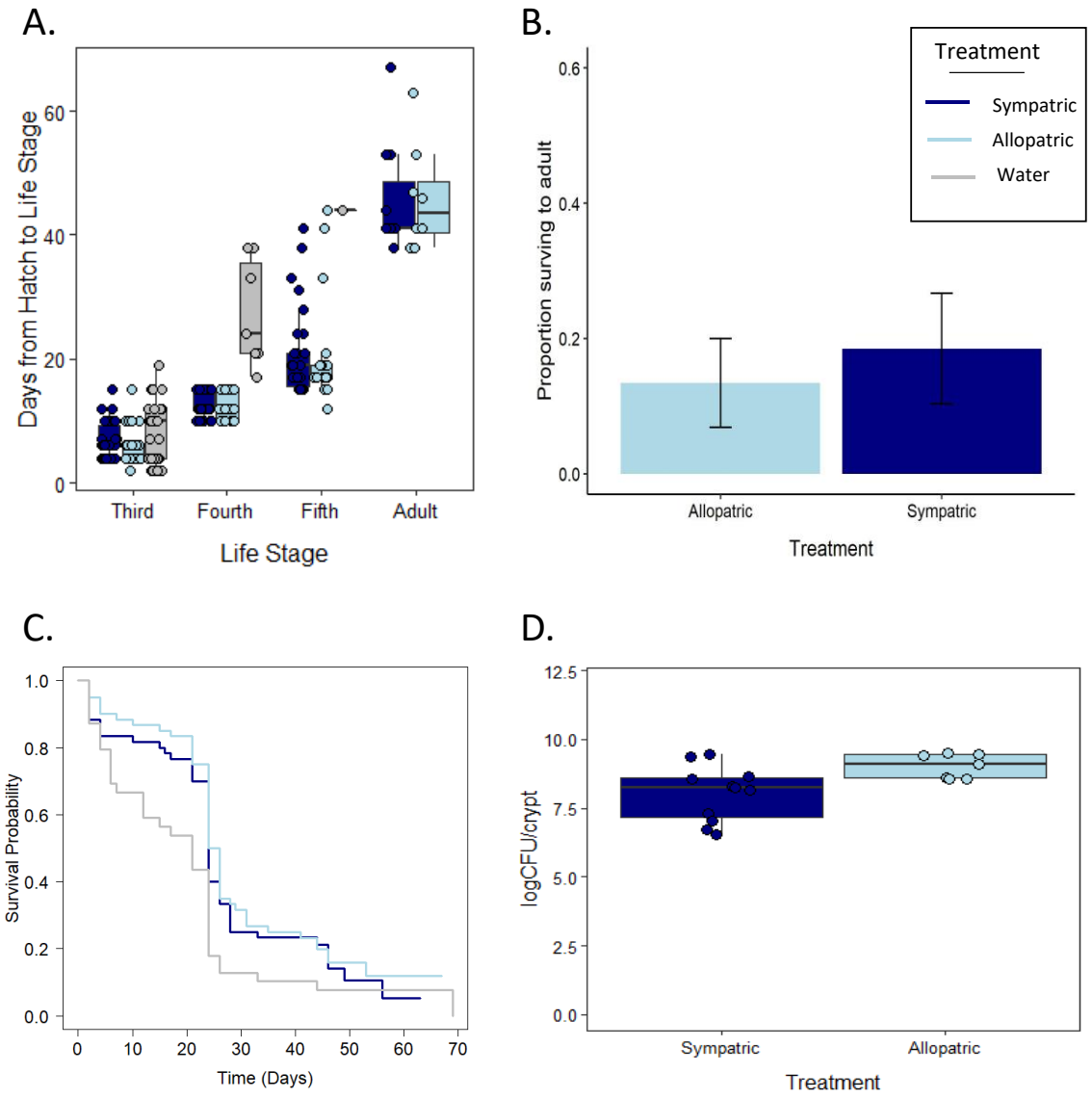


Figure 8. Host fitness at the large geographic scale. Plot A shows host development rate across all life stages when paired with a sympatric versus an allopatric symbiont. Development rate to adult varied in response to host origin (χ^2 = 10.49, p = 0.001) but did not vary in response to symbiont origin nor an interaction between host and symbiont origin. Plot B shows the proportion of bugs surviving to adult which did not vary in response to hosts receiving a sympatric versus allopatric symbiont. No bugs receiving water survived to adult, so they are not depicted here. Plot C shows survival across all experimental treatments. Survival did not vary in response to host origin, symbiont origin, nor an interaction between host and symbiont origin. Survival over time did not vary across bugs receiving water versus those receiving a symbiont. This trend was driven by bugs that became developmentally "stuck" at a juvenile life stage but took a long time to die. Plot D shows symbiont fitness (logCFU/crypt) for sympatric and allopatric treatments. We observed a significant effect of symbiont ($F_{1,15}$ = 8.52, p = 0.01) on symbiont fitness but no effect of host nor an interaction between host and symbiont fitness.

Table 1. Statistics for host development rate to adult and survival for reciprocal inoculations at three geographic scales. We performed cox proportional hazard models to test for effects of geographic origin of host and symbiont and an interaction between host and symbiont geographic origin on time to adult (development rate) and survival to adult (survival). If a significant interaction was observed, we performed a linear contrast to test whether host fitness varied in response to sympatric versus allopatric symbionts.

Geographic Scale	Test	Effect	df	χ^2	p-value
Small	Development Rate	Symbiont	3	41.67	< **0.001**
		Host	3	1.69	0.64
		Host*Symbiont	9	18.27	**0.03***
		***sympatric-allopatric contrast (p = 0.23)			
	Survival	Symbiont	3	20.70	**0.0001**
		Host	3	11.53	**0.009**
		Host*Symbiont	9	11.26	0.258
Intermediate	Development Rate	Symbiont	2	2.79	0.25
		Host	2	12.89	**0.002**
		Host*Symbiont	4	8.28	0.08
	Survival	Symbiont	2	5.41	0.07
		Host	2	0.42	0.81
		Host*Symbiont	4	3.61	0.46
Large	Development Rate	Symbiont	1	0.13	0.72
		Host	1	10.49	**0.001**
		Host*Symbiont	1	3.07	0.08
	Survival	Symbiont	1	0.03	0.87
		Host	1	1.51	0.22
		Host*Symbiont	1	0.43	0.51

Specialization is not observed between host species and associated symbiont strains

We tested for specialization between symbionts and the host species from which they originated. We predicted we would observe higher fitness interactions between hosts and conspecific-derived symbionts relative to symbionts derived from a heterospecific host. Symbiont origin did not affect host development rate to adult across *A. tristis, A. andresii*, or *A. scorbutica* (Table 2; Figures 9, S8). Across all host species, we did not detect a significant effect of symbiont origin for host survival (Table 2; Figures 9, S8). Moreover, the proportion of hosts surviving to adult

did not vary in response to receiving a symbiont from a conspecific host versus a heterospecific-derived symbiont (Figure 9). Similarly, symbiont fitness (CFUs/crypt) did not vary in response to host species (*A. tristis*: $F_{2,17} = 1.93$, $p = 0.18$; *A. scorbutica*: $F_{2,17} = 2.88$, $p = 0.08$; *A. andresii*: $F_{2,35} = 2.56$, $p = 0.09$; Figures 10, S9). These results suggest hosts from each species can mutualistically interact with a shared generalist symbiont.

Table 2. Statistics for host development rate to adult and survival for reciprocal inoculations between three host species and symbionts derived from conspecific versus heterospecific hosts. We performed cox proportional hazard models to assess rate of development to adult and survival.

Test	**Host**	**Effect**	**df**	χ^2	**p-value**
Development Rate	*A. tristis*	Symbiont	2	0.04	0.98
	A. scorbutica	Symbiont	2	0.09	0.95
	A. andresii	Symbiont	2	1.84	0.40
Survival	*A. tristis*	Symbiont	2	0.03	0.99
	A. scorbutica	Symbiont	2	1.23	0.54
	A. andresii	Symbiont	2	3.48	0.18

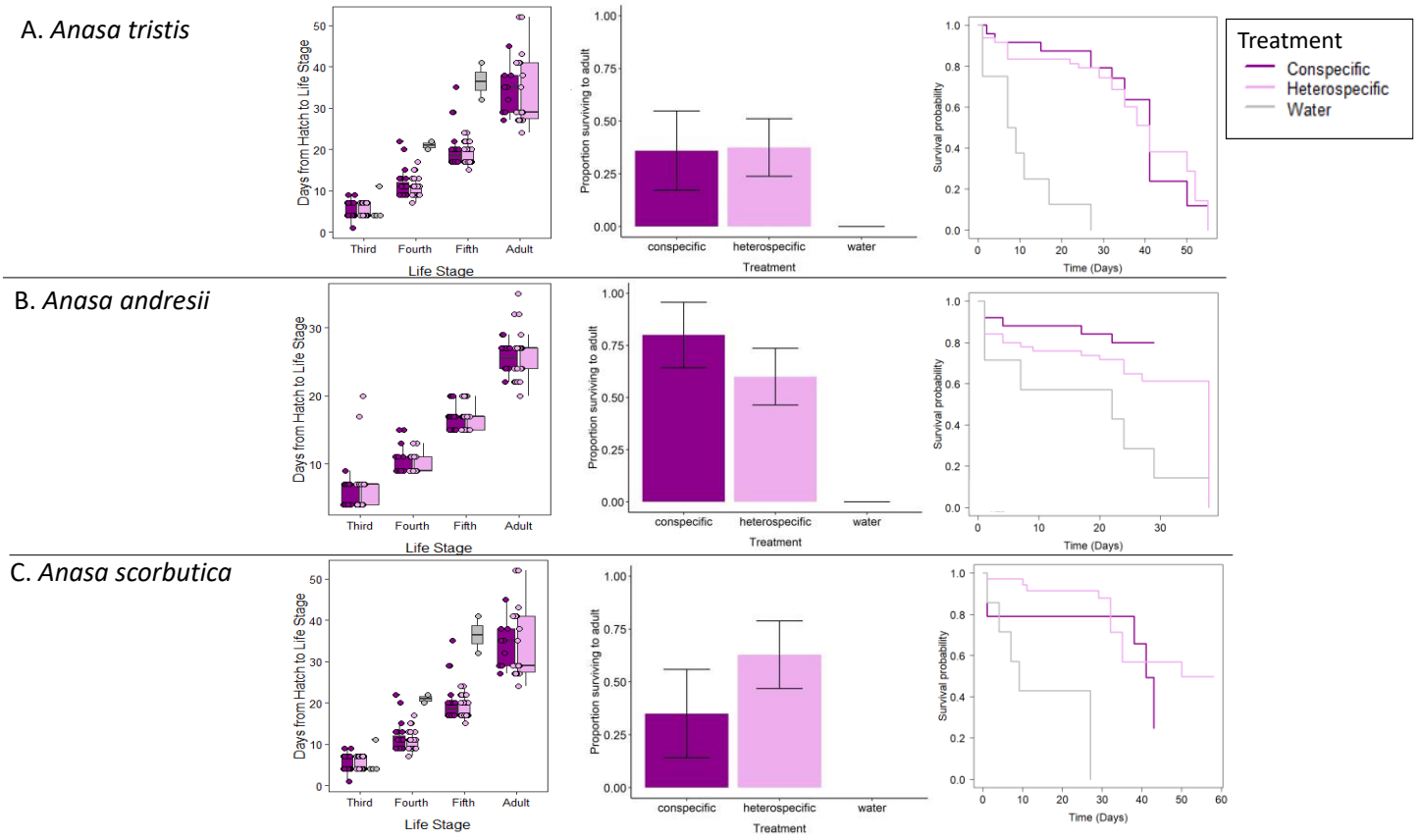


Figure 9. Host fitness data from reciprocal inoculations to test for specialization across host species with symbiont strain. Plots A-C show host development rate, proportion of hosts surviving to adult, and host survival curves (left to right) when hosts of each species were paired with symbionts derived from a conspecific versus heterospecific symbiont: *A. tristis* (A), *A. andresii* (B), and *A. scorbutica* (C). We observed no effect of symbiont on host development rate to adult nor host survival (see figure S8 for pairwise combinations of host species and symbiont strain) We observed no effect of conspecific versus heterospecific symbiont origin on the proportion of hosts surviving to adult across species. difference between treatments. Overall, aposymbiotic bugs receiving water experienced slower development and reduced survival.

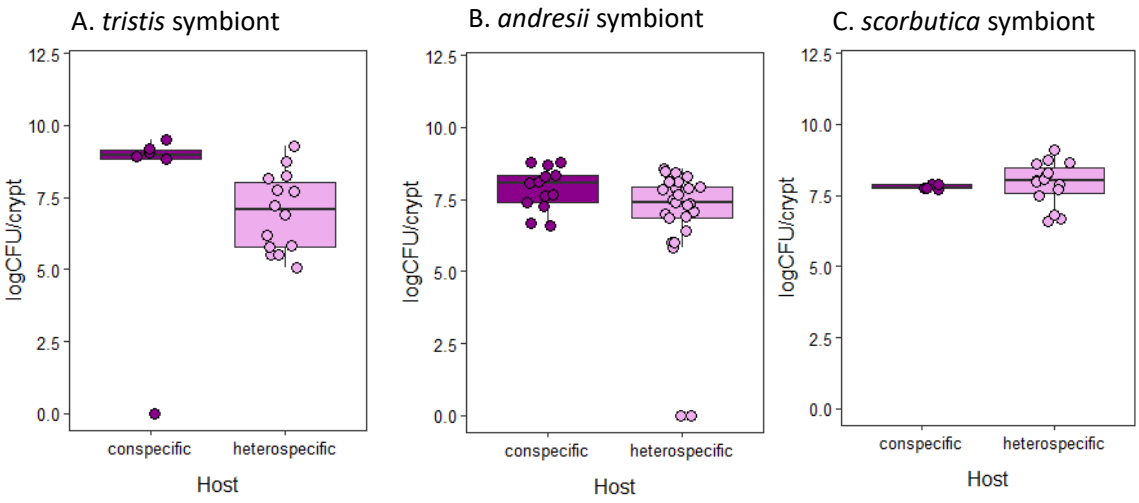


Figure 10. Symbiont fitness (logCFU/crypt) when symbionts were paired with hosts that were conspecific or heterospecific to the hosts from which they were derived. Symbiont fitness was measured by dissecting the crypts of bugs that survived to adult during host fitness assays and counting the number of CFUs per crypt for each host species: *A. tristis* (A), *A. andresii* (B), *A. scorbutica* (C). We observed no effect of host species on symbiont fitness (see Figures S9 for all pairwise combinations).

Discussion

In this study, we tested for patterns of specialization consistent with pairwise and diffuse coevolution within a horizontally transmitted mutualism. We assessed evidence for pairwise coevolution by testing for local adaptation across three geographic scales. We observed no specificity between sympatric host and symbiont lineages. Moreover, we observed no evidence of population structure across *A. tristis* symbiont populations, indicating local adaptation is unlikely within this interaction. We then tested for specialization between three host species, *A. tristis*, *A. scorbutica*, and *A. andresii*, with their associated *Caballeronia* symbionts but observed no evidence for specialization. Our results strongly demonstrate a lack of host-symbiont specificity in these interactions consistent with pairwise coevolution. Instead, we observe evidence of generalist, beneficial symbionts likely under selection from a range of hosts. These

dynamics suggest symbionts are under selection for certain fixed traits either through one-sided selection by hosts or through the coupling of the evolutionary trajectories of *Anasa* insects through reciprocal selection with their shared generalist *Caballeronia* symbionts under diffuse coevolution.

We argue that diffuse coevolution should be the focus of future investigation in this other and animal-microbe mutualisms. For the *Anasa-Caballeronia* symbioses, while hosts are not specialized to specific strains of *Caballeronia*, this interaction undoubtedly entails some degree of host specialization. Hosts require *Caballeronia* symbionts for survival and development (Acevedo *et al*. 2021). Hosts also exhibit morphological specialization to *Caballeronia* as demonstrated through the evolution of crypts for the sequestration of the symbiont. Crypt formation is induced by *Caballeronia* symbionts, which are the primary occupants of the crypts (Acevedo *et al*. 2021).

Dependence on *Caballeronia* symbionts is observed across a large range of Coreid insects, which nearly all sequester the symbiont in crypts (reviewed in Kaltenpoth and Flórez 2020). Moreover, colonization of crypts by non-symbiotic bacteria is highly restricted across these interactions (Ohbayashi *et al*. 2015; Itoh *et al*. 2019; Kikuchi *et al*. 2020). The evolution of crypts for the sequestration of specific symbionts suggests the symbiont likely exerts selection on its hosts. Moreover, in contrast to the species-specificity demonstrated by their hosts, *Caballeronia* symbionts exhibit less dependency and species-specificity toward their Coreid hosts (Kikuchi *et al*. 2007, 2011*b*; Garcia *et al*. 2014; Hosokawa *et al*. 2016). These asymmetric dynamics between a range of specialist hosts interacting with shared generalist symbionts are similar to those observed between plants and their pollinators, which are often driven by diffuse coevolution (Bascompte *et al*. 2003, 2013; Thompson 2006).

Furthermore, the genetic and phenotypic dynamics demonstrated in this study are consistent with diffuse coevolution. Diffuse coevolution between multiple hosts and a shared pool of symbionts reduces the strength of selection across pairwise interactions, while favoring the evolution of generally beneficial symbiont traits (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997; Inouye and Stinchcombe 2011). As a result, phenotypic variation across pairwise interactions is not expected. We do not observe phenotypic variation across pairwise interactions. However, we do observe geographic genetic variation across symbiont strains, indicating the potential for reciprocal selection between *Anasa* insect species and their microbial symbionts. In general, diffuse coevolution between generalist symbionts and a range of hosts may increase the probability that hosts will form successful mutualistic interactions each generation across a large geographic range.

Diffuse coevolution involving generalist interactions may be important for mutualists within an agricultural setting. Specialization can be costly if opportunities for repeated interactions between host and symbiont lineages are limited. Insects adapted to an agricultural setting must contend with sporadic availability of plant resources, variable crop variety, and enumerable pest mitigation methods. These environmental characteristics may force insects to migrate between fields, increasing opportunities for gene flow between populations. Moreover, agricultural practices such as selling crops, relocation of farm equipment, and disposal of organic waste may inadvertently disperse both metabolically active and overwintering insects. Our genetic analysis of *A. tristis* hosts isolated across three different states in the Eastern U.S. provides no evidence of genetic differentiation across these populations. This mixing of host lineages across a large geographic scale likely selects against specialization because hosts are unlikely to encounter the same local symbiont strains each generation. Accordingly, agricultural insects, such as *A. tristis*,

that depend on environmentally acquired microbial symbionts are likely under selection for generalism.

We provide evidence of the potential for diffuse coevolution in the interaction between *Caballeronia* spp. and its insect hosts. However, we do not demonstrate direct evidence for diffuse coevolution. Future work should directly test the role of diffuse coevolution for the maintenance of horizontally transmitted mutualisms. This can be accomplished using experimental and evolutionary genetics approaches. Empirical methods rely on demonstrating that the strength of selection on the symbiont is altered by the presence or absence of a host species (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997). Evolutionary genetics techniques can be used to determine whether the evolutionary trajectories of each pairwise symbiotic interaction are correlated due to shared underlying genetic interactions with the common partner (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997; Inouye and Stinchcombe 2011; Ossler and Heath 2018).

Previous work has employed evolutionary genetics techniques to test for evidence of diffuse coevolution between legumes with their horizontally transmitted mycorrhizal and rhizobial symbionts (Ossler and Heath 2018). A genetic correlation linking the evolutionary trajectories of these interactions was not observed. In general, the results of this study are consistent with those observed across the horizontally transmitted mutualism between legumes and their rhizobial symbionts. For example, previous work has demonstrated that generalist legume hosts that can interact with multiple symbionts benefit by exhibiting increased geographic ranges (Harrison *et al*., 2018). Moreover, previous work has demonstrated there is no geographic variation within rhizobial species (Harrison *et al*. 2017*b*) nor evidence for local adaptation (Harrison *et al*. 2017*a*). However, the interaction between legumes and rhizobia generally exhibits a high degree

of context-dependency (Heath and Tiffin 2007), and local adaptation has been observed under low nutrient environmental conditions (Rekret and Maherali 2019). In general, deciphering whether coevolution generally plays a role across horizontally transmitted interactions may be challenging because they frequently exhibit context-dependence and are often mediated by genetic interactions across many loci. Experimental evolution may provide a useful tool for assessing the implications of coevolution within mutualism (Hoang *et al*. 2016); however, recent work in this area has led to conflicting conclusions (Rafaluk-Mohr *et al*. 2018; Hoang *et al*. 2022).

Finally, we do not ignore the fact that coevolution may not contribute to this interaction. It is possible that cooperation is maintained by hosts exerting strong selection on symbionts for beneficial traits. Recent work has suggested that mutualisms evolve through host exploitation of symbionts (Nakajima *et al*. 2013; Lowe *et al*. 2016; Sørensen *et al*. 2019), and whether symbionts generally benefit within mutualistic interactions if often unknown (Garcia and Gerardo 2014). However, a substantial amount of work has demonstrated that mutualism stability depends on selection for the maintenance of host partner choice (Visick and McFall-Ngai 2000; West *et al*. 2002; Nyholm and McFall-Ngai 2004; Bshary and Grutter 2005; Bever *et al*. 2009*b*; Heath and Tiffin 2009; Gubry-Rangin *et al*. 2010), suggesting symbionts must also impose selection on their hosts to maintain long-term cooperative dynamics (Foster and Kokko 2006; Brown and Akçay 2019). The genetic variation across symbiont strains demonstrated through our whole genome analysis indicates the potential for selection to maintain partner choice and facilitate fitness alignment between host and symbiont through diffuse coevolution. Identifying and characterizing the genes underlying this interaction will be necessary to determine whether diffuse coevolution or one-sided selection underlie this interaction.

Coevolution often plays an important role in the maintenance of interspecific interactions. Horizontally transmitted mutualisms often exemplify characteristics that are consistent with pairwise coevolution; however, little empirical evidence has directly demonstrated evidence of reciprocal evolutionary change for both host and symbiont. Moreover, few studies have considered how community dynamics alter coevolutionary interactions within symbiotic mutualisms. We tested for evidence of pairwise and diffuse coevolution by measuring patterns of host-symbiont specificity and observed no evidence for pairwise coevolution. Rather, our results suggest that interactions with generalist symbionts produce the same fitness outcomes regardless of host or symbiont origin, demonstrating the potential for diffuse coevolution, which should be a focus of future work aiming to elucidate the role of coevolution within mutualism.

Supplemental information

Figure S1. Pangenome analysis of symbiont strains isolated from IN (blue), GA (purple), and NC (green). Dark regions show the presence of gene clusters, and faded regions indicate the absence of gene clusters. The box to the right shows the relative number of gene clusters across genomes, relative number of genes present in only one genome (singleton genes), relative number of genes per kbp, redundancy, genome completion, relative GC-content, and total length of the sequence. Variation can be observed across the genomes of symbionts isolated from across their geographic range.

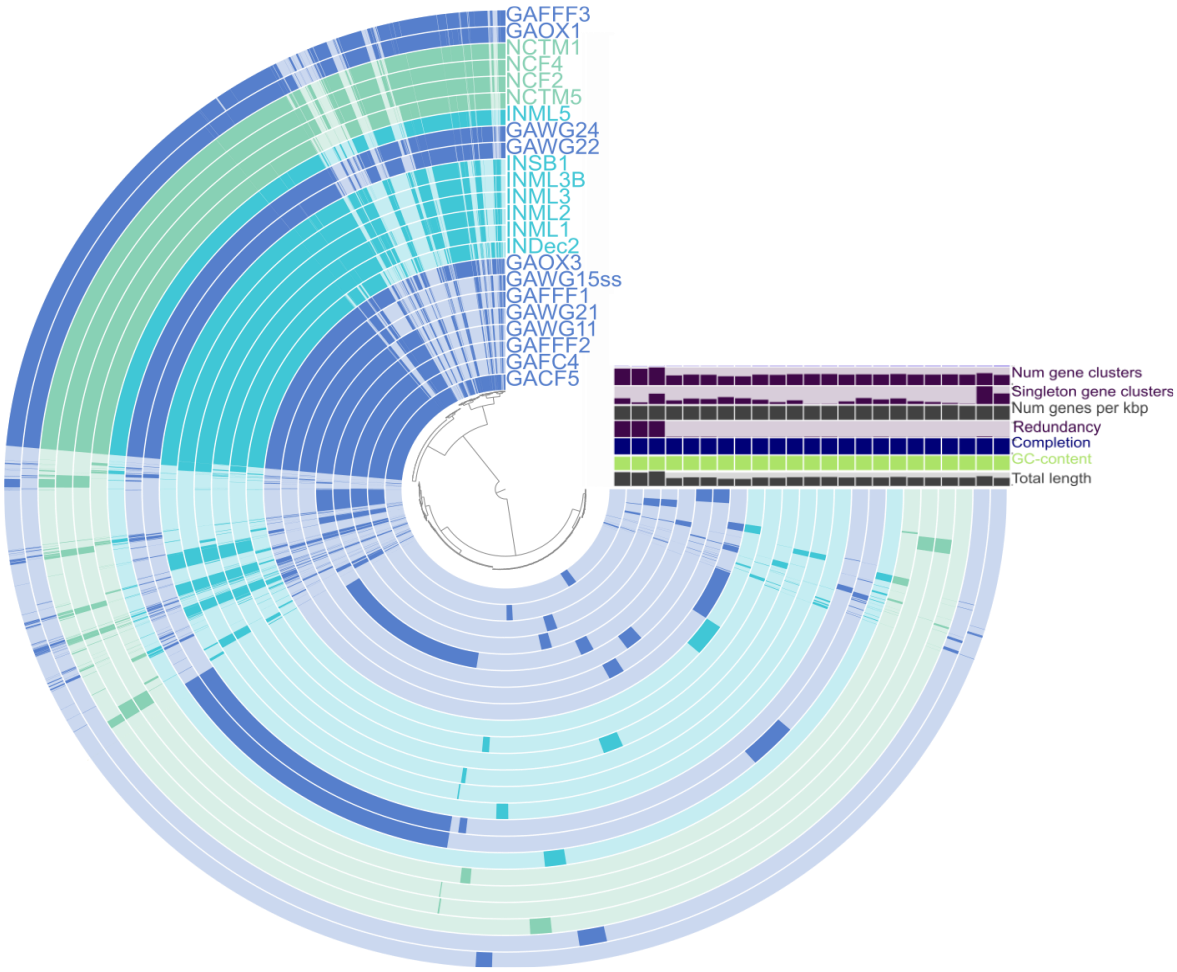


Figure S2. Pangenome analysis of symbiont strains isolated from three different host species *A. tristis* (AT; yellow), *A. andresii* (AA; orange), and *A. scorbutica* (AA; pink). Dark regions represent the presence of gene clusters and faded regions represent the absence of gene clusters. The box to the right shows the relative number of gene clusters across genomes, relative number of genes present in only one genome (singleton genes), relative number of genes per kbp, redundancy, genome completion, relative GC-content, and total length of the sequence. Variation can be observed across the genomes, particularly by the presence of gene clusters in *A. scorbutica* that are not present in *A. tristis* or *A. andresii* strains. Overall, little variation exists outside of these regions of the genome.

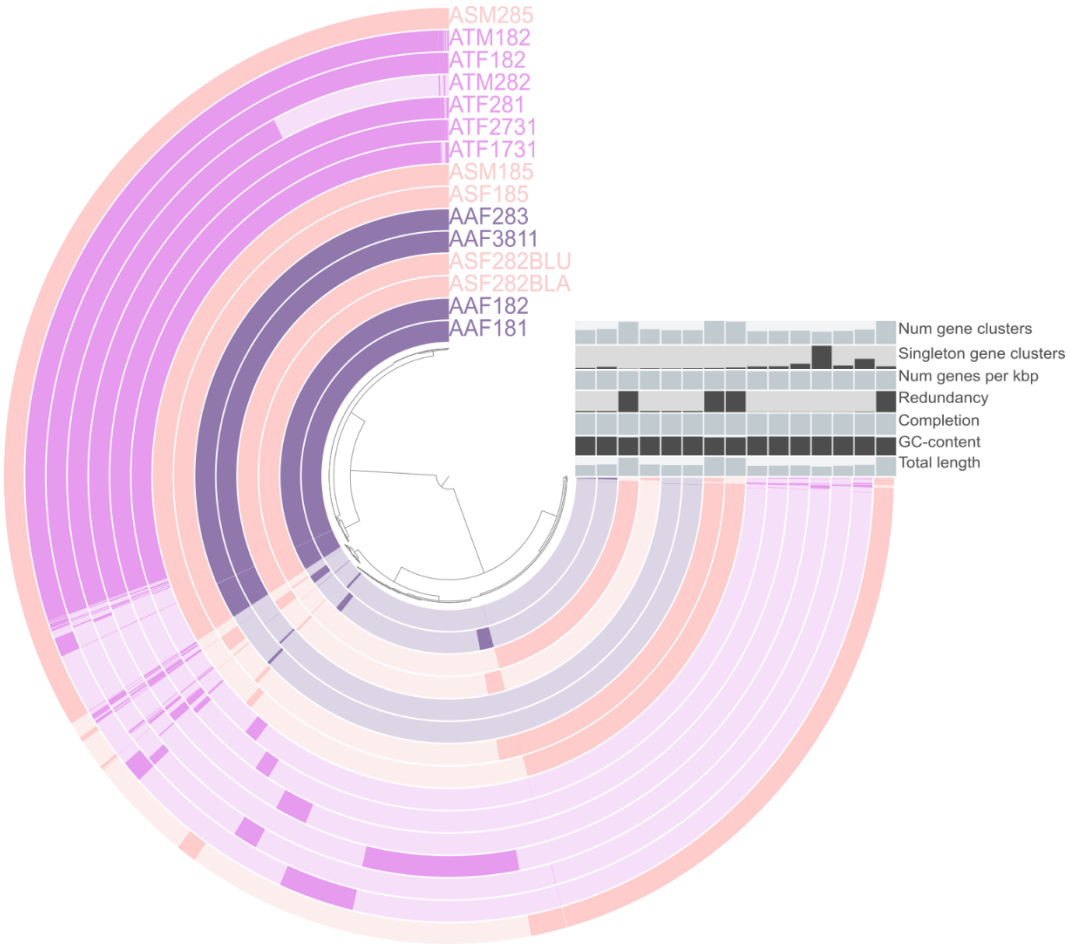


Figure S4. Host development rate and survival for all pairwise combinations of host and symbiont isolated across four sites at the small geographic scale: CF (A), FFF (B), Ox (C), and WG (D). We observed a significant effect of symbiont origin ($\chi^2 = 41.67$, $p < 0.001$) and an interaction between host and symbiont origin ($\chi^2 = 18.27$, $p = 0.03$) for host development rate to adult. This interaction was not driven by differences in the effect of sympatric versus allopatric symbionts on host fitness ($p = 0.23$). We observed a significant effect of symbiont origin ($\chi^2 = 20.70$, $p = 0.0001$) and host origin ($\chi^2 = 11.53$, $p = 0.009$) on host survival. Overall, we find no evidence for genetic specificity between host and symbiont.

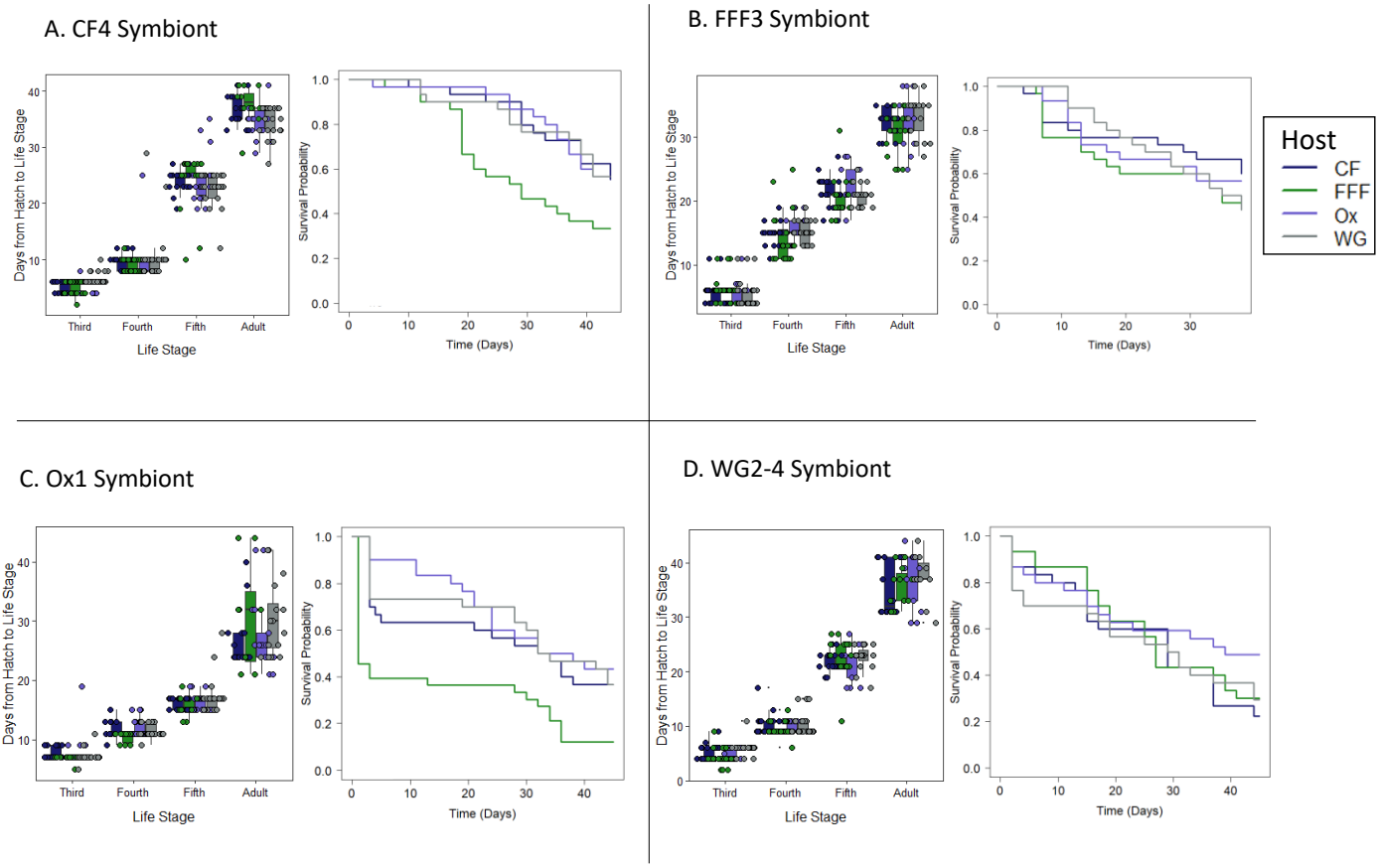


Figure S5. Symbiont fitness (logCFU/crypt) for the small geographic scale. We measured the fitness for each individual symbiont, GACF4 (A), GAFFF3 (B), GAOx1 (C), and GAWG2-4 (D), when paired with hosts from each population. Symbiont fitness varied in response to symbiont origin ($F_{3,166} = 20.20$, $p < 0.001$) and host origin ($F_{3,169} = 3.09$, $p = 0.03$). Symbiont fitness did not vary in response to an interaction between host and symbiont origin.

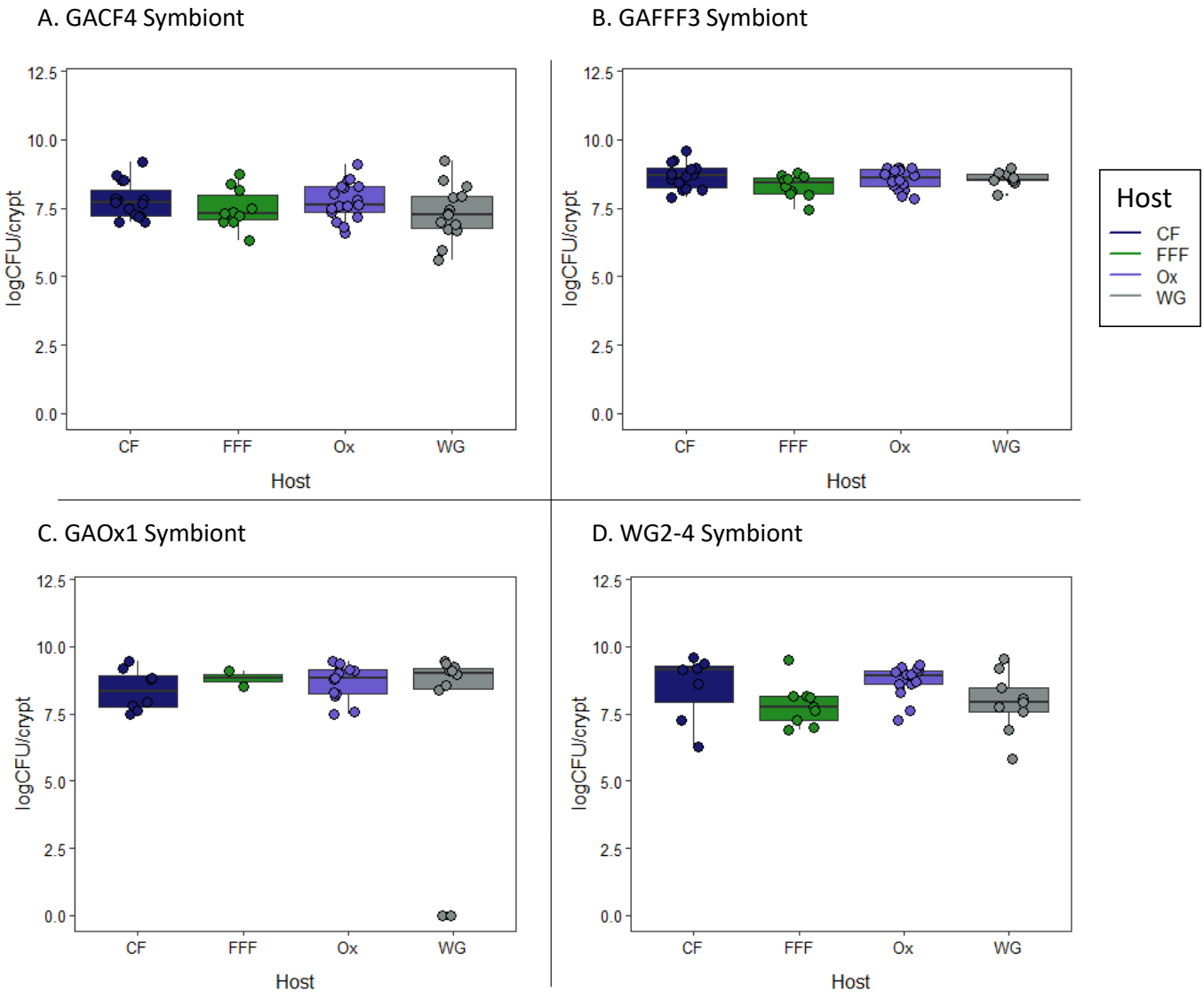


Table S1. Reciprocal inoculation to test the effect symbiont on host survival and development rate at the small geographic scale when inoculations were performed synchronously. We performed a linear contrast to test whether interactions resulted from an effect of sympatric versus allopatric symbionts on host fitness.

Test	Effect	df	χ^2	p-value
Development Rate	Symbiont	3	5.60	0.13
	Host	1	2.58	0.12
	Host*Symbiont	3	2.44	0.49
Survival	Symbiont	3	3.40	0.33
	Host	1	0.03	0.86
	Host*Symbiont	3	8.92	**0.03***

*sympatric-allopatric contrast (p = 0.90)

Figure S6. Host development rate and survival at the small geographic scale for the FFF (A) and CF (B) host populations when inoculations with symbionts from each population were performed synchronously. Development rate did not vary in response to the origin of host, symbiont, nor an interaction between the origin of host and symbiont. We observed no effect of host nor symbiont origin on host survival. We did observe an interaction between host and symbiont origin for host survival (χ^2 = 8.92, p = 0.03), but this was not driven by differences in the effect of sympatric versus allopatric symbionts on host fitness (p = 0.90). Plot C shows the overall development rate and survival for sympatric versus allopatric combinations of host and symbiont.

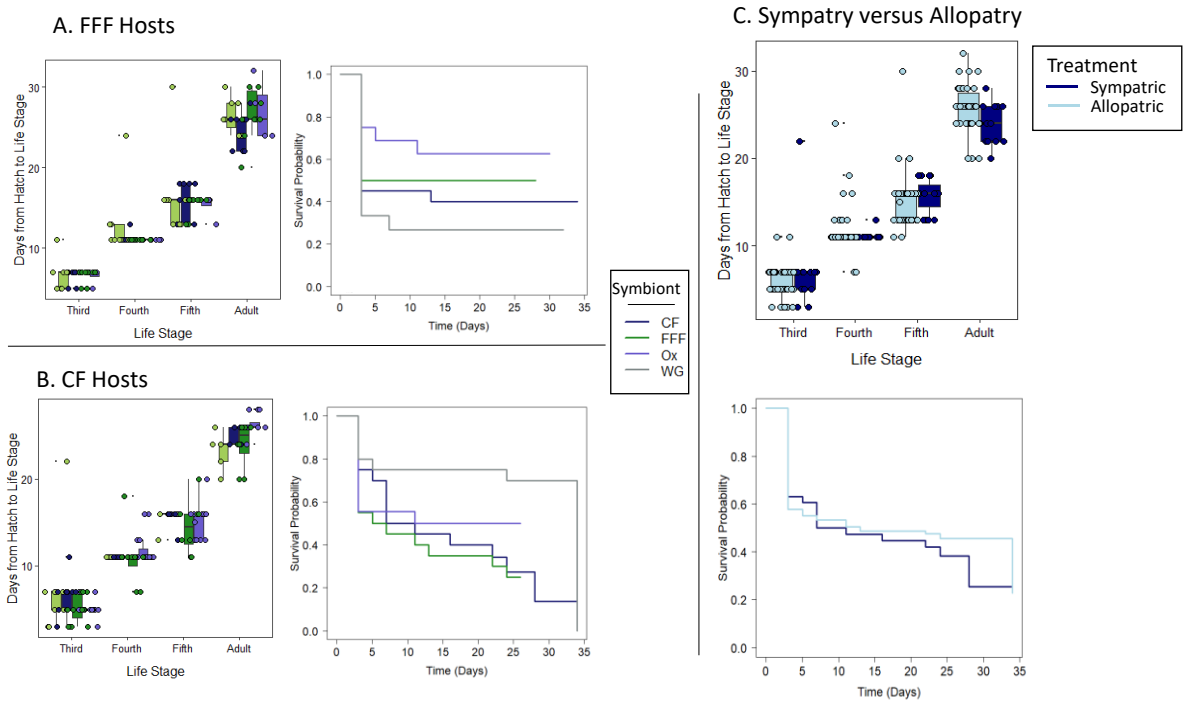
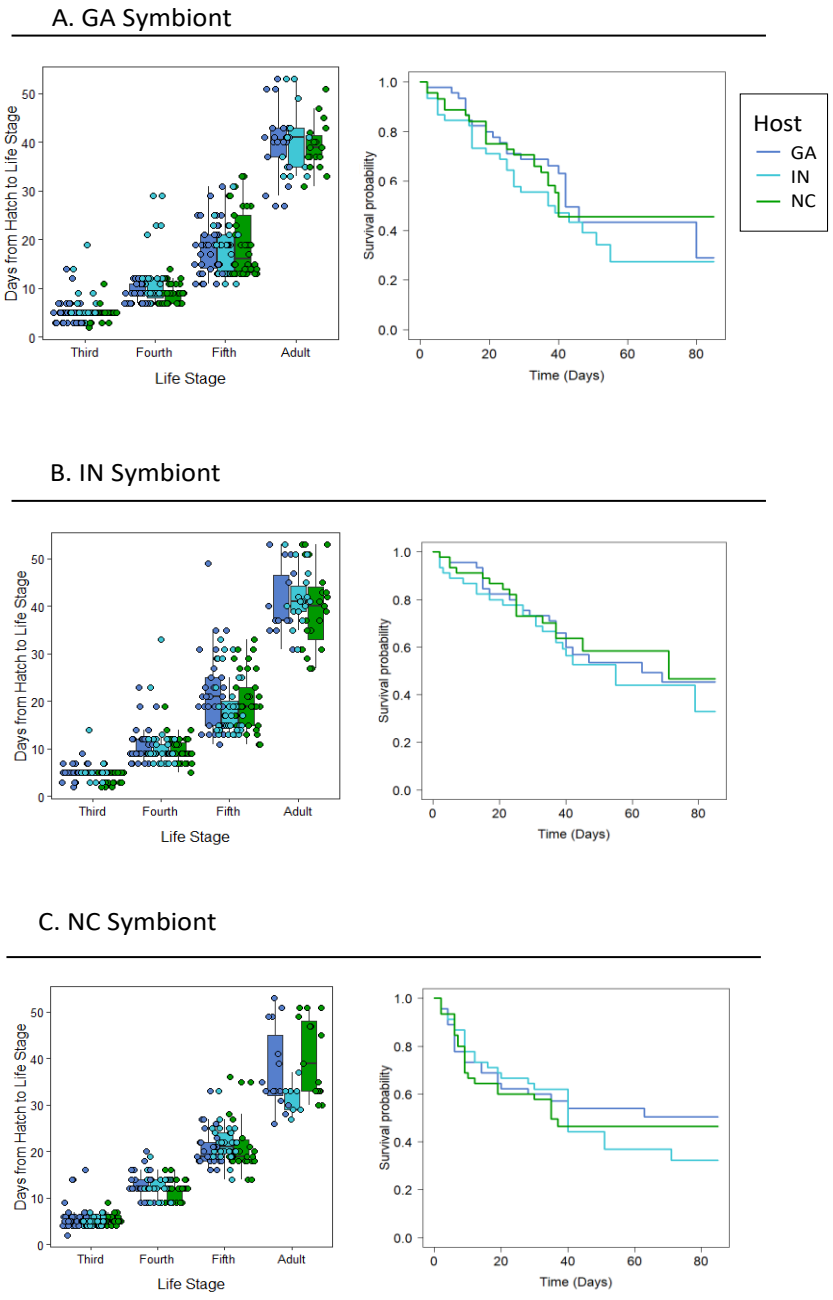


Figure S7. Host development rate and survival for all pairwise combinations of hosts and symbionts that were isolated from GA (A), IN (B), and NC (C). We detected a significant effect of host geographic origin on host development rate (χ^2 = 10.49, p = 0.001). We did not observe an effect of symbiont nor an interaction between host and symbiont geographic origin. Host survival did not vary in response to geographic origin of host, symbiont, nor an interaction between host and symbiont geographic origin. Overall, we observe no difference in host fitness in response to receiving a sympatric versus an allopatric symbiont.



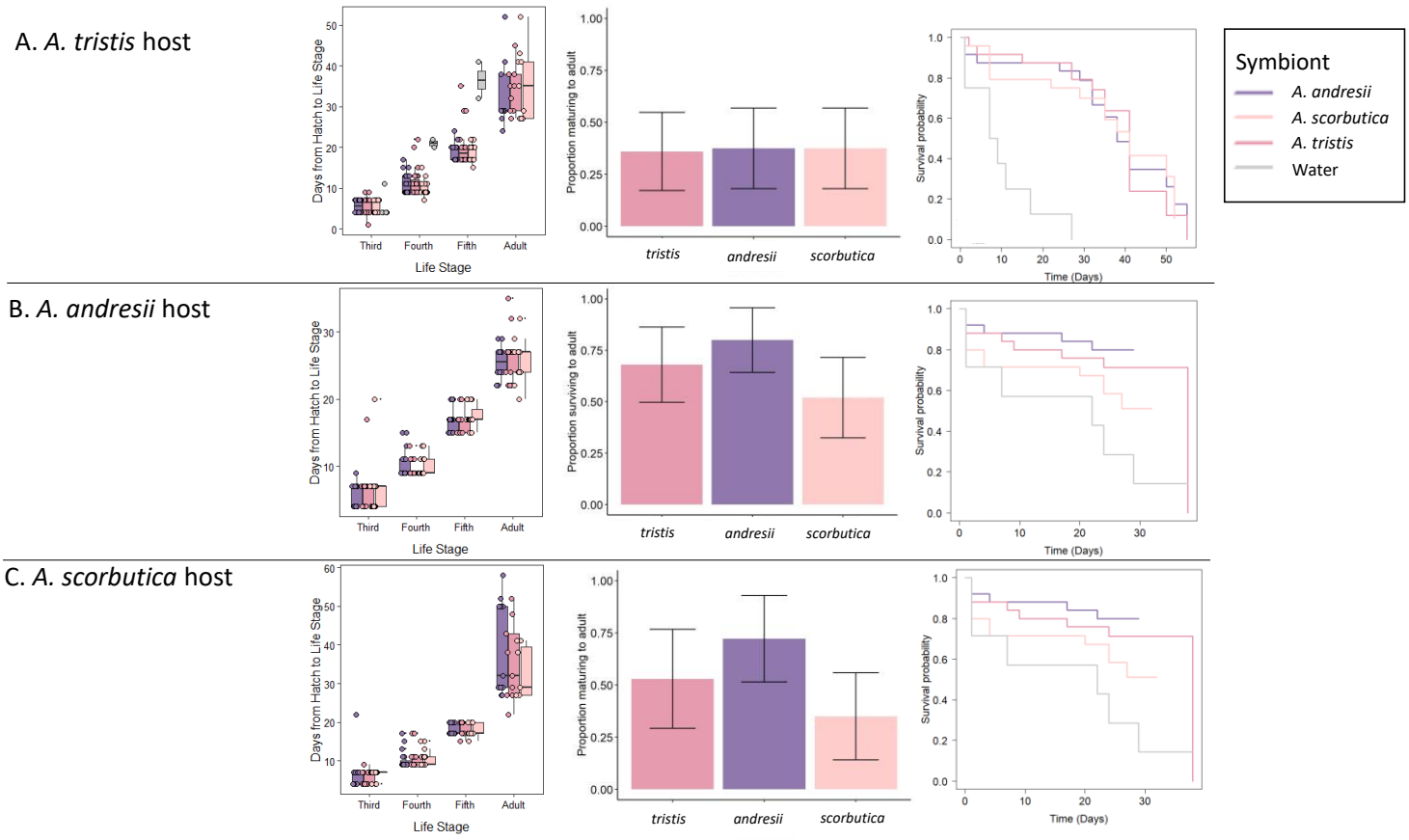


Figure S8. Host fitness data for all pairwise combinations of host species and symbiont strain. Plots A-C show host development rate across all developmental life stages for each host species: *A. tristis* (A), *A. scorbutica* (B), and *A. andresii* (C). We did not observe an effect of symbiont origin on host development rate. Plots D-F show host survival for each host species: *A. tristis* (D), *A. scorbutica* (E), and *A. andresii* (F). We did not observe a significant effect of symbiont origin on survival for any host species. Overall, we found no evidence for specialization.

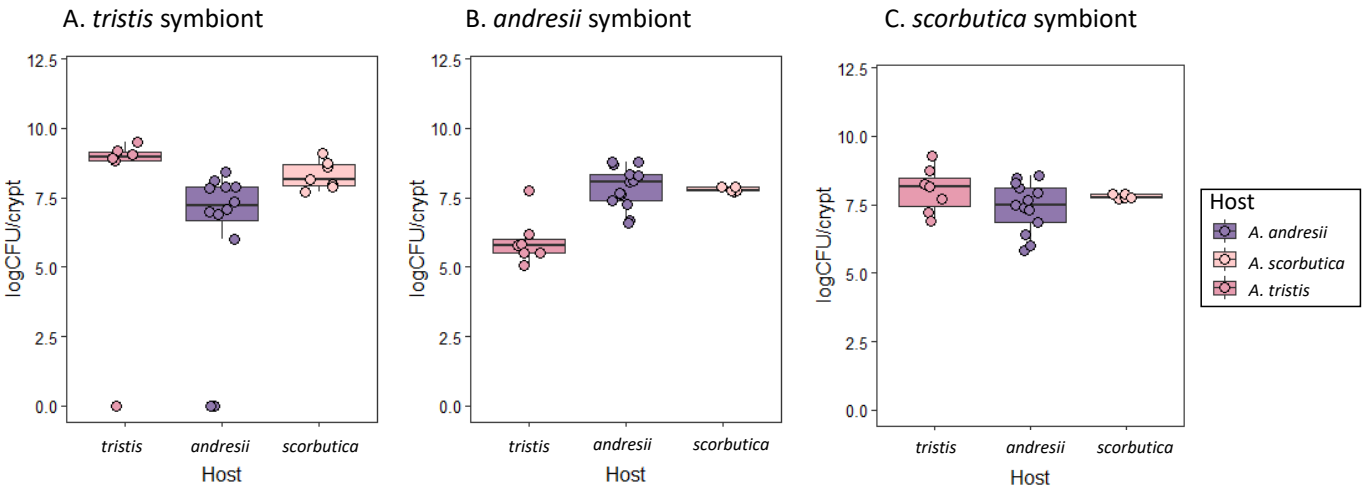


Figure S9. Symbiont fitness (logCFU/crypt) for all pairwise combinations of host species and symbiont origin. The fitness of symbionts isolated from each host species is shown when symbionts were paired with *A. tristis* (A), *A. scorbutica* (B), and *A. andresii* (C). We observed no effect of host species on symbiont fitness.

CHAPTER IV

HOST-ASSOCIATED SYMBIONT TRANSMISSION RAPIDLY REDUCES COOPERATIVE SYMBIONT TRAITS

Kayla S. Stoy, Erika A. Diaz, Levi T. Morran, and Nicole M. Gerardo

Abstract

Symbiont transmission mode is predicted to have important implications for the persistence of mutualistic interactions. The maintenance of cooperation between hosts and symbionts depends on the alignment of host and symbiont fitness interests. Under vertical transmission, symbiont evolution is tightly coupled to the host environment, which may facilitate fitness alignment. In contrast, horizontally transmitted symbionts spend time in the external environment, partially decoupling their evolution from the host. The decoupling of symbiont evolution from the host environment may misalign host and symbiont fitness interests if selection favors symbiont traits for exploitation or survival apart from the host. Despite this, horizontally transmitted mutualisms are common in nature. Here, we tested whether transmission environment alters the maintenance of cooperative symbiont traits. We experimentally evolved a *Paraburkholderia* symbiont of *Anasa tristis* hosts through several selection environments. Symbionts were passaged between *A. tristis* hosts, between *A. tristis* hosts and soil, through soil, or through standard culture media. We observed a rapid reduction in cooperative symbiont traits for treatments where symbionts were passaged through hosts. In contrast, symbionts passaged solely through soil exhibited fewer deleterious effects on their hosts. The rapid loss of cooperative traits across host-evolved bacteria likely resulted from one of two processes. First, passage through hosts may have selected for exploitative symbionts. Alternatively, passaging through hosts may have led to host-induced bottlenecking of symbiont populations, resulting in loss of important

cooperative traits. Contrary to expectations, these results indicate the potential for horizontal transmission to maintain cooperative symbiont traits by decoupling symbiont evolution from the host environment, thus limiting opportunities for symbionts to evolve exploitative traits and undergo large bottlenecks.

Introduction

The persistence of mutualistic interactions between hosts and their microbial symbionts depends on maintaining the alignment of host and symbiont fitness. While maximizing their own fitness, both host and symbiont must exert selection on their partner for cooperative traits and the inhibition of exploitation (Trivers 1971; Nowak and May 1992; Noe and Hammerstein 1994; Doebeli and Knowlton 1998; Sachs *et al*. 2004). According to evolutionary theory, symbiont transmission mode can alter the strength of selection to maintain cooperation across host-symbiont interactions (Anderson and May 1982; Ewald 1987; Bull 1994; Frank 1996; Herre *et al*. 1999; Wade 2007; Drown *et al*. 2013). Under vertical transmission, parents directly passage their microbial symbionts to their offspring. This transmission mode couples host and symbiont fitness interests because symbiont transmission depends on host survival and reproduction. Vertical transmission should then favor cooperation and limit exploitation. In contrast, hosts may horizontally acquire their microbial symbionts from other unrelated hosts or from the environment. This transmission mode potentially misaligns host and symbiont fitness because symbionts survive without their hosts. Transitions toward parasitism may result from symbionts evolving to exploit their hosts without deleterious consequences for their own fitness. Despite these predictions, horizontal transmission is common across mutualisms (Lee and Ruby 1994*a*; Wilkinson 1997; Simms 2002; Kikuchi *et al*. 2011; Henry *et al*. 2013; Chrostek *et al*. 2017; Hartmann *et al*. 2017; Li *et al*. 2017; Acevedo *et al*. 2021). Whether transmission mode alters the

direction of selection for cooperative symbiont traits remains a relatively untested question within natural host-symbiont interactions.

Mounting evidence suggests that there are conditions under which horizontal transmission may have beneficial ramifications for the long-term persistence of mutualism (Mikheyev *et al*. 2006; O'Fallon 2008; Shapiro and Turner 2014; Hartmann *et al*. 2017; Brown and Akçay 2019; Breusing *et al*. 2022). For example, Shapiro and Turner (2014), predict that horizontal transmission can facilitate the evolution of increased fitness benefits for hosts if horizontal transmission and symbiont-conferred benefits are correlated with one another. For example, reef-building corals benefit by delaying symbiont acquisition to avoid oxidative and light stress during early developmental stages (Hartmann *et al*. 2017). Similarly, horizontal transmission likely benefits hydrothermal vent snails that experience high rates of dispersal by allowing them to acquire locally adapted microbial symbionts (Breusing *et al*. 2022). Within these interactions, symbiont-conferred benefits are directly correlated with horizontal transmission.

Partner choice likely underlies correlations between horizontal transmission and symbiont-conferred benefits (Shapiro and Turner 2014). Partner choice can facilitate fitness alignment within horizontally transmitted mutualisms because hosts choose not to interact with exploitative symbiont genotypes, putting them at a disadvantage relative to cooperative symbionts (Visick and McFall-Ngai 2000; West *et al*. 2002; Nyholm and McFall-Ngai 2004; Bshary and Grutter 2005; Bever *et al*. 2009*b*; Heath and Tiffin 2009; Gubry-Rangin *et al*. 2010; Friesen 2012). Symbionts that are acquired by hosts often reside in symbiotic organs that enrich their growth and minimize competition with other microbes, giving cooperative symbiont genotypes a fitness advantage (Fronk and Sachs 2022). Therefore, partner choice can couple horizontal transmission and the evolution of cooperative symbiont traits by maintaining strong selection on symbionts

for cooperation. Moreover, horizontal transmission can maintain variation within symbiont populations (reviewed in Stoy *et al*. 2020), which may provide opportunities required for selection to prime host partner choice (Foster and Kokko 2006; Akçay 2017). This may strengthen the efficacy of host-driven selection for symbiont-conferred benefits (O'Fallon 2008; Shapiro and Turner 2014), potentially increasing the overall rewards achieved through the interaction (Shapiro *et al*. 2016).

Horizontal transmission may also benefit mutualisms in other ways. For example, it reduces the probability of symbiont population bottlenecks and genome degradation, relative to symbiont populations that are vertically transmitted (O'Fallon 2008; Bennett and Moran 2015). This can have important implications for both host and symbiont fitness. First, population bottlenecks that lead to deleterious mutations through Muller's ratchet can result in the loss of essential functions important for both hosts and symbionts (Bennett and Moran 2015). Second, reductions in standing genetic variation within symbiont populations can reduce the efficacy of selection on these populations (O'Fallon 2008). This may result in less rapid responses to selection from hosts relative to symbiont populations in which variation is maintained through horizontal transmission (Shapiro and Turner 2014; Shapiro *et al*. 2016). Moreover, these symbionts may be less effective at responding to selection outside of the mutualism (Morran *et al*. 2016). This may be costly if hosts experience a change in their external environments, with the consequence that their symbionts no longer possess environmentally relevant traits (Wernegreen 2012).

Experimental evolution is a powerful tool to assess the implications of specific selection pressures within species interactions. While the use of experimental evolution to understand mutualistic interactions has recently increased (Martinez *et al*. 2016; Morran *et al*. 2016; Shapiro *et al*. 2016; Batstone *et al*. 2020; Hoang *et al*. 2021), it has generally been an underutilized tool

for studying mutualism (Hoang *et al*. 2016). Experimental evolution may be especially useful to assess the effects of selection pressures faced by symbionts across the selection environments commonly associated with different symbiont transmission modes. In this study, we use experimental evolution to directly test whether transmission environment alters selection for the maintenance of cooperative symbiont traits. We leverage a naturally occurring interaction between an insect host *Anasa tristis* and its moderately beneficial bacterial symbiont *Paraburkholderia* (Acevedo *et al*. 2021). *A. tristis* squash bugs require bacterial symbionts for survival and development to adulthood. In nature, *A. tristis* are required to select symbionts from their environment each generation, and most commonly partner with *Caballeronia* spp. bacteria, though other bacteria also form symbioses with the bugs (Acevedo *et al*. 2021). This transmission pathway introduces opportunities for diverse selection pressures to shape the evolutionary trajectory of the symbiont. Using experimental evolution, we can directly test the relative effects of selection from hosts versus selection from the environment and determine the consequences of these disparate selection pressures for the maintenance of cooperative symbiont traits.

To accomplish this, we experimentally passaged the bacterial symbiont *Paraburkholderia* through four selective environments. *Paraburkholderia* has a weakly beneficial effect on squash bug host fitness relative to *Caballeronia* spp., the most prevalent symbiont of *A. tristis* (Acevedo *et al*. 2021). As such, selection on cooperative traits during experimental evolution may readily drive observable increases in symbiont-conferred host benefits. We passaged symbionts under frequent *A. tristis* host exposure (host-to-host transmission), between *A. tristis* hosts and soil (environmentally-mediated host transmission), through non-sterile soil (environmental passage), or through standard culture media (LB). We then tested the implications of experimental

evolution through each environment by assaying host fitness when paired with evolved versus ancestral symbionts as well as symbiont colonization and growth within hosts.

Materials and Methods

Study System

The squash bug *Anasa tristis* is an agricultural pest of cucurbits crops. Squash bugs develop through five instar stages before reaching adulthood. During the second instar, squash bugs environmentally acquire bacterial symbionts (Acevedo *et al*. 2021). Environment-mediated transmission between co-localized bugs has been demonstrated in the lab. Transmission can occur through feeding on the fecal matter of neighboring squash bugs (Villa and Chen, *in prep*), although likely also occurs through passaging in plants and soil. Squash bugs harbor these symbionts in specialized regions of the gut, referred to as the crypt, which is primarily colonized by bacteria within the *Burkholderiaceae* family (Acevedo *et al*. 2021). The most prevalent crypt symbiont of *A. tristis* is *Caballeronia* spp., which provides squash bugs with important fitness benefits, including rapid development and increased survival to adulthood. However, squash bugs can also receive weak fitness benefits from a closely related *Paraburkholderia* symbiont that also colonizes host crypts (Acevedo *et al*. 2021). This symbiont provides reduced fitness benefits compared to *Caballeronia*; however, relative to aposymbiotic bugs, those harboring *Paraburkholderia* exhibit more rapid development to adulthood.

For this study, we chose to experimentally evolve the *Paraburkholderia* symbiont. Because it provides squash bugs with weak fitness benefits, we predicted greater evolutionary potential for directional selection to drive the evolution of observable increases in cooperative traits across our experimental treatments. Specifically, we experimentally evolved *Paraburkholderia* strain

SMT4a, which was derived from soil at a site containing coreid insects (Garcia *et al*. 2014). This strain can be cultured on standard culture media. SMT4a was previously fluorescently labeled with Green Fluorescent Protein (GFP), including a marker for kanamycin resistance, using a triparental mating protocol (Kikuchi and Fukatsu 2014; Acevedo *et al*. 2021).

Squash bugs used for experimental evolution were originally collected at Oxford Organic Farm in Oxford, Georgia and maintained as a laboratory stock in environmental chambers at Emory University. Chambers were maintained at 21 ℃, 50% humidity, and a 16/8-hour day/night cycle. Laboratory stocks were reared on and fed yellow crookneck squash plants. To prepare hosts for experimental evolution, eggs were collected and surface sterilized with 70% ethanol and 10% bleach. Emerging first instars nymphs were fed surface sterilized organic zucchini wrapped in parafilm. Nymphs were used in experimental evolution once they reached second instar.

Experimental Evolution of the *Paraburkholderia* Symbiont

We designed experimental evolution treatments to increase symbiont-conferred host benefits by selecting for symbionts that potentially facilitated rapid host development. We chose to select for more rapid host development because previous work indicates this is the largest fitness benefit conferred to hosts by their symbionts (Acevedo *et al*. 2021). Across all experimental treatments, only bacterial symbionts were evolved across passages. Symbionts were evolved through four experimental treatments (Figure 1). The effects of symbiont transmission in a consistent host environment were tested within the Host-to-Host Transmission (HTH) treatment, where symbionts were serially passaged between *A. tristis* hosts. The effects of alternating selection from hosts and the external environment were tested within the Environmentally-Mediated Host Transmission (EMH) treatment, and symbiont passaging alternated between *A. tristis* hosts and non-sterile soil from a local community garden. The effects of constant selection from the

environment outside of the host were tested using the Environmental Passage (EP) treatment, where symbionts were serially passaged through non-sterile soil. Finally, symbionts were serially passaged through Luria Broth (LB), which was included as a control for genetic drift and stochasticity introduced by the lab culturing methods. Between each passage and across all treatments, bacteria were cultured on LB agar plates to select colonies for the next passage. Each experimental treatment was independently replicated four times to produce four replicate symbiont populations per treatment. All replicate lineages began with an isogenic clone of SMT4a, requiring all variation across treatments to evolve through *de novo* mutation. Selection was conducted over six passages (total of three passages in each of the two selective environments for the HT treatment).

Transfer and Selection of Symbionts

To begin experimental evolution across all treatments, SMT4a was revived from glycerol stocks by streaking onto yeast glucose agar plates containing kanamycin (YG-kan) and grown at 28 °C for 48 hours. Liquid cultures were prepared by selecting a single colony and placing into LB containing kanamycin (LB-kan) and grown overnight with shaking at 28 °C. Overnight cultures were diluted 1:7 in LB-kan and incubated at 28 °C with shaking for two hours. Symbiont feeding solutions (10mL) for host inoculation were prepared by diluting the two-hour liquid cultures with sterile molecular water to ~$2x10^7$ cells/mL. Blue dye (1%) was added to each solution to allow for visual confirmation of the feeding solution in squash bug guts. For the HTH and EMH treatments, feeding solutions were poured over sterile dental cotton swabs placed into 35mm Petri dishes. The dishes were wrapped in parafilm to prevent spilling or squash bug drowning, allowing the bugs to feed from the cotton swab only. Hosts were starved for 24 hours prior to symbiont inoculation. Feeding solutions were provided to 10 hosts from the HTH and EMH

treatments for 24 hours. Feeding solutions were then removed, replaced with surface sterilized zucchini wrapped in parafilm, and hosts were reared in plastic vented containers until reaching fourth instar.

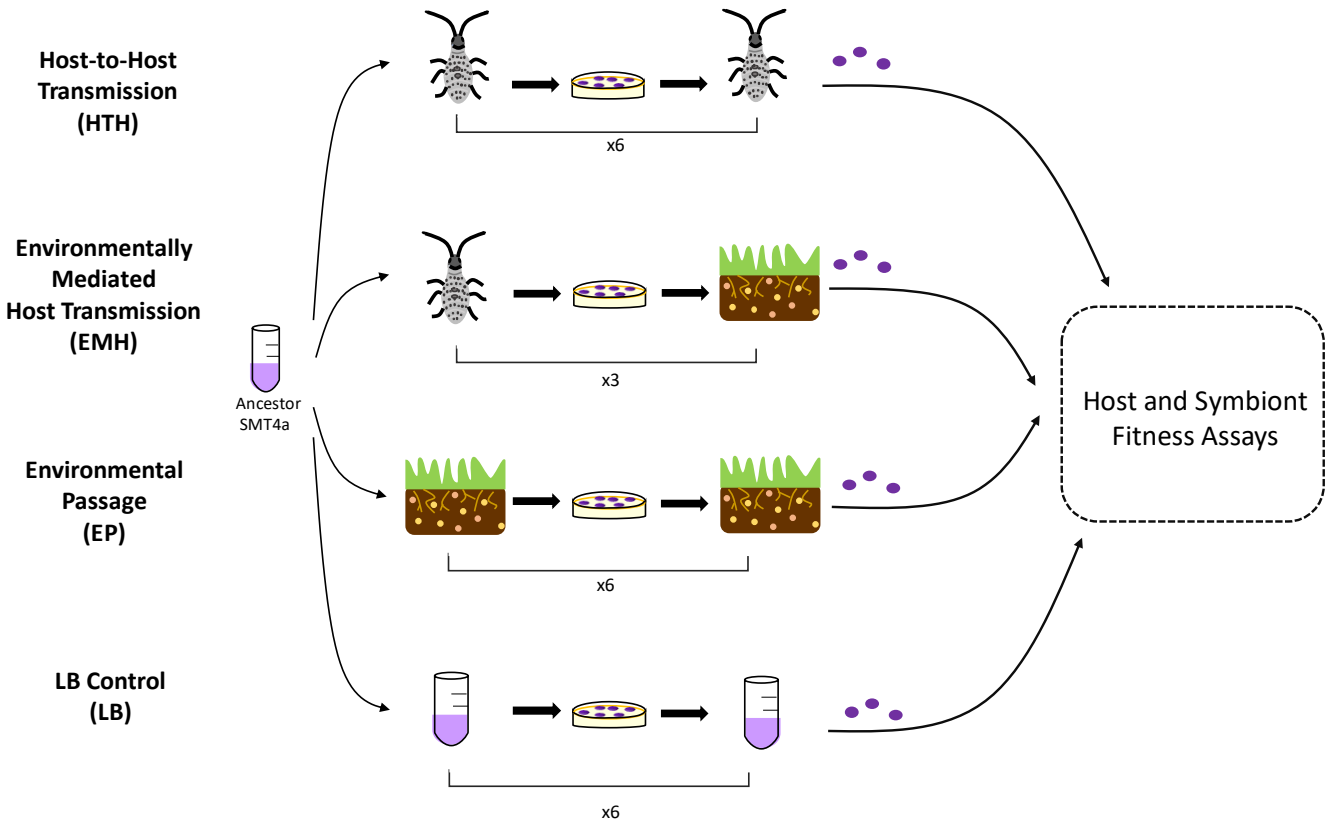


Figure 1. Experimental Evolution Design. GFP-labeled symbionts were passaged through four different transmission environments. In the Host-to-Host (HTH) treatment, symbionts were passaged between squash bug hosts. In the Environmentally-Mediated Host Transmission EMH) treatment, symbiont passaging alternated between host and non-sterile soil. For the Environmental Passage (EP) treatment, symbionts were passaged through non-sterile soil. A control (LB) treatment was included where symbionts were passaged through LB. Each treatment including a total of six symbiont passages (resulting in a total of three passages in each transmission environment for the HT treatment). Each treatment was independently replicated four times. Host fitness assays included measures of survival and development rate, and symbiont fitness included measures host colonization.

To select more rapid development in the HTH and EMH treatments, symbionts were isolated from the crypts of the first two hosts to reach fourth instar in each replicate. This time point was selected because previous works shows the *Caballeronia* symbiont begins having an effect on host development rate during this instar stage (Acevedo *et al*. 2021). Symbionts were isolated from crypts by dissection. Specifically, hosts were anesthetized using CO_2 and surface sterilized with 100% ethanol. Sterilized nymphs were then rinsed with Carlson's solution and allowed to dry. The crypt was removed, placed in 500uL Carlson's solution, and homogenized using a pestle. Crypt homogenate (50uL) was streaked onto YG-kan plates to isolate single colonies. To minimize the effects of population bottlenecks, we selected 50 GFP-labeled colonies from each crypt homogenate for passage to the next round of selection (50 colonies/crypt x 2 host crypts = 100 total colonies). Colonies were placed into LB-kan and grown overnight with shaking at 28 °C. Each overnight culture was prepared into glycerol stocks and stored at -80 °C until the next round of selection. For the HTH treatment, symbionts were then passaged to hosts in feeding solutions. For the EMH treatment, symbionts were inoculated into non-sterile soil (see below). For the EMH treatment, the complete transmission cycle (between hosts and soil) was repeated three times.

For environmental passage, fresh, non-sterile soil was collected from a site containing yellow crookneck squash plants in the Emory Educational Garden in Atlanta, Georgia. SMT4a for the EP treatment was revived from glycerol stocks, as described previously. Symbiont solutions were prepared as described previously, and soil (10g) was inoculated with ~$5x10^8$ cells/mL of SMT4a. Depending on the dryness of the soil, 500-600ul of water was added and mixed vigorously with a spatula. After inoculation, the symbiont was allowed to grow in soil for 14 days. GFP-labeled symbionts were recovered by collecting 1g of soil in an Eppendorf tube and

adding 600ul of Carlson's solution. Tubes were vortexed gently for 2 minutes and left to settle for at least 5 minutes. The supernatant was collected and streaked onto YG-kan in duplicate. We selected 100 GFP-labeled colonies for the next round of selection. Colonies were grown in LB-kan overnight with shaking at 28 °C. For the EP treatment, symbionts were then re-inoculated into soil. For the EMH treatment, symbionts were passaged to hosts in feeding solutions. Glycerol stocks were prepared for each passage and stored at -80 °C.

As a control, SMT4a was evolved through LB-kan for six passages. SMT4a was revived from glycerol stocks, as described previously. The symbiont was then grown in LB-kan overnight with shaking at 28 °C. Overnight cultures were streaked onto YG-kan agar plates to obtain single colonies. We selected 100 colonies for the next round of selection. Glycerol stocks were prepared for each passage.

Host fitness assays

To quantify the effects of symbiont evolution on host fitness, we measured host survival and development rate to adult. We picked a single representative symbiont colony from each replicate symbiont population to assay host fitness. Evolved symbionts were revived from glycerol by streaking onto LB plates. Hosts for fitness assays were starved overnight prior to symbiont inoculation. We performed inoculations using feeding solutions, as described for the experimental evolution. We inoculated thirty starved second instar nymphs with evolved symbionts from each replicate lineage using bacterial feeding solutions (n = 30 nymphs/replicate x 4 replicate lineages = total of 120 nymphs/treatment). We also inoculated thirty second instar nymphs with ancestral SMT4a for each replicate lineage (n = 30 nymphs/replicate x 4 replicate lineages = total of 120 ancestral inoculations). Finally, we included aposymbiotic controls for assays of each replicate lineage by starving nymphs overnight and providing feeding solutions

containing water but no symbiont. We prepared a total of thirty aposymbiotic control hosts per replicate lineage (n = 30 nymphs/replicate x 4 replicate lineages = total of 120 aposymbiotic controls). Feeding solutions were provided to hosts for 24 hours before replacement with surface sterilized zucchini wrapped in parafilm. Zucchini was replaced every two to three days. Host survival and developmental stage were recorded every two to three days until hosts reached adulthood.

Symbiont colonization assays

To quantify the effects of experimental evolution on symbiont colonization of hosts, we measured symbiont titers within host crypts and a nearby region of the gut, referred to as the M3 mid-gut. The M3 region is located at the anterior end of the crypt. To enter the crypt, symbionts must pass through the M3, which may serve as a filter. Microscopy and dissection of the gut demonstrates that SMT4a heavily colonizes the M3 region and may provide hosts some benefit by establishing in this region. While the symbionts are often associated within the crypt, research has shown other Hemiptera insects also harbor symbionts important for their fitness in the M3 region (Sudakaran *et al*. 2012; Salem *et al*. 2013), indicating symbiont colonization in multiple regions of the gut can have important fitness implications. Therefore, symbiont fitness was measured as the number of colony forming units (CFUs) per adult squash bug crypt and M3 (each measured separately). Experimental bugs that reached adulthood were surface sterilized in 70% ethanol. The crypt and M3 were manually dissected, placed separately in 1x PBS, and crushed. The homogenates were then serially diluted to 10^{-3}. Five replicates of each homogenate dilution were drop plated onto LB agar and allowed to air dry. Plates were incubated for 48 hours at 28 °C, and the number of GFP-labeled colonies was counted. When possible, calculations for the number of CFUs/crypt were performed using the dilutions containing more than twenty

CFUs/drop. However, titers within crypts were sometimes low, requiring calculations to depend on dilutions where CFUs/drop were less than twenty. CFUs per crypt and M3 were calculated by taking the average across all five replicate drops for a single dilution.

Statistics

We performed statistical analysis to assess the effects of experimental evolution on symbiont conferred host benefits relative to the ancestor. Therefore, aposymbiotic bugs were not included in statistical analyses. We performed mixed effects Cox proportional hazard models using the R package 'survival' to assess whether survival and development rates varied across symbiont treatments. For both, symbiont treatment was included as a fixed effect and replicate lineage was included as a random effect. For survival analysis, death was considered an event, and bugs were censored once reaching adult. For development rate analysis, we considered reaching adulthood as an event, and bugs that died before reaching adulthood were censored. Occasionally bugs were accidentally killed while collecting data, and these bugs were censored. When effects of treatment were observed, we performed post-hoc analysis to assess differences from ancestor using the 'treatment versus control' function in the R package 'emmeans.' Host survival was further analyzed using a Chi-squared test to determine whether the proportion of bugs surviving to adulthood and between developmental life stages varied across experimental treatments. Effects of treatment were further assessed using Tukey's post-hoc analysis. We used a mixed effect generalized linear model to test whether the logCFU/crypt and logCFU/M3 varied in response to experimental treatment. Treatment was included as a main effect and replicate as a random effect. Statistics were performed in R, version 4.1.0.

Results

We designed our experimental evolution to impose selection on symbionts for increased symbiont-conferred benefits across host-associated treatments. Specifically, we selected for more rapid development to fourth instar. However, we observed no differences between treatments for rate of development to fourth instar or to adult (Figure 1A).

Symbiont evolution had a large effect on host survival (Figure 2B-D). We observed a significant effect of treatment on host survival over time ($\chi^2 = 39.98$, df = 5, $p < 0.0001$). Relative to hosts paired with ancestral symbionts, hosts paired with any experimentally evolved symbionts exhibited reduced survival. However, relative to those paired with the ancestor, significant differences in survival were only observed for hosts paired with the HTH and EMH evolved symbionts (HTH-Ancestor: $p = 0.0001$; EMH-Ancestor: $p = 0.0002$) (Figure 2B-D). Across all replicate lineages, host-associated symbionts had deleterious effects on host survival relative to the ancestor. Overall, EP symbionts did not significantly differ in their effect on host survival relative to the ancestor. However, EP symbionts varied widely in their effects on host survival across replicate lineages. Relative to the ancestral symbiont, EP symbionts from replicate lineages two and four exhibited deleterious effects on host survival, while EP symbionts from lineages one and three improved or maintained similar effects on host survival.

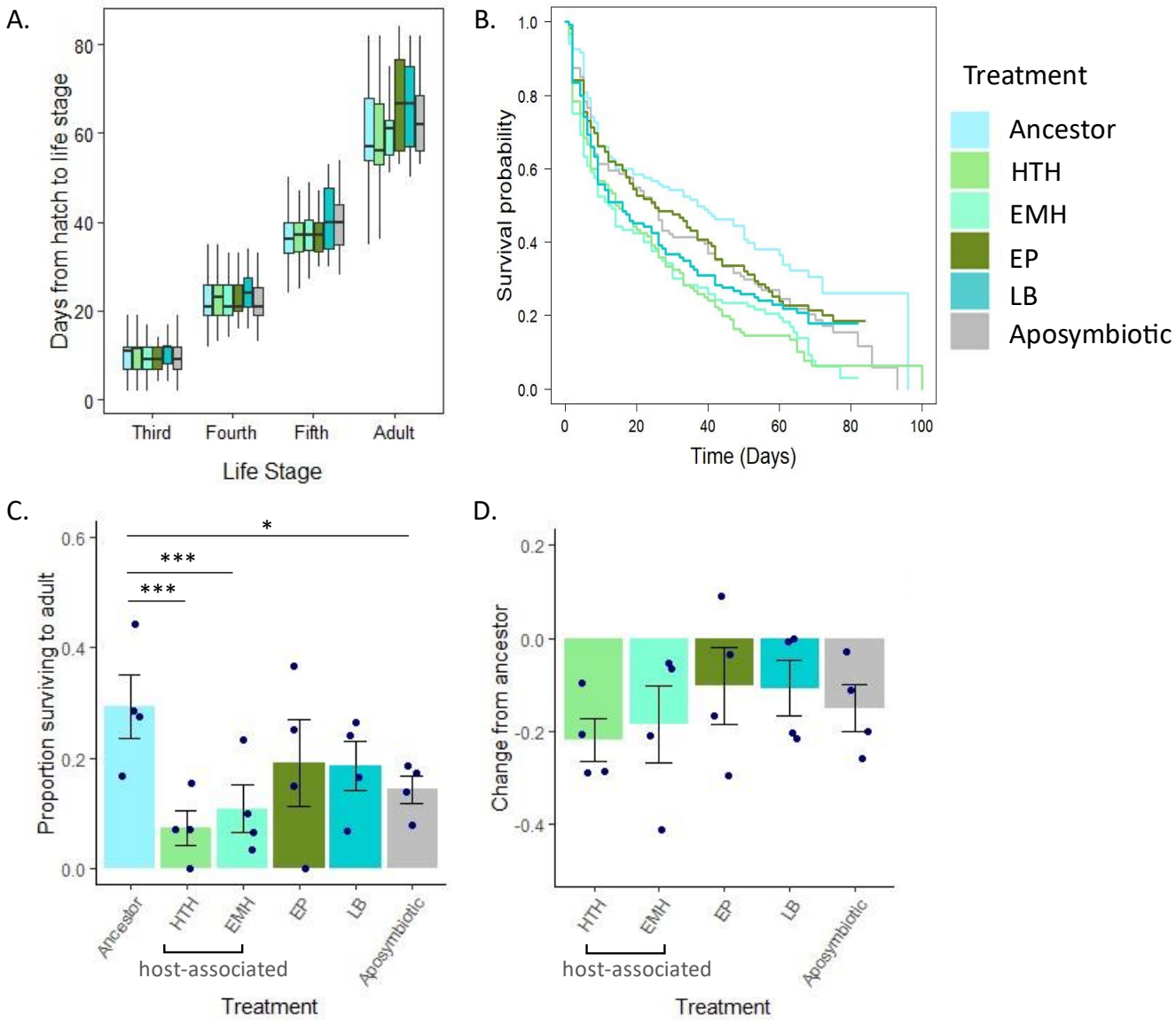


Figure 2. Host fitness data. We assessed the effects of symbiont treatment on host development rate and survival. (A) Host development time across all life stages. We observed no differences in development rates across experimental treatments. (B) Survival curves for hosts paired with each symbiont. Survival for hosts paired with symbionts evolved under host-to-host transmission (HTH) and environmentally-mediated host transmission (EMH) exhibited significantly reduced survival compared to the ancestor. (C) Proportion of hosts surviving to adult when paired with symbionts from each treatment. (D) Difference in the proportion of hosts surviving to adult for each experimental treatment relative to the ancestor. Survival to adult was significantly reduced for hosts without symbionts (Aposymbiotic) and those paired with HTH and EMH symbionts. For plots C and D, error bars represent the standard error across the four replicate lineages. Points represent the proportion of bugs surviving to adult (C) and change in survival to adulthood relative to the ancestor (D) for each individual replicate lineage.

We also observed a significant effect of treatment for the proportion of hosts surviving to adult ($\chi^2 = 22.99$, df = 5, $p = 0.0003$). This effect resulted from hosts paired with HTH and EMH symbionts exhibiting significantly lower survival to adulthood than hosts paired with the ancestral symbiont (HTH-ancestor: $p < 0.0001$; EMH-ancestor: $p = 0.0013$). Finally, while we observed effects of treatment for overall survival, we did not observe treatment-level effects on the rate of survival between instar stages (Figure S1), indicating we did not select for increased symbiont virulence at any particular host developmental life stage.

Overall, we observed high rates of host colonization across symbionts replicates (60-83%) (Figure 3). Rates of colonization across all replicates varied between the M3 midguts (54-83%) and crypts (47-80%). Symbiont titers were higher in the M3 region of the gut than the crypt ($F_{1,70} = 9.23$, $p = 0.003$) (Figure 3). However, we observed no significant differences in the colony forming units per M3 or per crypt across experimental treatments. These results indicate that we did not select for increased adult colonization.

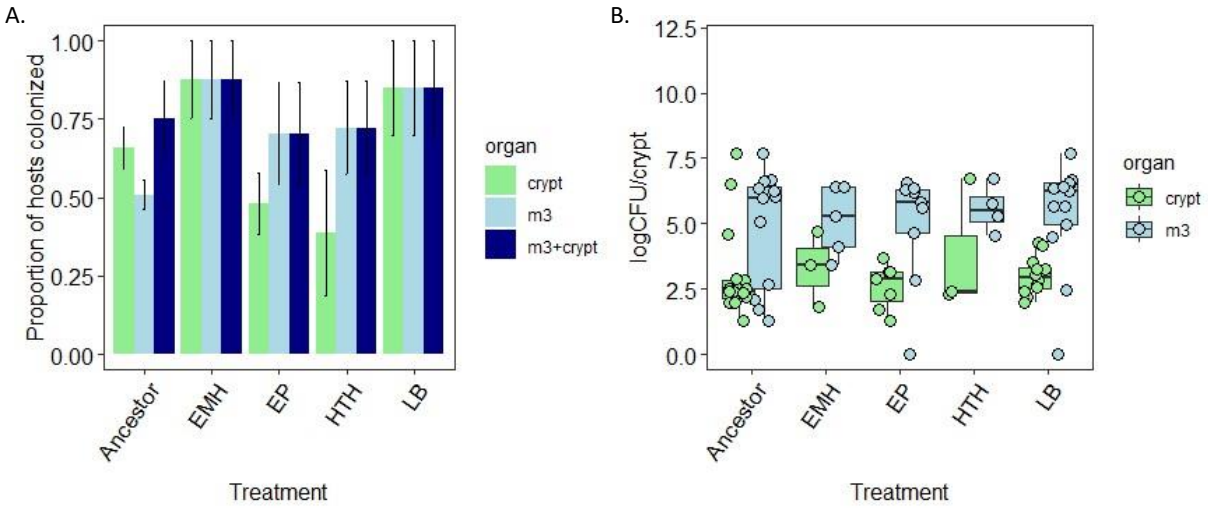


Figure 3. Symbiont colonization and within host titers. Hosts that matured to adult were dissected and assessed for the presence of the GFP-labeled symbiont. (A) Proportion of dissected adults from each treatment for which the symbiont was recovered from the crypt (green) or the m3 midgut (light blue). Overall proportion of symbiont-positive bugs shown in dark blue. Error bars represent the standard error across the four replicate lineages. (B) Symbiont titers in host crypts (green) and the m3 midgut (light blue). Symbiont titers were significantly higher within the m3 midguts compared to the crypts. Symbiont was not recovered from aposymbiotic bugs, which are not shown here.

Discussion

We used experimental evolution to directly test whether transmission environment alters selection for the maintenance of cooperative symbiont traits. The squash bug symbiont *Paraburkholderia* was experimentally evolved through four environments to test the effects of consistent selection from hosts (HTH), alternating selection from hosts and the environment (EMH), consistent selection from the environment (EP), and stochastic processes (LB). We imposed selection for increased symbiont cooperation by passaging symbionts associated with hosts exhibiting the fastest rates of development to fourth instar. Despite imposing selection for increased cooperative symbiont traits, symbionts across all experimentally evolved treatments

exhibited deleterious effects on host survival relative to the ancestor. Surprisingly, symbionts evolved with hosts, both within the HTH and EMH treatments, exhibited the most pronounced evolution of deleterious traits for host fitness. In contrast, symbionts that evolved apart from hosts under environmental transmission (EP) and through culture media (LB), decreased host fitness relative to the ancestor but evolved deleterious traits less rapidly than those evolved with hosts. Our results demonstrate that fitness alignment is not a guaranteed consequence of host-associated transmission and suggest potential benefits of decoupling symbiont evolution from the host environment.

One potential benefit of decoupling symbiont evolution from the host is decreased opportunities for symbionts to evolve exploitative traits. Previous research using plants and rhizobial bacterial symbionts has demonstrated that symbionts can be under selection for host exploitation (Porter and Simms 2014). Within our experimental evolution, host-associated treatments (HTH and EMH) most rapidly evolved deleterious traits affecting host survival. In contrast to the host-associated treatments (HTH and EMH), environmentally passaged (EP) symbiont evolution was completely decoupled from the host environment. Therefore, these symbionts were not under selection for exploitation, and they exhibited fewer deleterious effects on host fitness.

Within host-associated treatments, symbionts were given repeated opportunities to adapt to their hosts, though pathways by which hosts could prevent exploitation were limited. Specifically, our study limited opportunities for hosts to invoke partner choice. Effective partner choice requires hosts to have opportunities to select between symbionts of varying quality (Foster and Kokko 2006; Akçay 2017). We attempted to maintain variation in symbiont populations that arose through *de novo* mutation or through interaction with other microbes (in soil environments) by selecting a large number of colonies for each passage. However, because variation was largely

required to arise *de novo* and passage through hosts likely resulted in large within-host transmission bottlenecks, variation within symbiont populations, and thus opportunities for hosts to invoke partner choice, were likely limited.

Additionally, hosts were not co-passaged with symbionts, preventing coevolution. Experimental evolution between legumes and rhizobia has demonstrated that reciprocal selection between host and symbiont genotypes may be more important for the evolution of cooperative symbiont traits than partner choice (Batstone *et al*. 2020). Therefore, we may observe different outcomes if host and symbiont have opportunities to coevolve. We attempted to artificially impose selection for increased cooperative symbiont traits by selecting symbionts associated with the fastest developing hosts. However, across our experimental treatments, we did not observe changes in the rate of host development, indicating selection may not have been effective. By inhibiting host evolution, symbionts experienced weak selection, if any, for increased cooperation and reduced exploitation.

Alternatively, the deleterious consequence of symbiont evolution within the host environment may have resulted through genetic drift. Host-associated symbiont populations likely experienced large population bottlenecks during host colonization. Opportunities for the introduction of genetic variation within host-associated symbiont populations was then limited further because these symbionts spent little time decoupled from the host environment. Within the HTH treatment, opportunities for horizontal gene transfer through interactions with other microbial species were completely eliminated. Variation could only be introduced through *de novo* mutation within the host environment. Horizontal gene transfer was possible within the EMH treatment, but if variation was introduced, it may have been quickly eliminated through within-host bottlenecks during the next round of selection.

In contrast, symbionts evolved within the EP and LB treatments exhibited fewer deleterious effects on host fitness. These symbionts were decoupled from the host environment and did not undergo within-host transmission bottlenecks, potentially maintaining genetic variation within these replicates. The effects of EP symbionts on host survival varied greatly across replicate lineages. This variation likely resulted from selection within the soil environment, possibly through competition with other microbes, that incidentally selected for symbiont traits that benefitted hosts within some replicates while selecting for traits that harmed hosts in others. Evolution within the soil environment likely resulted in interactions with other microbes, potentially increasing opportunities for horizontal gene transfer. As a result of decoupling symbiont evolution from the host environment, these symbiont populations were more likely to evolve and maintain genetic variation. However, these symbionts populations began with little genetic variation. Therefore, the reduction of symbiont-conferred host benefits within the EP treatment relative to the ancestor may also have resulted from genetic drift, but, at a slower rate than in host-associated symbiont populations undergoing large within-host bottlenecks.

Conclusion

Overall, this experimental evolution demonstrates that symbiont transmission environment can alter the direction of selection for cooperative symbiont traits. Surprisingly, we observe a rapid loss of cooperative traits across host-associated symbiont evolution treatments, including both Host-to-Host (HTH) and Environmentally-Mediated Host (EMH) transmission. In contrast, symbionts evolved within the Environmental Passage (EP) treatment exhibited fewer deleterious effects on host fitness. The rapid loss of cooperative traits across host-associated symbiont treatments likely resulted through opportunities for symbionts to evolve exploitative traits or through the loss of important functions through genetic drift. We cannot definitively state which

of these evolutionary forces underlies changes in symbiont-conferred benefits. Genetic variation was likely very low across all treatments, increasing the potential for genetic drift to underlie symbiont evolution. Within-host populations bottlenecks likely increased the rate at which deleterious traits evolved for symbionts evolved in the host environment. Although, it is also feasible that the rapid evolution of deleterious symbiont traits across host-associated treatments resulted through a combination of genetic drift and selection for exploitation. In the future, we plan explore these alternative explanations further by assessing genomic changes to experimentally evolved symbionts. Future work should also further explore the effects of transmission environment on the evolution of cooperative traits, specifically considering the consequences of partner choice and coevolution.

Supplemental information

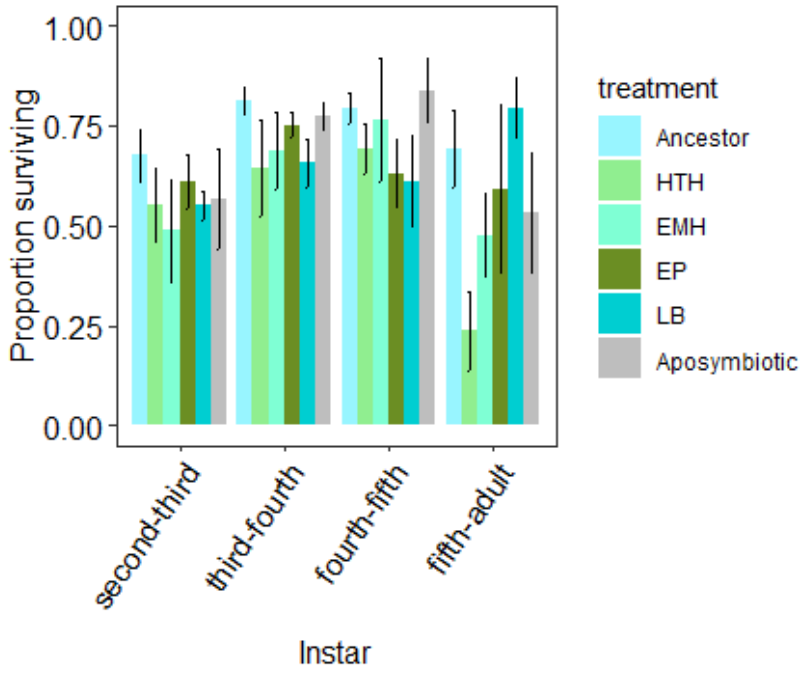


Figure S1. Proportion of hosts surviving from one instar to the next. We observed no effect of treatment on the proportion of hosts that survived from one treatment to the next.

CHAPTER V

CONCLUSION

This dissertation examines the eco-evolutionary conditions that facilitate the persistence of mutualistic interactions between hosts and their microbial symbionts. The stability of mutualistic interactions depends on the alignment of host and symbiont fitness. This is theorized to occur most readily under vertical transmission, which aligns host and symbiont fitness interests. In contrast, fitness interests may become misaligned under horizontal transmission because symbionts do not depend on their hosts for transmission. Despite these predictions, ancient horizontally transmitted interactions are common in nature. How these horizontally transmitted interactions persist despite the potential costs remains an unsolved problem in evolutionary biology. I addressed this question by leveraging a naturally-occurring interaction between the squash bug *Anasa tristis* and its horizontally transmitted bacterial symbionts. Specifically, I tested whether coevolution contributes to the persistence of the horizontally transmitted mutualism between *A. tristis* and its symbionts. I then directed tested whether transmission environment, like those experienced under horizontal and vertical transmission, alters the direction of selection for cooperative symbionts traits.

The role of coevolution in horizontally transmitted interactions

Pairwise coevolution, reciprocal adaptation between two species, has been the focus of many studies examining species interactions. This form of coevolution may stabilize mutualisms if co-localized lineages of host and symbiont exert strong selection on one another for cooperative traits across generations. If tight pairwise coevolution underlies an interaction, the interacting species will exhibit patterns of host-symbiont specialization. Diffuse coevolution, reciprocal adaptation between a range of species with a shared common partner, may also drive the maintenance of cooperative traits between hosts and symbionts. However, less empirical work has tested for evidence of diffuse coevolution across symbiotic interactions. Under diffuse coevolution, mutualistic interactions are primarily shaped by

asymmetric interactions between generalists and specialists. In Chapter III, I tested for patterns of host-symbiont specialization consistent with pairwise versus diffuse coevolution. I found no evidence for specialization consistent with pairwise coevolution between *Anasa* insect hosts and their *Caballeronia* symbionts. Instead, I observed generalist dynamics consistent with diffuse coevolution. Furthermore, I found no evidence for population structure across the geographic range of *A. tristis*, suggesting a high level of gene flow between populations. These results indicate that specialization may be costly within this horizontally transmitted mutualism by reducing the probability that hosts will find and associate with compatible symbionts. Rather, hosts and symbionts are likely under selection for generalism, which may facilitate host dispersal over a large geographic range. In contrast to their hosts, *Caballeronia* symbionts exhibit patterns of geographic variation across their natural range. Therefore, hosts may benefit by horizontal transmission and generalism, which potentially allow them to acquire symbionts that are locally adapted to the environment. The overall strength of selection for cooperative symbiont traits may be maintained through frequent interactions with a range of insect hosts.

The effects of transmission environment on cooperative symbiont traits

Vertical transmission is theorized to stabilize mutualistic interactions by facilitating fitness alignment and increasing opportunities for coevolution. In contrast, horizontal transmission can disrupt partner fidelity, potentially misaligning fitness interests and reducing opportunities for coevolution. Despite a long history of theory highlighting the potential costs of horizontal transmission, few studies have empirically tested whether transmission mode alters selection for cooperative symbiont traits. In chapter IV, I use experimental evolution to directly test whether transmission environment, like those experienced by symbionts under vertical and horizontal transmission, alters the direction of selection for cooperative symbiont traits. I experimentally evolved the *Paraburkholderia* symbiont of *A. tristis* under frequent host exposure, exposure to both hosts and the environment, and through the environment without host exposure. I found that host-associated evolution produced symbionts that rapidly reduced host fitness. In contrast, environmentally passaged symbionts exhibited fewer deleterious effects on their hosts. The

reduction of symbiont-conferred benefits under host-associated evolution likely occurred through selection for exploitative traits or due to population bottlenecks that resulted in the accumulation of deleterious mutations through genetic drift.

A growing body of evidence has suggested potential benefits of environmental symbiont acquisition. Environmental transmission partially decouples symbiont evolution from the host environment, which may reduce the strength of selection on symbionts to evolve exploitative traits. Environmental transmission may also allow hosts to acquire environmentally relevant, locally adapted symbionts. Finally, environmental transmission can reduce the probability of symbionts to undergo within host transmission bottlenecks that can lead to the loss of beneficial symbiont traits. The maintenance of genetic variation within symbiont populations may also increase the efficacy of selection on symbiont populations from hosts and their external environments, potentially facilitating the evolution of cooperative symbiont traits.

Future directions

In general, the causes and consequences of processes that maintain genetic and phenotypic variation within mutualism, such as horizontal transmission, have yet to be understood. Future work in the field of mutualism must focus on developing an evolutionary genetics framework to understand the coevolutionary dynamics underlying host-symbiont mutualisms. This dissertation identifies a potential role for diffuse coevolution to maintain stability across mutualistic interactions between three insect host species with their shared *Caballeronia* symbionts. However, it does not demonstrate direct evidence for diffuse coevolution. Future work within this system should aim to identify the exact mechanisms of benefit exchange between *Anasa tristis* and *Caballeronia*, determine the genes underlying the interaction, and test for evidence of genetic polymorphism at these specific sites within the genome to better assess whether genetic variation exists across these interactions. Once the mechanisms and genes underlying benefit exchange have been identified, we can test whether diffuse coevolution maintains the interactions

between *Anasa* insects and *Caballeronia* by assessing whether phenotypic traits across each pairwise interaction are genetically correlated.

Experimental evolution should also be used to investigate the consequences of pairwise versus diffuse coevolution within mutualistic interactions. This can be accomplished by comparing the evolutionary trajectories of mutualistic partners that are co-passaged with tight partner fidelity versus those that are co-passaged with reduced partner fidelity. Under tight partner fidelity, mutualists will see the same partner each passage, increasing opportunities for pairwise coevolution. Under loose partner fidelity, mutualists will alternate between partners each passage, decreasing opportunities for pairwise coevolution but increasing opportunities for diffuse coevolution. Theory suggests that the evolution of increased cooperative symbiont traits should evolve under pairwise coevolution because specialized partners can select for increased rewards relative to generalists. However, future work should also consider the conditions under which specialized versus generalist interactions are favored and whether there are eco-evolutionary tradeoffs associated with each strategy. This may provide important insights into the eco-evolutionary conditions under which we may observe mutualistic interactions that are stabilized by pairwise coevolution versus diffuse coevolution.

Furthermore, more work should test for evidence of pairwise and diffuse coevolution in natural host-symbiont populations. These efforts should include targeted approaches to quantify genetic polymorphism across natural populations and patterns of host-symbiont specificity over geographic and temporal timescales. The eco-evolutionary consequences of pairwise versus diffuse coevolution in host-symbiont mutualistic interactions should be explored using empirical approaches, including experimental evolution, to test whether altering community composition changes the stability and evolution of mutualisms. Moreover, we should aim to disentangle the roles of indirect coevolutionary cascade from diffuse coevolutionary interactions and consider the differential implications of these processes for the stability of mutualism. Once concerted efforts have been made to test for evidence of coevolution across mutualistic interactions, we should assess whether the prevalence of coevolution varies across vertically

versus horizontally transmitted interactions. It is possible that the prevalence of coevolution may not vary across these interactions, but the form of coevolution does.

Finally, many mutualisms are characterized by quantitative traits, such that many genes underlie the interaction. In contrast, many of the evolutionary genetics models used to understand coevolutionary interactions are based on interactions at few loci. This theoretical framework stems from host-parasite interactions, which are more frequently dependent on interactions at a few loci rather than quantitative interactions. Future work should consider the implications of quantitative traits for long-term coevolutionary host-symbiont interactions. For example, species interactions dependent on quantitative genetic interactions may evolve at slower rates and/or may be evolutionary constrained if multiple loci required for the interaction experience differential selection outside the interaction. Mutualistic interactions also often exhibit greater context-dependency than host-parasite interactions, which may further complicate and obscure the (co)evolutionary consequences of interactions mediated by many loci. Developing an evolutionary genetics framework for understanding the coevolutionary dynamics associated with host-symbiont mutualisms will be an important step forward to address these questions and improve understanding for how host-symbiont mutualisms persist.

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Fellowships, Grants, and Awards

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2017-2022	Graduate Research Fellowship, National Science Foundation
2017-2022	Laney Fellowship, Emory University Laney Graduate School
2014	Outstanding Honors Thesis Award, Indiana University Department of Biology
2014	Phi Beta Kappa Induction, Indiana University
2013	Hutton Honors College Research Grant, Indiana University Hutton Honors College
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2011	JE Leonard Scholarship, Indiana University

Peer-Reviewed Publications

*Undergraduate mentee

9. **Stoy, K.S.**, Diaz, Erika, Morran, L.T., Gerardo, N.M. (2022). Host-associated symbiont transmission rapidly reduces cooperative symbiont traits. *In prep.*

8. **Stoy, K.S.**, Chavez, J., *De Las Casas, V., Berasategui, A., Morran, L.T., Gerardo, N.M. (2022). Evaluating coevolution in a horizontally transmitted mutualism. *Submitted*.

7**.** Mendiola, S.Y., **Stoy, K.S.**, DiSalvo, S., Wynn, C., Civitello, D.J., Gerardo, N.M. (2021). Competitive exclusion of phytopathogenic *Serratia marcescens* from squash bug vectors by the facultative endosymbiont *Caballeronia*. *Applied and Environmental Microbiology*. DOI: 10.1128/AEM.01550-21

6. Acevedo, T.S., Fricker, G.P., Garcia, J.R., Alcaide, T., Berasategui, A., **Stoy, K.S.**, Gerardo, N.M. (2021): The importance of environmentally-acquired bacterial symbionts for the squash bug (*Anasa tristis*), a significant agricultural pest. *Frontiers in Microbiology.* DOI: 10.3389/fmicb.2021.719112

5. **Stoy, K.S.**, Gibson, A.K., Gerardo, N.M., Morran, L.T. (2020): A need to consider the evolutionary genetics of host-symbiont mutualisms. *Journal of Evolutionary Biology.* DOI:10.1111/jeb.13715

4. Gerardo, N.M., Hoang, K.L., **Stoy, K.S.** (2020): Evolution of animal immunity in the light of beneficial symbioses. *Philosophical Transactions of the Royal Society B.* 375:20190601. http://dx.doi.org/10.1098/rstb.2019.0601

3. Gibson, A.K., **Stoy, K.S.**, Lively, C.M. (2018): Bloody-minded parasites and sex: the effects of fluctuating virulence. *Journal of Evolutionary Biology*. DOI: 10.1111/jeb.13253.

2. Vergara, D., Fuentes, J.A., **Stoy, K.S.**, Lively, C.M. (2016): Evaluating Shell Variation Across Different Populations of Freshwater Snail. *Molluscan Research*. DOI: 10.1080/13235818.2016.1253446.

1. Gibson, A.K., **Stoy, K.S.**, Gelarden, I.A., Lively, C.M., Morran, L.T. (2015): The evolution of reduced antagonism – a role for host-parasite coevolution. *Evolution*. 69(11): 2820-2830.

Peer-Reviewed Science Communication

1. **Stoy, K.S**. and Gibson, A.K. (2018). Bloody-minded parasites and sex. *The Science Breaker.* https://doi.org/10.25250/thescbr.brk130

Presentations

2021	Evaluating the role of coevolution in a horizontally transmitted mutualism. Contributed talk, Evolution Conference. *Virtual*.
2021	Evaluating the role of coevolution in a horizontally transmitted mutualism. Invited Talk, University of Virginia, Department of Ecology and Evolutionary Biology Seminar Series. *Virtual*.
2018	Evaluating pathways of horizontal transmission of an insect microbial mutualist. Poster, Southeastern Population Ecology and Evolutionary Genetics Conference. Charlottesville, VA.
2015	The evolution of reduced antagonism – a role for host-parasite coevolution. Contributed Talk, Midwest Ecology and Evolution Conference. Bloomington, IN.

Teaching

2022	Teaching Assistant: Undergraduate Biology Research for Credit, Emory University
2021	Teaching Assistant: Undergraduate Biology Research for Credit, Emory University
2019	Teaching Assistant: Foundations of Modern Biology I, Emory University
2017	Teaching Assistant: Evolutionary Biology, Emory University

Undergraduate Mentoring

2020-2022	Madison Hopkins, Emory University (Research for credit)
2019-2021	Valeria de las Casas, Emory University (Honors thesis)
2019-2020	Ritika Manik, Emory University (Volunteer)
	Ishani Vyas, Oglethorpe University (Volunteer)
2018	Heather Beavers, Emory University (Volunteer)

Training

2021	High Throughput Genomic Analysis. Canadian Bioinformatic Workshops. Virtual.
2019	Guarda Evolutionary Biology Summer School. Guarda, Switzerland.

Service

2020-2022	Seminar Committee; Department of Biology, Emory University
2018-2022	Seminar Committee; Population Biology, Ecology, and Evolution Program Emory University (Member 2018; President 2019-2022)

Outreach

2022	Volunteer at Carrie Steele-Pitts House for at-risk youth. Led discussions about stress management, preparing for scholarships, and studying for finals in college.
2022	Judge at Georgia State Science and Engineering Fair. Athens
2019 - 2020	After school tutor and mentor for K-12 children at East Atlanta Kids Club, an afterschool program for underrepresented children from under-resourced, low-income, and primarily minority school districts. This volunteerism has been suspended due to the pandemic but will resume when permitted.
2019	Volunteer at an NSF-sponsored science camp for children from the Edgewood, Atlanta neighborhood, a low-income community and under-resourced school district
2019	Lunch and informal talk with undergraduates about research careers - Merry Lea Environmental Learning Center at Goshen College, Albion, IN
2017-2019	Elementary school hands-on “Mycrobes” science outreach sponsored by Gerardo Lab.
2018	Judge at Georgia State Science Fair. Athens, GA. March 2018.
2010-2014	University Coalitions for Global Health (Secretary 2012-2013; President 2013-2014).