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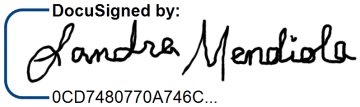
Signature: 

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The microbial symbiont of an agricultural insect pest affects pathogen vectoring across scales

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An abstract of
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Abstract

The microbial symbiont of an agricultural insect pest affects pathogen vectoring across scales
By Sandra Y Mendiola

Many insects form intimate associations, known as symbioses, with select microbes. Some symbioses can influence traits that contribute to vector competence—an insect vector's ability to harbor and transmit a pathogen. Leveraging symbioses to mitigate the burden of agricultural vector-borne diseases has gained traction in recent years; however, implementation of these tactics requires a deeper understanding of the impact of symbionts on pathogen vectoring in individual insects and, more importantly, their implications for population-level pathogen transmission.

My dissertation uses the squash bug (*Anasa tristis*) to address how symbiotic microbes influence pathogen vectoring by an insect host. *A. tristis* is a pest of commercial cucurbit crops and the only confirmed vector of *Serratia marcescens*, causative agent of Cucurbit Yellow Vine Disease (CYVD). *A. tristis* forms symbiotic associations with bacteria in the genus *Caballeronia*, which colonizes specialized structures in their gut, known as crypts. Previous work has shown that *Caballeronia* accelerates host development and decreases mortality relative to symbiont-free (aposymbiotic) individuals.

My dissertation employs a multiscale approach to study the interactions between *A. tristis*, *Caballeronia*, and *S. marcescens.* Within individual insects, I demonstrated that *Caballeronia* has a marked effect on *S. marcescens* infection, with symbiotic individuals having shorter infections of lower intensity than their aposymbiotic counterparts. I also investigated how *Caballeronia* influences insect gene expression and its consequences for pathogen establishment. To see the impact of these findings on insect populations, I created a model of CYVD transmission that explicitly accounts for the presence of symbiotic and aposymbiotic bugs. I showed that aposymbiotic bugs contribute disproportionately to plant infections and high symbiont coverage in insects is needed for effective pathogen control.

To fully realize the potential of insect-microbe symbioses in combating vector borne disease, we must first understand how symbionts interact with pathogens within their insect hosts and ensure that patterns observed at the individual level have the desired effect when scaled to entire populations. My work investigating the influence of symbiotic microbes on *A. tristis* across scales provides a framework for the integration of vector biology and disease ecology to investigate the interactions between insects, pathogens, and symbionts.

The microbial symbiont of an agricultural insect pest affects pathogen vectoring across scales

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Lastly, I want to thank myself for seeing this through. There were many moments when this seemed too daunting to continue, but I have persisted, in no small part thanks to the many people I have mentioned here. Lo hicimos! Adelante!

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Chapter I: Introduction

Complex interspecies interactions are ubiquitous in our world, as evidenced by the prevalence of symbioses—defined as persistent interactions between individuals of different species. Symbioses are diverse, encompassing corals and photosynthetic algae, legumes and nitrogen-fixing bacteria, and even humans and their gut microbes. Beneficial microbial symbionts are particularly common in insects, whose success as one of the most diverse groups of animals on Earth can be attributed to the myriad services provided by their symbionts. Symbiotic microorganisms can have marked effects on their insect hosts. Within insects, microbial symbionts have been shown to serve a variety of beneficial roles, such as satisfying nutritional requirements (Douglas 1998), conferring increased resistance to abiotic stressors (Neelakanta et al. 2010), or protecting against parasitoids (Brownlie and Johnson 2009). Associations with defensive microbes can also be a significant line of defense for insects against pathogens (Łukasik et al. 2013, Miller et al. 2021).

The surge in microbiome research has drawn attention to the ways that insects' own microbial communities can be exploited as tools for controlling insect pests and disease vectors [Table 1]. The widespread nature of insect-microbe symbioses, their importance in insect ecology, and their specificity make them ideal candidates for novel insect control methods. Proposed methods of symbiont-mediated vector control range from disrupting symbioses that are critical for insect survival to deploying symbiotic microbes capable of affecting insects' vectoring capacity. The potential for mediating host-pathogen interactions has made insect symbionts the subject of much interest, especially in cases where insect hosts act as vectors that can transmit pathogens to humans, wildlife, and agricultural crops.

Symbiotic microbes can interact with an invading pathogen directly or indirectly, often making them important players in driving host infection outcomes (Gerardo and Parker 2014).

Studies transplanting one or a few key symbiont species between hosts have successfully created pathogen resistant phenotypes in previously susceptible insects (Moreira et al. 2009, Koch and Schmid-Hempel 2011, Gonella et al. 2018). In fact, the insect microbiota has emerged as a strong determinant of vector competence, an insect's ability to harbor and transmit pathogens, in several systems (Cirimotich et al. 2011, Weiss and Aksoy 2011).

Their influence on vector competence presents the opportunity to exploit symbiotic associations for vector-borne disease control (Rio et al. 2004, Douglas 2007, Zindel et al. 2011, Crotti et al. 2012). Thus far, research into symbiont-mediated vector control methods has focused on interventions for human pathogens. The utility of such approaches has been established most notably for dengue and Chagas disease (Beard et al. 2002, Moreira et al. 2009, Caragata et al. 2016). More recently, the use of symbiotic microbes to reduce the impact of vector-borne diseases in agriculture has garnered much discussion (Chuche et al. 2017, Arora and Douglas 2017, Gonella et al. 2020), but research has not yet progressed to deployment in the field.

Vector-borne pathogens pose significant threats to agricultural productivity. The increasing incidence of insecticide resistance and growing concern for environmental impacts associated with the use of broad-spectrum insecticides have led to a search for more targeted and sustainable ways to mitigate vector-borne disease, making vectoring insects ideal candidates to consider for symbiont-mediated vector control. Despite many well-characterized symbioses in insect pests (Douglas 1998, Kikuchi et al. 2011, Prado and Zucchi 2012), progress comparable to that in the control of vector-borne human pathogens has been slow. Though many symbioses show promise for vector control in the laboratory, field applications remain elusive. The application of symbiont-mediated vector control techniques in agriculture has been hindered by

the complex, multipartite interactions between plants, pathogens, vectors, and symbionts, which complicate predictions of symbiont impact on agricultural disease.

The overarching goal of this dissertation is to understand the tripartite interactions between insect vectors, their symbionts, and the pathogens that they transmit, as well as the consequences of those within-host processes on vector competence and broader-scale pathogen transmission. I investigate these questions using the squash bug *Anasa tristis* DeGeer (Hemiptera: Coreidae).

A. tristis are well-documented pests of commercial cucurbit crops across North and Central America (Beard 1940). *A. tristis* survives almost exclusively on cucurbit plants, including several commercially important crops, such as squash, zucchini, pumpkin, and watermelon (Bonjour and Fargo 1989, Doughty, Wilson et al. 2016). In addition to damaging crops through feeding, *A. tristis* is the only confirmed vector of *Serratia marcescens*, causative agent of Cucurbit Yellow Vine Disease (CYVD) (Bruton et al. 1998, Bextine et al. 2001). *A. tristis* undergo incomplete metamorphosis, with five distinct nymphal stages before reaching adulthood. Nymphs begin feeding in earnest at the second instar stage, after which they may acquire *S. marcescens* at any point. Infection with *S. marcescens* has been shown to persist through transstadial molts (Wayadande et al. 2005) and in overwintering adults (Pair et al. 2004).

A. tristis form symbiotic associations with bacteria in the genus *Caballeronia*—formerly classified in the genus *Burkholderia* (Dobritsa and Samadpour 2019)—which colonizes specialized structures, called crypts, in the squash bug's midgut. *A. tristis* acquire free-living *Caballeronia* symbionts *de novo* from the environment at every generation, most commonly in the second instar stage. *Caballeronia* symbionts are common among heteropteran insects (Kikuchi et al. 2011, Itoh et al. 2014, 2014) and have been shown to confer various fitness benefits to their hosts, including:

insecticide resistance (Kikuchi et al. 2012), enhanced innate immunity (Kim et al. 2015), and increased growth and fecundity (Lee et al. 2017). In *A. tristis*, association with *Caballeronia* accelerates development and decreases mortality relative to aposymbiotic (symbiont-free) individuals (Acevedo et al. 2021).

Tripartite interactions between insects, symbionts, and pathogens are multi-faceted and complex. Investigating these interactions and their potential for disrupting disease transmission is an important first step in crafting successful symbiont-mediated vector control tactics. The squash bug system is well-suited for studying insect-symbiont-pathogen interactions: 1) squash bugs can be reared year-round in the lab; 2) *Caballeronia* and *S. marcescens* are both cultivable and amenable to experimental and genetic manipulation; 3) environmental acquisition of *Caballeronia* allows for rearing of symbiont-free bugs or for the introduction of symbiont strains in a controlled fashion.

The continued spread and rise of CYVD incidence across the United States and its devasting impact on crop yields have highlighted the need for additional squash bug control methods, particularly for organic farmers (Doughty et al. 2016). My dissertation investigates the potential to use *Caballeronia* symbionts to disrupt the transmission of *S. marcescens* by squash bugs. I investigate how association with *Caballeronia* affects the capacity for *S. marcescens* to infect and persist in *A. tristis*, what the implications of these effects are for pathogen transmission at the population level, and how *Caballeronia* may affect host gene expression to enable differences in pathogen infection outcomes.

Summary of Dissertation Chapters

In Chapter II, I give an overview of symbiont-mediated vector control and review current advances in vector control that make use of symbiotic microbes. I further emphasize the importance of integrating empirical and theoretical research when developing symbiont-

mediated vector control strategies. This approach ensures that candidate symbionts for vector control that show promise under laboratory settings will have the intended outcome when deployed in the field.

Chapter III delves into how symbiont status affects the potential of *A. tristis* to vector phytopathogenic *S. marcescens*. In this chapter, I infect symbiotic and aposymbiotic (symbiont-free) individuals with *S. marcescens* then track the prevalence and intensity of *S. marcescens* infection in individuals over time. Through these methods, I show that symbiotic individuals have pathogen infections of much lower intensity and shorter duration than their aposymbiotic counterparts, a pattern that was generalizable across several symbiont strains. Furthermore, I show that reversing the order of exposure to symbiont and pathogen yields the same results. Thus, any insect receiving symbiotic bacteria, regardless of pre-existing pathogen infection, will have a low intensity pathogen infection that is cleared rapidly. Meanwhile, symbiotic bacteria show no signs of hindrance in host colonization ability due to the presence of pathogenic bacteria, reaching the same titers in the hosts across all conditions tested.

In Chapter IV, I expand on the results of Chapter III to model how the individual-level trends in pathogen infection outcomes translate to a field setting. For this chapter, I built and parameterized a model of Ordinary Differential Equations that captured Cucurbit Yellow Vine Disease transmission dynamics while keeping track of a squash bug vector population that consisted of symbiotic and aposymbiotic individuals that were either susceptible to or infected with *S. marcescens*. The model treated symbiotic individuals as the baseline while altering parameters for aposymbiotic bugs' pathogen clearance and transmission rates based on findings in Chapter III. I also varied symbiont coverage in the population from high (~90%) to perfect (100%) to further test the impact of aposymbiotic bugs on pathogen transmission at the field

level. Subsequent sensitivity analysis of CYVD infection simulations showed that symbiont coverage in the population, followed by the transmission rate, explained the most variance in total plant infections at the end of the field season. Despite making up a small portion of the total population, aposymbiotic bugs contributed disproportionately to pathogen transmission, and almost full symbiont coverage in the population ($\geq$ 97%) was necessary to keep plant infection incidence low. Furthermore, while reducing aposymbiotic bugs' pathogen transmission rate reduced plant infections, enabling aposymbiotic bugs to clear pathogen infection as rapidly as symbiotic bugs had almost no effect.

In Chapter V, I seek to identify underlying differences in gene expression that may elucidate the mechanisms that lead to expedited pathogen clearance in symbiotic compared to aposymbiotic individuals. Using RNASeq, I looked at differences in aposymbiotic and symbiotic squash bugs when they were infected or uninfected with *S. marcescens*. Given known differences in the development of the symbiotic organs (crypts) between symbiotic and aposymbiotic bugs, I dissected the crypts out of individuals sampled so that gene expression could be examined separately for the symbiotic organs and the rest of the insect body. A principal component analysis of the gene expression data showed tissue type (crypts vs body) and symbiont status were the strongest determinants of variation in our data. Surprisingly, *S. marcescens* infection status had no impact on differences in gene expression across squash bugs, supporting a potential commensal relationship between phytopathogen and insect vector. Gene expression did differ significantly in symbiotic and aposymbiotic crypts as well as between the crypts and bodies of insects, regardless of symbiont status. Functional analysis of differentially expressed genes revealed that aposymbiotic bugs experienced heightened physiological stress while symbiotic bugs upregulated metabolic functions. I also observed downregulation of immune genes in the

crypts relative to the bodies of squash bugs. These results are consistent with the role of *Caballeronia* as a nutritional symbiont that triggers morphological and physiological changes in its insect host upon acquisition.

Table 1.1 insect symbioses with potential for pest or vector control

A. SYMBIONT-MEDIATED CONTROL IN PESTS

HOST SPECIES	SYMBIONT	SUSCEPTIBLE CROP(S)	INSECT PROCESS TARGETED	METHOD	STATUS	REFERENCES
Drosophila suzukii	*Saccharomyces cerevisiae*	Soft-skinned fruits	Survival	RNAi	Proof of concept[1]	(Murphy et al. 2016)
Acyrthosiphon pisum	*Buchnera aphidicola*	Various	Survival	RNAi	Proof of concept	(Chung et al. 2018)
Coptotermes formosanus	*Enterobacter cloacae*	Structural woods	Survival	Symbiont replacement	Proof of concept	(Zhao et al. 2008)
Riptortus pedestris	*Burkholderia*[3]	Legumes	Insecticide resistance	Symbiont replacement	Proposed[2]	(Kikuchi et al. 2012)
Megacopta punctatissima	*Candidatus Ishikawaella capsulata*	Legumes	Host plant use	Symbiont replacement	Proof of concept	(Hosokawa et al. 2007)

B. SYMBIONT-MEDIATED CONTROL IN DISEASE VECTORS

HOST SPECIES	SYMBIONT	PATHOGEN(S)	INSECT PROCESS TARGETED	METHOD	STATUS	REFERENCES
Thysanoptera	Enterobacteriales	Tospoviruses	Longevity	RNAi	Proof of concept	(Whitten et al. 2016)
Homalodisca vitripennis	*Pantoea agglomerans*	*Xylella fastidiosa*	Vector competence	Paratransgenesis	Proof of concept	(Arora et al. 2018)
Euscelidius variegatus†	*Asaia*	*Candidatus Phytoplasma vitis*	Vector competence	Symbiont replacement	Proof of concept	(Gonella et al. 2018)
*Aedes aegypti**	*Wolbachia*	Dengue virus	Vector competence	Symbiont replacement	Field release	(Moreira et al. 2009, Bian et al. 2010)
*Rhodnius prolixus**	*Rhodococcus rhodnii*	*Trypanosoma cruzi*	Vector competence	Paratransgenesis	Semi-field trial	(Beard et al. 1998, 2002)

[1] Method for control has been developed, but has not been tested outside the laboratory.
[2] Potential to use the identified symbiotic association for control.
[3] Now reclassified as *Caballeronia*.
† Not natural vector
* Human disease vector

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Chapter II: An integrative approach to symbiont-mediated vector control for agricultural pathogens

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Abstract

Vector-borne pathogens pose significant threats to agricultural productivity. Methods that exploit associations between insects and their symbiotic microbes, dubbed symbiont-mediated vector control, are emerging as viable alternatives to insecticides for the control of vector-borne agricultural plant pathogens. The development of methods for effective microbial manipulation, such as RNA interference and paratransgenesis, may facilitate symbiont-mediated vector control tactics aimed at either suppressing insect populations or at manipulating vector competence, an insect vector's ability to acquire, harbor, and transmit pathogens. As suppression strategies transition from the laboratory to the field, the need for methods to evaluate their viability and predict their outcomes is apparent. Mathematical models of symbiont impact on agricultural disease can inform the development of symbiont-mediated vector control. We propose an integrative approach, combining theoretical and empirical experiments to identify the best practices for achieving meaningful improvements to crop health and productivity.

Introduction

Insect pests play a substantial role in global crop losses, damaging crops directly by feeding, indirectly by vectoring plant pathogens, or through a combination of the two. As of 2017, insects are estimated to reduce annual worldwide crop yields by 20% with crop losses valued at more than 470 billion USD [1]. Without suitable interventions, these numbers will likely increase as global temperatures rise [2]. Pesticides have long been used to mitigate insect-associated crop losses; however, the rising incidence of pesticide resistance [3] and the negative environmental impacts associated with their use have spurred a search for alternative pest control measures, including the genetic modification of insect pests [4] and plants [5].

The use of symbiotic microbes to control insect vector populations and the pathogens they transmit, dubbed symbiont-mediated vector control, has also emerged as a promising tactic for reducing crop losses [6**,7**,8]. Symbiotic associations influence various traits that contribute to pathogen transmission, including vector development, longevity, and competence—an insect's ability to acquire, maintain, and transmit a given pathogen. Furthermore, the specificity and necessity of many insect-microbe symbioses ensures minimal unintended effects to the environment and positive selection for insects to maintain their symbiotic associations. Significant strides have already been made in symbiont-mediated vector control of human pathogens [9**], most notably for mosquito-borne diseases [10-12].

Here, we discuss ways to leverage insect-microbe symbioses to disrupt the transmission of vector-borne agricultural plant pathogens. We then highlight how mathematical models coupling vector population dynamics, symbiont interactions, and disease ecology can aid in evaluating symbiont-mediated vector control tactics throughout development and deployment.

Overview of symbiont-mediated vector control

Insects form long-term intimate associations, known as symbioses, with select microbes. Insect-microbe symbioses are widespread among agricultural insect pests and vectors [13] and can exert both positive and negative influences on insect host phenotypes. While parasitic symbionts exploit their hosts and can lower host fitness, mutualistic symbionts can supplement nutritionally poor diets [14] or increase host resistance to abiotic stressors [15] and natural enemies [16,17]. Microbial symbionts have thus become an attractive target for the manipulation of insect traits relevant to the vectoring of agricultural pathogens.

Symbiont-mediated vector control falls into two broad categories: suppressing vector populations and reducing vector competence (**Figure 2.1**; see [6**,7**] for a more in-depth review). Both of these approaches may use either naturally-occurring or modified symbionts to achieve their goals. Suppressing vector populations to mitigate crop losses assumes that, all else being equal, having fewer insect vectors both reduces pathogen transmission and alleviates associated crop damage from insect feeding. Much like pesticides or biocontrol agents, this approach focuses on reducing vector fitness. It is particularly effective against vectors that also cause substantial feeding damage to crops. From a practical standpoint, horizontally-transmitted symbionts acquired from the environment are often better suited for population suppression tactics. In contrast, vertically-transmitted symbionts, which depend on host reproduction for propagation, would limit their own spread by harming juveniles or reproductive adults, thus reducing their effects on vector populations. Symbionts can also directly target an insect's vector competence and inhibit pathogen transmission without population suppression. Symbionts can influence vector competence through their interactions with the insect vector itself and/or with co-infecting microbes within the vector [18]. This approach is better suited to cases where

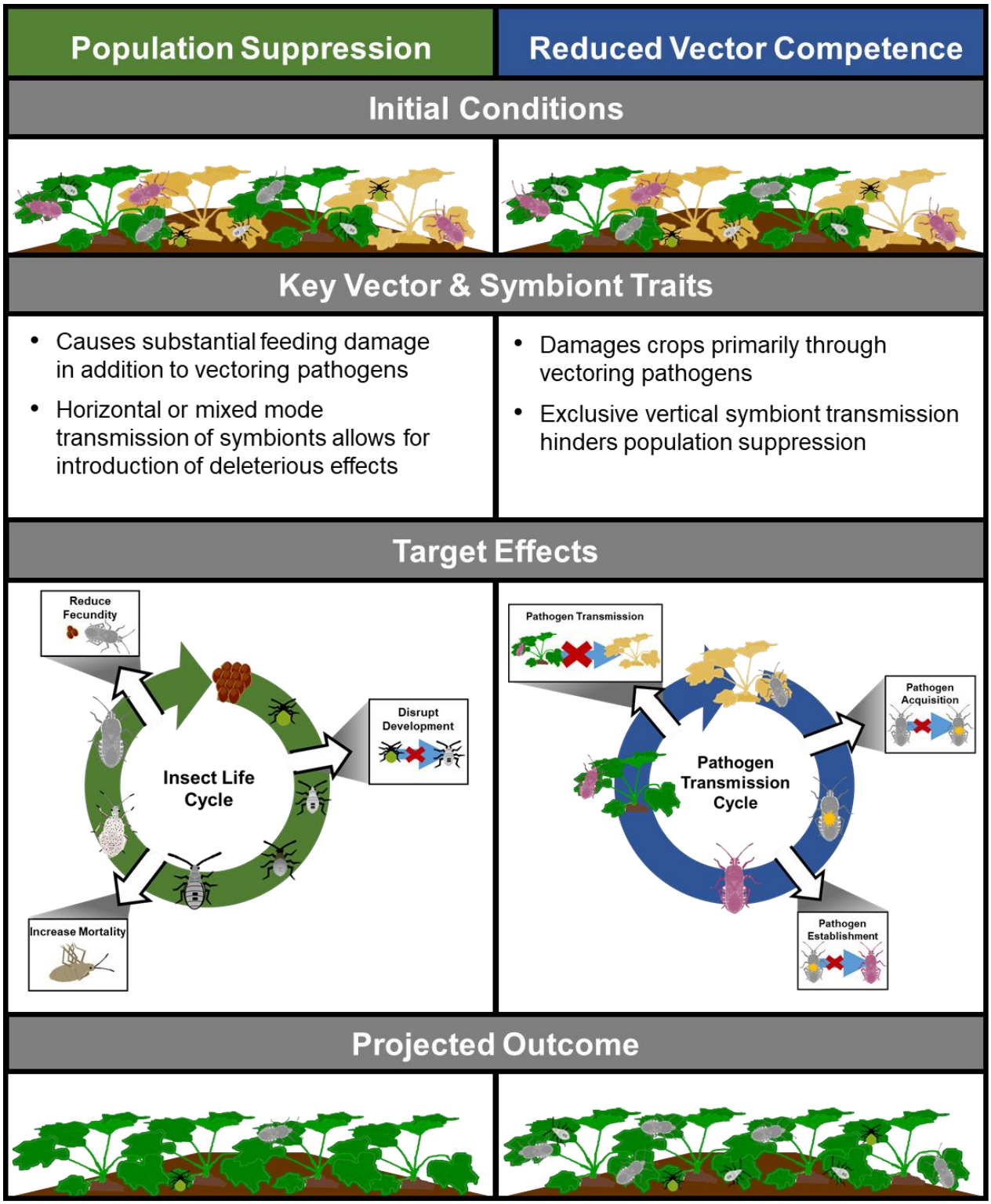

Figure 2.1. Symbiont-mediated vector control falls into two broad categories: population suppression and reduced vector competence. We illustrate the impact of each type of vector control using healthy plants (green), diseased plants (yellow), and infected (pink) and uninfected (gray) insect vectors. The optimal approach is determined by a variety of factors, including initial conditions and the key traits of the vector species and symbionts involved. Both categories seek to mitigate disease prevalence by targeting different traits in the insect life or pathogen transmission cycle.

offspring h inders population suppression techniques, or it is desirable to preserve the ecological function of the insect. Promising strategies for symbiont-mediated vector control

Natural variation across symbiont populations can be a rich source of candidate symbionts for vector control or vector competence reduction, but without a way to identify promising candidates *a priori*, such approaches require extensive screening of symbionts. One promising strategy is symbiont transplants between closely related species, as in the case of *Wolbachia* endosymbionts transplanted to *Aedes aegypti* mosquitoes [11]. Similar transplants in vectors of agricultural pathogens have also shown promise. Exogenous *Asaia* bacterial symbionts transplanted from other leafhopper species interfere with the establishment of *Candidatus* Phytoplasma vitis, causative agent of grapevine flavescence dorée, in the leafhopper *Euscelidius variegatus*, effectively reducing pathogen colonization of insects under laboratory conditions [19*]. Advances in our understanding of symbiont-insect interactions are needed to enrich these searches for symbionts with the greatest potential for disease control. Alternatively, symbionts can be genetically modified to interfere with insect development. Technologies such as RNA interference (RNAi) can be used to disrupt the expression of insect [20] or symbiont [21*] genes to achieve these effects. The use of symbionts to deliver double-stranded RNA (dsRNA) has already been proposed as an alternative to microinjection and plant-mediated RNAi to achieve sustained RNAi in insects [22**]. These approaches are particularly tractable for symbionts amenable to laboratory transformation. Engineered symbionts targeting essential insect genes to increase host mortality have already seen some success. For example, symbiotic yeast used to deliver dsRNA targeting gamma tubulin, a component of the cell cytoskeleton, reduced the fecundity and survival of *Drosophila suzukii*, a pest of soft-skinned fruits [23*]. Symbionts can also indirectly increase host mortality by targeting insect genes necessary for the maintenance of

obligate symbionts that are critical for host survival and reproduction. For example, silencing genes that enable the persistence of the obligate nutritional symbiont *Buchnera aphidicola* in the bacteriocytes of pea aphids, *Acyrthosiphon pisum*, resulted in decreased *Buchnera* abundance, significantly slowing aphid growth [21*]. Although this example involved oral administration of dsRNA through an artificial diet, future directions could make use of facultative symbionts to deliver dsRNA and disrupt obligate symbioses.

In addition to targeting the insect host, researchers can modify symbionts to express antipathogen properties, a technique known as paratransgenesis. To combat the agricultural pathogen *Xylella fastidiosa*, the causative agent of Pierce's disease in grapes, *Pantoea agglomerans*, bacterial symbionts of the glassy-winged sharpshooter, the vector of *X. fastidiosa*, were engineered to secrete antimicrobial peptides with demonstrated activity against *X. fastidiosa* [24*]. Vectors with modified symbionts were refractory to pathogen acquisition, reached peak pathogen loads less than five percent that of control vectors, and consistently failed to transmit *X. fastidiosa* to plants [24*]. Field tests employing novel methods of symbiont dispersal relying on microparticles [25] are forthcoming. If effective, this could be the first successful use of paratransgenic symbionts against a vector-borne agricultural plant pathogen.

The last few years have seen an increased interest in identifying either naturally-occurring or genetically modified symbionts that could be used for vector control [26-28]. As interest in this field grows and field applications become feasible, the need for methods to evaluate the potential and value of these techniques in reducing vector-borne disease transmission in agricultural systems is apparent.

Integrative research to advance symbiont-mediated vector control

Identifying or engineering symbionts with promising population suppression or vector competence-reducing effects on individual insects is the first step in generating novel symbiont-mediated vector control tactics. Ecological experiments and theoretical models are crucial for determining how symbiont effects on vectors scale up and for evaluating their long-term feasibility under varying ecological conditions and potential for evolutionary change. Integrating empirical outcomes with theoretical models has been critical for the use of *Wolbachia* endosymbionts to control dengue transmission by *Aedes aegypti* mosquitoes, the best developed example of symbiont-mediated vector control in any system to date (see [29*,30*]). Progress in this system provides a useful template for how theory and experiments are needed to develop symbiont-mediated vector control for agricultural plant pathogens.

Mathematical models can help address many of the questions raised throughout the development of novel symbiont-mediated vector control tactics (**Figure 2.2**). For example, models can help assess whether, and under what conditions, symbionts can establish and persist in wildtype vector populations. These processes will vary substantially based on the transmission mode of the symbionts in question. For vertically-transmitted symbionts, researchers will be interested in how vector survival and fecundity influence symbiont persistence and spread. From an evolutionary perspective, making symbionts too detrimental to their host's fitness can lead to selection against the symbiosis. Any costs associated with maintaining vertically-transmitted symbioses can strongly influence symbiont persistence in vector populations, particularly when benefits are not consistently high. For horizontally-transmitted symbionts, understanding the ability of the microbe to transmit to new hosts or survive in the environment will be key. Additionally, candidate symbionts for population suppression may require repeated introductions

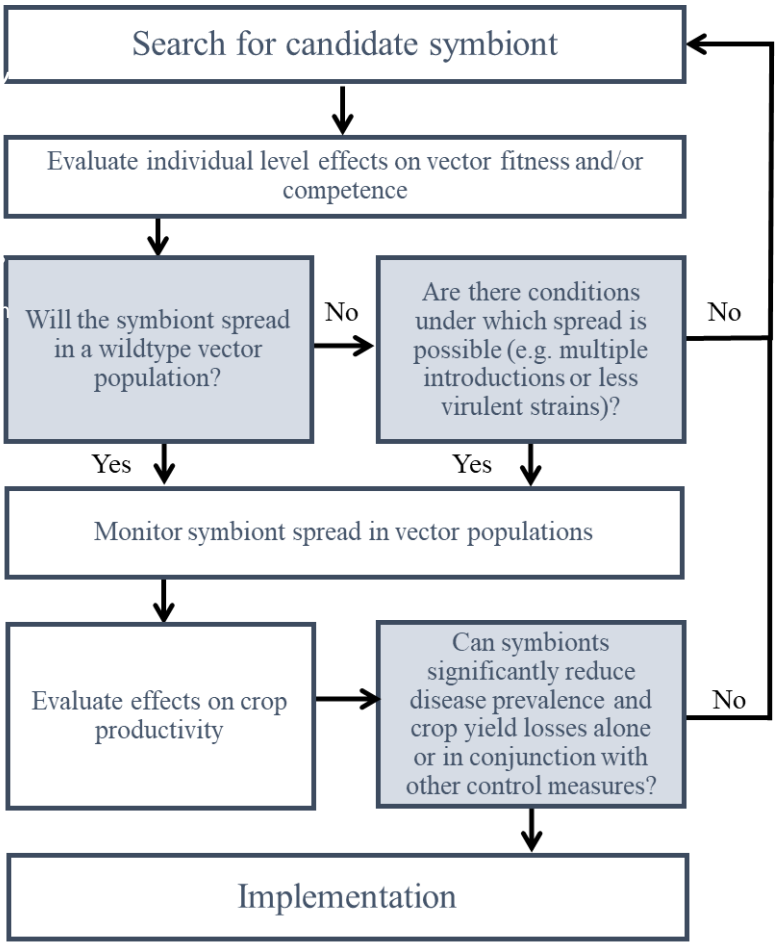

Figure 2.2. Schematic detailing how and where models come into play throughout the development of novel symbiont-mediated vector control techniques. Shaded boxes provide guiding questions that models can help answer during this process, and white boxes list necessary empirical steps. Arrows connect interacting steps with answers to the questions posed where relevant.

to counteract strong selection against their persistence. Theoretical models can help optimize the timing and magnitude of these introductions to enhance symbiont persistence [31] by accounting for seasonal variation in vector abundance and population growth, for example [32].

Once a symbiont has demonstrated an ability to spread in a vector population, models can focus on assessing its likely impact on disease incidence and crop yield losses. Laboratory experiments on individual effects detailing symbiont functionality, particularly with regard to effects on vector fitness or competence, should be scaled up to reflect population-level effects. Assessing the impact of candidate symbionts on crop yield will require integrating models of vector and symbiont population dynamics with models of disease spread in plants that incorporate explicit terms translating insect-inflicted damage into impact on yield. Vector population models should track the spread of the candidate symbiont in the wildtype vector population and include relevant stages of insect development, as well as uninfected and infected

insect classes. Vector population dynamics can be incorporated into models of disease dynamics through vector feeding rates and relative contributions to pathogen transmission. In cases where symbionts are used for population suppression, the detrimental effects of the symbiont can be reflected in the rates at which insects mature and their probability of survival and reproduction. If symbionts are used to reduce vector competence, the rate at which insects move to the infected class or the probability that they successfully transmit a pathogen can be lowered depending on which component of vector competence is being affected.

The relationship between disease, plant damage, and yield losses will differ for each pathogen and crop species. Crop loss simulation models have long informed the prioritization of crop protection measures by the yield-reducing effects of various harmful organisms [33], and existing models can provide a framework for incorporating the yield-reducing effects of pathogens on crops. Crop loss models, however, typically focus on accrued damage to plants and do not incorporate the dynamics of the pests inflicting damage [34**]. Coupling models of symbiont-mediated vector control with existing crop loss model frameworks will increase the predictive power of models used to evaluate the impact of symbionts on crop yield.

Models built throughout the development of novel symbiont-mediated vector control methods may reveal specific symbiont traits or dissemination strategies that are more or less likely to succeed for specific crops. These model predictions can guide experiments simulating field releases. Data on prevalence of disease and crop yield obtained from these experiments can validate these models or identify gaps in knowledge to improve the existing models and predictions, for instance, by revealing the need to incorporate previously omitted ecological factors. This may include consideration of variables outside the insect-symbiont-pathogen relationship, such as abiotic environmental factors, herbivory from general crop pests, and

coinfection with other pathogens. Furthermore, models can be modified to reflect the use of other biological or chemical pest controls implemented in succession or in combination with symbiont-mediated vector control tactics as part of larger pest management programs. Promising empirical and theoretical results at this stage will ease the transition to and implementation of full-scale field releases.

Conclusion

The successful manipulation of symbiotic microbes to control insect pests and suppress vector-borne pathogens *in vivo* has opened up new opportunities for the control of vector-borne agricultural plant pathogens. The diversity of insect symbiotic associations, the myriad of ways in which symbionts can be altered, and the various methods of symbiont dispersal make highly tailored pathogen interventions possible. Integrating theory into the development of symbiont-mediated vector control tactics can help advance the field in meaningful ways. Theoretical and empirical assessments are both critical for determining whether large-scale implementation of symbiont-mediated vector control will lead to desirable outcomes. Although researchers must still contend with other barriers to deploy symbiont-mediated vector control tactics in the field—most prominently public perceptions of the risk associated with genetically modified organisms—here, we have provided a model of integrative research that can effectively guide the investment of time and resources into the most useful control methods.

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Chapter III: Competitive Exclusion of phytopathogenic *Serratia marcescens* from squash bug vectors by the gut endosymbiont *Caballeronia*

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Abstract

Many insects harbor microbial symbiotic partners that offer protection against pathogens, parasitoids, and other natural enemies. Mounting evidence suggests that these symbiotic microbes can play key roles in determining infection outcomes in insect vectors, making them important players in the quest to develop novel vector control strategies. Using the squash bug *Anasa tristis*, we investigated how the presence of *Caballeronia* symbionts affected the persistence and intensity of phytopathogenic *Serratia marcescens* within the insect vector. We reared insects aposymbiotically and with different *Caballeronia* isolates, infected them with *S. marcescens*, then sampled the insects periodically to assess the intensity and persistence of pathogen infection. Squash bugs harboring *Caballeronia* consistently had much lower-intensity infections and cleared *S. marcescens* significantly faster than their aposymbiotic counterparts. These patterns held even when we reversed the timing of exposure to symbiont and pathogen. Taken together, these results indicate that *Caballeronia* symbionts play an essential role in *S. marcescens* infection outcomes in squash bugs and could be used to alter vector competence to enhance agricultural productivity in the future.

Introduction

Vector-borne diseases have posed significant threats to agricultural productivity as well as human and wildlife health for millennia (1, 2). Controlling populations of insect vectors has traditionally played a key role in human efforts to reduce the burden of vector-borne diseases, often to great effect (3). However, the negative environmental impacts associated with insecticides (4)—the most common type of vector control—and rising incidences of pesticide resistance (5) have spurred a search for alternative control measures. The successful introduction of *Wolbachia* endosymbionts into *Aedes aegypti* mosquitoes for the control of dengue virus (6) highlights the potential for the development of other symbiotic microbes to control insect vector populations and the pathogens they transmit, a tactic known as symbiont-mediated vector control (7, 8).

While factors such as temperature (9, 10), host genetic background (11, 12), and innate immunity (13) have long been known to influence vector competence—an insect's ability to acquire, maintain, and transmit pathogens (14)—mounting evidence suggests that symbiotic microbes can play key roles in determining pathogen infection outcomes in insect vectors (15, 16). Interactions between insects and pathogens do not happen in isolation; many insects harbor microbial symbiotic partners that offer protection against insect pathogens, parasitoids, and other natural enemies (17-20). These microbial partners also interact with vectored pathogens in a myriad of ways that can either facilitate or inhibit infection. In some insects, for example, proteins produced by symbionts are essential for supporting parasite survival (21, 22), increasing vector competence. In other cases, symbionts decrease insects' vector competence by interfering with the establishment of vectored pathogens (23, 24) or by promoting proper immune system development in their insect host and enabling a more robust response to subsequent pathogen

infections (25). In fact, some vectored pathogens must actively disrupt the natural insect microbiota in order to successfully establish themselves (26).

A symbiont's ability to coexist with or competitively exclude coinfecting pathogens within its insect host can dictate pathogen infection outcomes that can affect vector competence. Knowledge of these dynamics is an important early step in assessing the potential for symbiont-mediated vector control. Here, we explore how symbiotic microbes affect the dynamics of a phytopathogen infection in the squash bug (*Anasa tristis* DeGeer), an insect pest of agricultural importance. The squash bug is the primary vector of Cucurbit Yellow Vine Disease (CYVD) (27), caused by phytopathogenic lineages of *Serratia marcescens* (28, 29). Although other insects have been shown to acquire *S. marcescens* in artificial feeding systems, the squash bug is the only insect confirmed to transmit *S. marcescens* in the field (30). CYVD leads to significant yield losses in squash, pumpkin, and related crops (31). The phytopathogenic lineages of *S. marcescens*, unlike their entomopathogenic counterparts, have limited influence on insect fitness when ingested (**Supplementary Fig 3.1**).

Like other stink bugs and their relatives, squash bugs form symbiotic associations with bacteria in the genus *Caballeronia*, formerly contained within the genus *Burkholderia* (32-34). *Cabelleronia* symbionts are acquired *de novo* from the environment at each host generation, allowing us a straightforward way to manipulate symbiont acquisition. Symbionts are housed in a specialized region of squash bugs' posterior midgut known as the crypts and are typically acquired early in the insect life cycle (35). Previous work has established that successful host colonization by *Caballeronia* results in accelerated development and decreased mortality relative to aposymbiotic (symbiont-free) individuals (36). Leveraging the natural characteristics of this system, we investigated the impact of symbiont colonization on the intensity and persistence of

S. marcescens infections in squash bugs and looked for evidence of priority effects. We provide evidence that the symbiont *Caballeronia* prevents successful, long-term establishment of *S. marcescens* in its *A. tristis* vector, regardless of whether it is the first to colonize the insect or not.

Results

We conducted two experiments to determine how *Caballeronia* symbionts interacted with phytopathogenic *S. marcescens*. First, we determined whether the presence of *Caballeronia* symbionts influenced the outcome of *S. marcescens* infection in squash bugs. We then tested for priority effects by varying exposure to pathogen and symbiont.

Symbionts reduce the persistence and intensity of pathogen infections in squash bugs

We found a clear effect of *Caballeronia* symbiont colonization status on both the persistence and intensity of *S. marcescens* (strain Z01) infection (**Figure 3.1**). Nearly all aposymbiotic bugs retained Z01 infection throughout the experiment, resulting in no temporal trend in pathogen prevalence among aposymbiotic bugs (GLM coefficient for days post acquisition (DPA) in aposymbiotic bugs = 0.02, SE = 0.21, $P = 0.91$). There were, however, significant declines in the prevalence of infection among symbiont-positive bugs over time (GLM coefficient for DPA in symbiont positive bugs = -0.65, SE = 0.12, $P = 4.09 \times 10^{-8}$). Furthermore, prevalence of infection with Z01 declined significantly faster in symbiont-positive squash bugs relative to aposymbiotic bugs (difference in slope = -0.68, SE = 0.24, $p = 5 \times 10^{-3}$) (**Figure 3.1A**).

Among squash bugs that were infected, those harboring *Caballeronia* symbionts had initial Z01 titers that were 1 000-fold lower than aposymbiotic bugs (difference in GLM intercept from aposymbiotic bugs = -4.13, SE = 0.36, $p < 2 \times 10^{-16}$). Differences in titer between the two groups persisted over time, with symbiont positive bugs retaining significantly lower pathogen

titers (difference in DPA coefficients = -0.74, SE = 0.09, $p = 2.76 \times 10^{-15}$). While symbiont positive squash bugs experienced significant reductions in infection intensity over time (GLM coefficient for DPA in symbiont positive bugs= -0.67, SE = 0.07, $p < 2 \times 10^{-16}$), we did not identify a significant temporal trend in Z01 titer for aposymbiotic bugs (GLM coefficient for DPA in symbiont negative bugs = 0.07, SE = 0.07, p = 0.30) (**Figure 3.1B**).

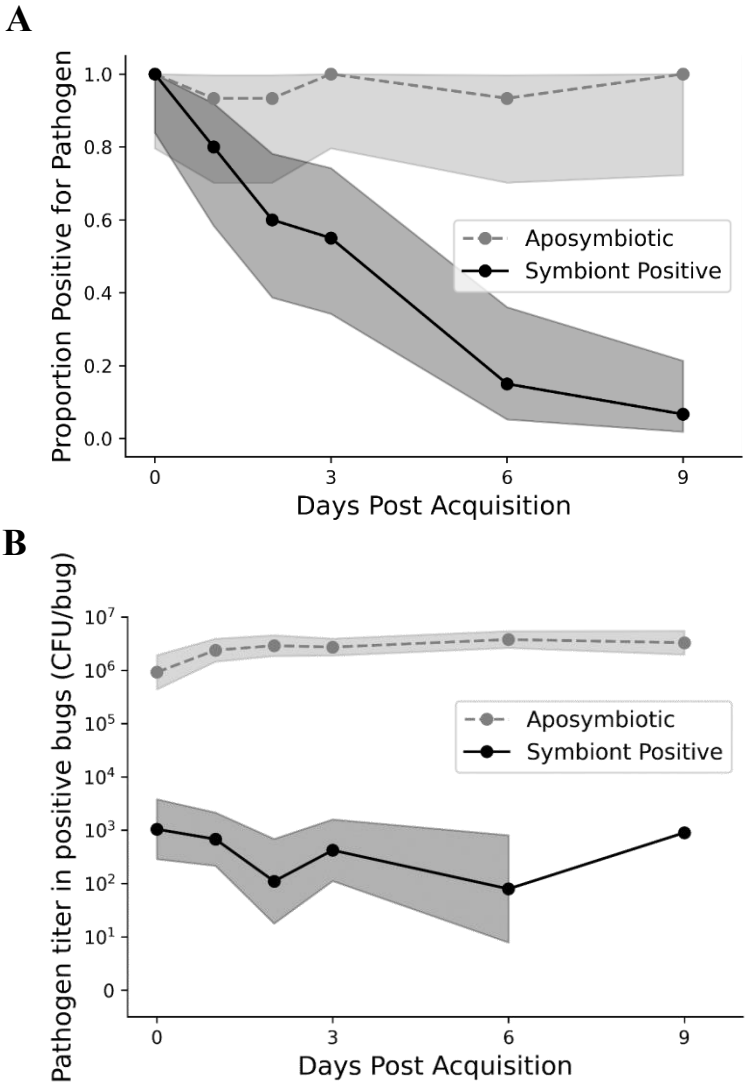

Figure 3.1. Symbiont status affects persistence and intensity of *Serratia* infection (A) The proportion of individual bugs that tested positive for *S. marcescens* at a given time point across aposymbiotic and symbiont positive treatments. For the aposymbiotic treatment, each point represents 15 squash bugs except for 9 dpa which represents 10 individuals. For the symbiont positive treatment, each point represents 20 squash bugs. (B) *S. marcescens* titer recovered from infected squash bugs over time. This data includes only non-zero values. Points represent up to 15 individuals for the aposymbiotic treatment or up to 20 for the symbiont positive treatment, with later time points typically representing fewer individuals particularly in symbiont positive bugs. Shading in both panels indicates 95% confidence intervals where possible to calculate.

Accounting for symbiont strains and batches of aposymbiotic bugs showed similar patterns of Z01 clearance between aposymbiotic and symbiont-positive bugs, with no significant

differences among different symbiont strains or among batches of aposymbiotic bugs (**Supplementary Fig 3.2a**). Variation was more apparent in our infection intensity data, particularly in initial titers. These differences largely disappeared over time, however, Apo3 remained somewhat anomalous within its treatment group. In the case of Apo3, the difference was driven by the fact that Apo3, which began at a lower titer than the other two aposymbiotic batches, rose in titer to a similar level during the course of the experiment. Individual differences in infection intensity between symbiont strains could be due to the drop in sampling power as bugs began clearing Z01 infections. However, it must be noted that, despite some statistically significant differences among *Caballeronia* strains, these differences were rather small, typically less than ten-fold, compared to the 1000-fold differences between symbiont positive and aposymbiotic bugs (**Supplementary Fig 3.2b**).

Time and symbiont status were strong predictor variables in our model of persistence data (full model: $R^2 = 0.62$, time model: $R^2 = .26$, treatment model: $R^2 = .34$), whereas symbiont status was by far the strongest predictor variable in our data on infection intensity (full model: $R^2 = 0.63$, time model: $R^2 = 0.04$, treatment model: $R^2 = 0.53$). Despite not accounting for specific symbiont strains or batch-to-batch variation among squash bugs, our model with consolidated treatments, still explained most of the variation in our Z01 infection intensity ($R^2 = 0.63$) and persistence ($R^2 = 0.62$) data.

Symbionts retain pathogen clearing properties regardless of order of exposure

After establishing the effect of symbiont presence on Z01 infection intensity and persistence, we investigated whether these dynamics would persist if the order of exposure to symbiont and pathogen were reversed. Although initial Z01 infection prevalence was lower in our symbiont first treatment than in either the pathogen only or pathogen first treatments, we

found that both treatments that received symbionts, regardless of order, cleared Z01 faster than the pathogen only treatment in which bugs remained aposymbiotic (symbiont first: difference in DPA coefficient from aposymbiotic treatment = -0.58, SE = 0.24, p = 0.02; pathogen first: DPA coefficient from aposymbiotic treatment = -0.46, SE = 0.17, p = 6.6 x 10^{-3}) (**Figure 3.2A**). Despite differences in initial prevalence, we found that the rate of clearance did not differ significantly between symbiont first and pathogen first treatments (difference in DPA coefficient = 0.12, SE = 0.28, p = 0.67).

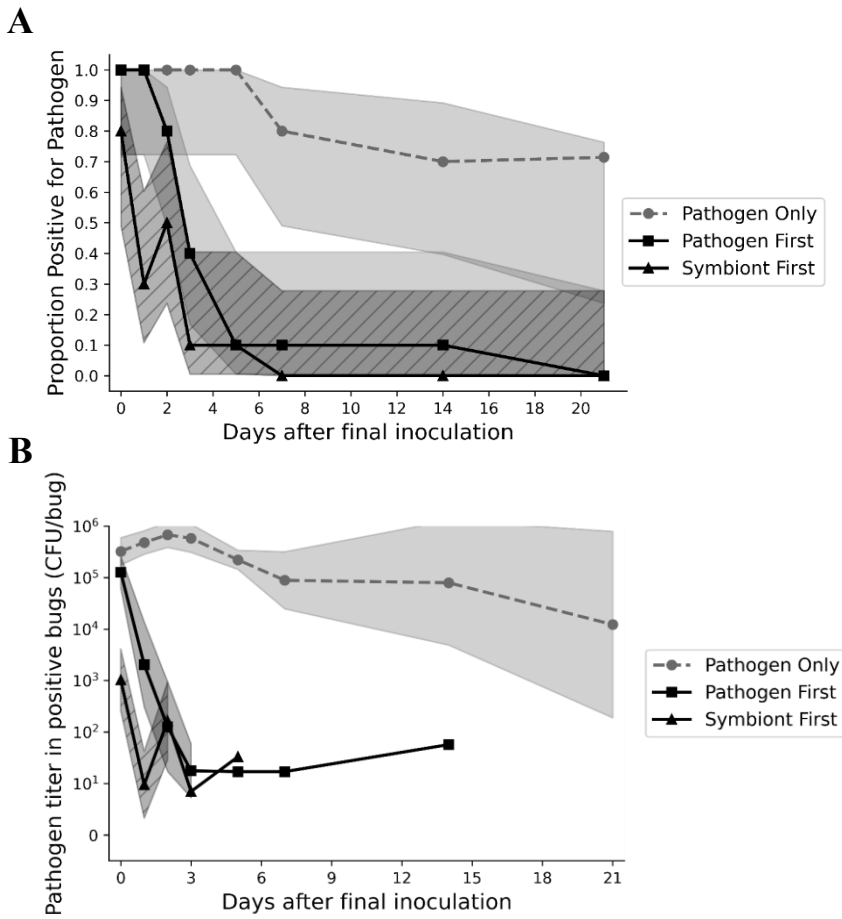

Figure 3.2. Order of infection does not impact the effect of symbiont colonization on pathogen persistence and intensity. (A) The proportion of individual bugs that tested positive for *S. marcescens* at a given time point. Pathogen only bugs remained aposymbiotic, but bugs in the pathogen first and symbiont first treatment were coinfected with symbiont and pathogen. Each point represents 10 individuals tested at that time point. (B) *S. marcescens* titer recovered from infected squash bugs over time. Only non-zero values were included in this analysis. Points represent up to 10 individuals in each treatment, with later time points typically representing fewer individuals. In both panels, shading indicates 95% confidence intervals where possible to calculate.

When we looked at Z01 infection intensity (**Figure 3.2B**), we found that both treatments that received symbionts, regardless of order, differed significantly from the pathogen only treatment in initial titers (symbiont first: difference in intercept from aposymbiotic = -4.45, SE =

0.53, $p < 2 \times 10^{-16}$; pathogen first: difference in intercept from aposymbiotic = -2.03, SE = 0.39, $p = 2.55 \times 10^{-5}$). The symbiont-first treatment had the lowest initial titers, consistent with findings from our previous experiment. These treatments also differed significantly from the pathogen only treatment in their rate of pathogen loss over time (symbiont first: difference in DPA coefficient from aposymbiotic treatment = -1.20, SE = 0.23, $p < 2.99 \times 10^{-7}$, pathogen first: difference in DPA coefficient from aposymbiotic treatment = -0.59, SE = 0.07, $p = 5.19 \times 10^{-16}$). Despite a higher initial titer than the symbiont first treatment, the pathogen first treatment declined in titer at a similar rate (difference in DPA coefficient treatment = 0.61, SE = 0.3, p = 0.08).

Symbiont titer and persistence is unaffected by previous pathogen exposure

Out of all 240 individuals exposed to *Caballeronia*, only 5 bugs were symbiont-negative when tested, a 98% success rate for symbiont colonization regardless of treatment. We found no significant differences in symbiont titers in either of the pathogen-exposed treatments compared to symbiont only controls over the course of the experiment

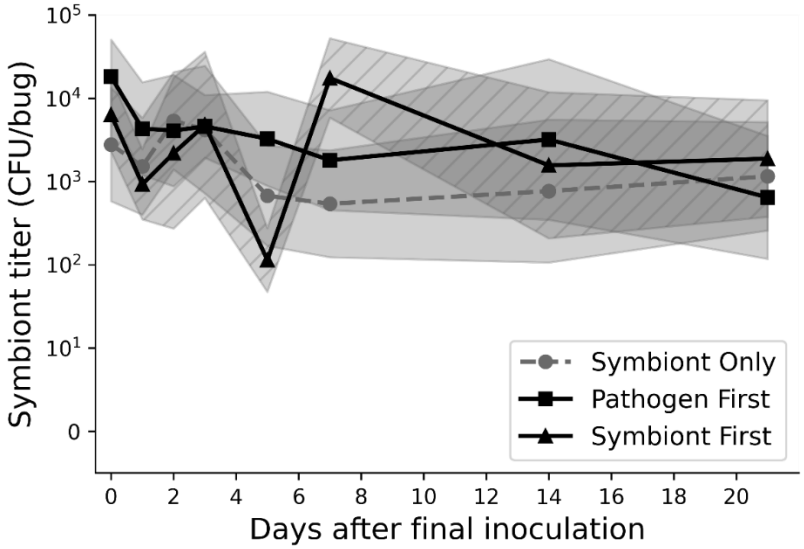

Figure 3.3. Order of infection does not affect symbiont titers. Symbiont titers recovered from squash bugs in each treatment. Only 2% of individuals across all treatments tested negative for the symbiont. Shading indicates 95% confidence intervals. No differences in symbiont infection intensity were detected between treatments.

(symbiont first: difference in intercept from symbiont only = 0.08, SE = 0.05, p = 0.13; pathogen first: difference in intercept from symbiont only = 0.07, SE = 0.04, p = 0.11) (**Figure 3.3**).

Discussion

We demonstrate that the presence of *Caballeronia* symbionts in squash bugs consistently results in the competitive exclusion of the plant pathogen *S. marcescens* resulting in lower intensity infections of shorter duration in symbiont-positive bugs compared to aposymbiotic individuals. Furthermore, we saw no evidence suggesting priority effects were in play for pathogen establishment. Patterns of competitive exclusion persisted regardless of whether insects were exposed to *S. marcescens* prior to symbiont establishment or after, and the ability of *Caballeronia* symbionts to successfully colonize their insect host was unaffected by previous pathogen exposure. Though there was some variability in their effects, we observed the same overall trends of *S. marcescens* persistence and infection intensity for all squash bug-associated *Caballeronia*. Differences observed among the symbiont strains were minimal compared to the much more striking differences between symbiotic and aposymbiotic insects. The magnitude of the differences we observed in *S. marcescens*-clearing abilities between aposymbiotic and symbiont-positive individuals is indicative of a significant within-host interaction between pathogen and symbiont.

The shorter persistence times of *S. marcescens* in symbiont positive bugs relative to aposymbiotic bugs could be driven, at least in part, by their much lower initial titers. Although it is possible that symbionts could alter insect feeding behavior, thus prompting less consumption of subsequently introduced bacteria, we think this unlikely given the failure of *S. marcescens* to maintain higher titers even when introduced prior to symbiont exposure. We think this pattern is

more indicative of fast and aggressive action mediated by *Caballeronia* symbionts to prevent the establishment or proliferation of *S. marcescens*.

Examples of defensive symbioses—whereby symbiotic microbes aid in the clearance or tolerance of co-infecting microorganisms—are plentiful among insects (18, 37-40) and can result in similar patterns of competitive exclusion of pathogens as those we observed. In carrion beetles, for example, symbiotic microbes outcompete entomopathogenic bacteria in vivo and make their hosts resistant to deadly larval infections if present prior to pathogen exposure (20). The antiviral effects of *Wolbachia* against RNA viruses that infect *Drosophila melanogaster* are also well-documented (18, 41). A general ecological framework of within-host microbial interactions can help us understand how symbionts confer protection to their hosts (42, 43).

There are three main, though not mutually exclusive, types of interactions that can result in symbiont-conferred protection within their host, symbionts and coinfecting pathogens or parasites may: 1) compete for a limiting resource (exploitative competition), 2) deal each other direct damage (interference competition), or 3) indirectly compete through a shared natural enemy, typically the host immune system (apparent competition) (44). Furthermore, we might expect the order and timing of organisms' arrival to influence the outcomes of these interactions (i.e., priority effects). Priority effects can play a dominant role in competitive interactions in cases where interacting organisms have high niche overlap (45, 46). Examples of niche pre-emption whereby an early arriving species sequesters or uses up resources required for the successful establishment of later arrivals are abundant in community development, particularly in the assembly of the human gut microbiome (47). Immune priming, a prominent example of apparent competition in host-symbiont-pathogen interactions, may also require an established symbiont presence in order to ward off subsequent pathogen infections (25).

Because our study does not address the mechanism through which *Caballeronia* symbionts and *S. marcescens* interact within the squash bug, we cannot say with certainty that our results stem from one specific type of interaction or another. It is possible that pathogen and symbiont compete for space or other limiting resources within the insect gut after they are ingested. Other insects are known to rely on various physical barriers within the gut to prevent pathogens from disseminating into the hemocoel and becoming more permanently established (48). Gut-colonizing symbionts can enhance these barriers by forming biofilms that effectively block pathogens from passing through (24, 26). However, this type of inhibitory priority effect is not seen in the squash bug, as evidenced by *S. marcescens'* failure to outperform *Caballeronia* when allowed to establish in insects beforehand. Work in a closely-related stink bug species, also possessing *Caballeronia* symbionts, suggests that apparent competition through the insect immune system could explain the pathogen-clearing patterns we observe in the squash bug. In the bean bug *Riptortus pedestris*, symbiont colonization leads to differential immune gene expression within the insect gut (49). Immune activation is a common mechanism for the regulation of symbiont titer among insects and could adversely affect co-infecting pathogens (50, 51), regardless of order of exposure. However, more work is needed to determine whether this is the case in squash bugs. It is also possible that symbiont-positive squash bugs may be able to mount a more robust immune response against *S. marcescens* given the general benefits conferred upon them by *Caballeronia* (36). If immune activation of the insect host is involved in the clearance of *S. marcescens*, the lack of priority effects observed suggest a very rapid immune response in individuals with pathogen exposure before symbiont establishment. Our results indicate that no matter the mechanism of interaction, *Caballeronia* symbionts maintain a competitive edge over *S. marcescens* within the squash bug.

Though primarily developed in the context of human health (52, 53), symbiont-mediated vector control tactics are now gaining traction among researchers hoping to curb the spread of agricultural pathogens (54, 55). The competitive exclusion of *S. marcescens* from squash bugs by their symbiotic bacteria is a promising result for further research in this area and could eventually lead to the development of novel methods for controlling Cucurbit Yellow Vine Disease (CYVD), which is transmitted by the squash bug and has become of increasing concern in the United States (31, 56-58). The absence of priority effects in *Caballeronia*-*S. marcescens* interactions is a notable trait in the system. Both the symbionts' ability to establish in insects and its ability to competitively exclude *S. marcescens* are maintained regardless of the insect's order of exposure to pathogen and symbiont. This could vastly simplify the timing of a *Caballeronia*-based intervention, should it be developed and deployed in the field. However, it must be noted that, though our results would suggest that symbiont-positive squash bugs are less likely to be competent, long-term vectors of *S. marcescens*, we did not test vector competence directly from insects to plants. If *Caballeronia* symbionts are to be used to mitigate CYVD transmission, further work should seek to confirm whether symbiont-positive bugs are in fact less likely to transmit *S. marcescens* to plants.

Because squash bugs damage plants through a combination of pathogen vectoring and heavy feeding (31), squash bug control strategies must also consider the fact that symbiont positive bugs develop faster and live longer than their aposymbiotic counterparts. An optimal control strategy must balance the risks of pathogen vectoring, where symbiont-positive bugs may be desirable, with the possibility of intense feeding damage if squash bug populations reach high densities, which is more likely if symbiont-positive bugs are more prevalent. Given the double

threat posed by squash bugs, it is likely that potential symbiont-mediated vector control methods will need to be deployed in conjunction with other pest management tactics for optimal results.

Materials & Methods

Insect rearing

We reared all insects in an environmental chamber held at constant temperature (27°C) and 60% relative humidity under a long day light cycle (16 hours light, 8 hours dark). We surface sterilized eggs collected from our existing *Anasa tristis* colony by alternately washing them in 70% ethanol and 10% bleach for one minute each and then rinsing with 70% ethanol for 10 seconds. Once eggs hatched, we transferred first instar nymphs to a sterile container and gave them slices of organic zucchini (*Cucurbita pepo*) fruit, surface sterilized with 70% ethanol and thinly wrapped in parafilm. We maintained insects in sterile containers with regular fruit changes until they molted to second instars.

Symbiont Effect on Pathogen Persistence

Symbiont Acquisition. We collected batches of 1-3-day-old second instars (n = 150) into sterile rearing boxes for administration of treatments. All *Caballeronia* strains used were isolated from the crypts of bugs collected from fields in Georgia, Indiana, or North Carolina. We prepared liquid diets consisting of either sterile water and 100 uL of filter-sterilized blue dye (aposymbiotic treatments) or of *Caballeronia* culture (either strain Sq4a, IN-SB1, NC-TM1, or GA-Ox1), sterile water, and 100 uL of blue dye (symbiont treatments) in 30 mm Petri dishes. All bacterial cultures were grown in standard Luria Bertani (LB) broth overnight in a shaking incubator at 28°C. We used OD600 measurements to standardize all bacterial diets to 2 x 10^7 CFUs per mL in a total volume of 5 mL. The blue food dye allowed us to visually confirm the uptake of liquid diets in the insects. Once all liquid components were mixed in the Petri dish, a

sterile dental swab was placed in the open dish before the dish was sealed with parafilm, leaving the dental swab protruding from one end so that the insects could feed freely. Insects had access to liquid diets for 24 hours before being placed back on fruit. Due to constraints in rearing large numbers of insects synchronously, only two of the symbiont treatment groups (GA-Ox1 and Sq4a) had a corresponding aposymbiotic control group (Apo2 and Apo3, respectively) that were started on the same day from the same batch of second instars. All other treatment groups—two symbiont treatments (IN-SB1 and NC-TM1) and one aposymbiotic treatment (Apo1)—were completed as independent batches. Insects matured to the third instar stage at varying rates depending on symbiont status and the strain of symbiont received (36).

Pathogen Acquisition. Third instars (n = 80), which had molted from second instar one to three days before, were collected from the established treatment groups and fed a strain of phytopathogenic *Serratia marcescens* (Z01) isolated from an infected zucchini and chromosomally labeled with red fluorescent protein (RFP)—henceforth referred simply as Z01. Fluorescent labeling was achieved via triparental mating with *E. coli* strains E1354 and E2072 carrying the pTNS3-asdEC and pmini-Tn7-gat-P1-rfp plasmids respectively (59). This protocol consistently produces site-specific insertion of fluorescent proteins in a the neutral attTn7 site (60, 61) 20-25 bp downstream of the glmS gene. The RFP positive conjugant was species confirmed via 16S sequencing and displayed consistent growth and morphology with the parental strain. Feedings were done via vacuum-infused zucchini cubes following established protocols (62). Briefly, liquid Z01 cultures were standardized to 2 x 10^7 CFUs per mL in approximately 25 mL of sterile water with 100 uL of blue dye, which was added in order to determine the success of the infusion. Slices of organic zucchini 6 mm thick were cut and quartered, placed in a vacuum flask, then submerged in liquid Z01 culture. The vacuum was

turned on, stoppered, and released repeatedly in 10 second intervals until zucchini slices were saturated. The slices were then removed from the flask, wrapped in a thin sheet of parafilm, and given to the squash bugs. Squash bugs had access to Z01-infused squash for 48 hours after which they were placed back on regular zucchini fruit.

Sampling. Once they had been fed Z01, insects were periodically sacrificed to determine the persistence (i.e., presence or absence) of *S. marcescens* and to quantify the intensity of infection (i.e., bacterial abundance among individuals that tested positive). Five individuals were sacrificed immediately after the 48-hour Z01 acquisition period (Day 0), every day for three days, then every three days after that.

At each time point, insects were surface sterilized for five minutes in 70% ethanol and allowed to dry. Whole insects were macerated with micropestles in 1.5 mL microfuge tubes filled with 200 uL of 1X phosphate buffered saline solution (PBS). Ten-fold serial dilutions up to 10^{-6} were prepared in 96-well plates. Aliquots of 20 uL were plated on standard LB agar in quadruplicate. Plates were incubated at room temperature for 48 hours before bacterial colonies were visualized and counted under a fluorescent microscope. The average number of colonies across replicates was then used to derive estimates of titer in CFUs per bug.

Sampling times were variable across treatments, with some treatments regrettably cut short due to the cessation of non-essential research activities in response to the COVID-19 pandemic. Here, we focus on data collected up to the last major time point we were able to sample from most treatments. This leaves us with data for most treatments through day nine, with the exception of the aposymbiotic control group (Apo3) corresponding to a trial with Sq4a, which was only sampled through day six.

Order of Exposure Effect on Pathogen Persistence

Bacterial acquisition. We collected batches of second instars (n = 90), which had molted one to three days prior, into sterile rearing boxes assigned to one of four treatments: symbiont only, pathogen only, symbiont first, and pathogen first. For this experiment, we used *Caballeronia* strain Sq4a labeled with green fluorescent protein (GFP) (36), as the symbiont and RFP-labeled Z01 as the pathogen. At the start, two treatments (symbiont only and symbiont first) received symbiont diets and two treatments (pathogen only and pathogen first) received pathogen diets. All bacteria were grown and standardized to concentrations of 2 x 10^7 CFUs/mL as before. All bacterial or sterile water feedings were done via vacuum-infused zucchini cubes for consistency. After 24 hours of access to their first bacterial diet, we placed insects on plain zucchini slices for 24 hours. We sacrificed five individuals, chosen at random, from each treatment to confirm the presence of Sq4a or Z01 before proceeding to the next feeding. We then gave insects in the symbiont only and pathogen only treatments zucchini infused with sterile water, insects in the pathogen first treatment symbiont diet, and insects in the symbiont first treatment pathogen diet. After another 24-hour period, insects were placed on plain zucchini fruit for the duration of the experiment. We chose to inoculate insects with both types of bacteria in the same life stage given the narrow window for successful symbiont establishment found in other bugs with *Caballeronia* symbionts (35).

Sampling. Following the final bacterial or sterile water diet, insects were periodically sacrificed to determine the persistence and intensity of symbiont and pathogen infection. Ten individuals were sacrificed immediately after the final inoculation (Day 0), every day for three days, at days five and seven, then weekly until day 21. We used the same sampling methods as before, this time plating samples in triplicate. Treatments with both Sq4a and Z01 were plated on regular LB for pathogen detection and on LB with 80 ug/mL of spectinomycin for symbiont detection.

Symbiont only and pathogen only treatments were only plated on one type of plate, LB with spectinomycin or standard LB, respectively. Colonies were visualized as before.

Statistical Analyses

All statistical analyses were run in R version 3.6.1. We used the package "glmmTMB" (63) to fit generalized linear models (GLMs) accounting for both treatment and time, in this case days post acquisition (DPA) of Z01 or days post final inoculation, to our data on the presence and intensity of Z01 infections in squash bugs, using the binomial and negative binomial error distributions, respectively. All individuals were included in analyses of persistence data, but analyses of Z01 infection intensity were restricted to non-zero values, because, by definition, infection intensity represents the abundance of a parasite within infected individuals. We used the function r.squaredLR in the package "MuMIn" to derive pseudo-R^2 estimates for our models to facilitate comparisons (64).

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Competing Interests

The authors declare no competing interests.

Supplementary Material

Figure S3.1

Anasa tristis survival following ingestion of phytopathogenic *S. marcescens*. Symbiont-positive insects fed *S. marcescens* during third instar then reared on plants showed no significant difference in survival when compared to *S. marcescens*-free individuals. This figure shows the average proportion of insects that survived over the course of 2 months across 12 replicates for each treatment. The gray bar indicates the time frame during which replicates were exposed to *S. marcescens*.

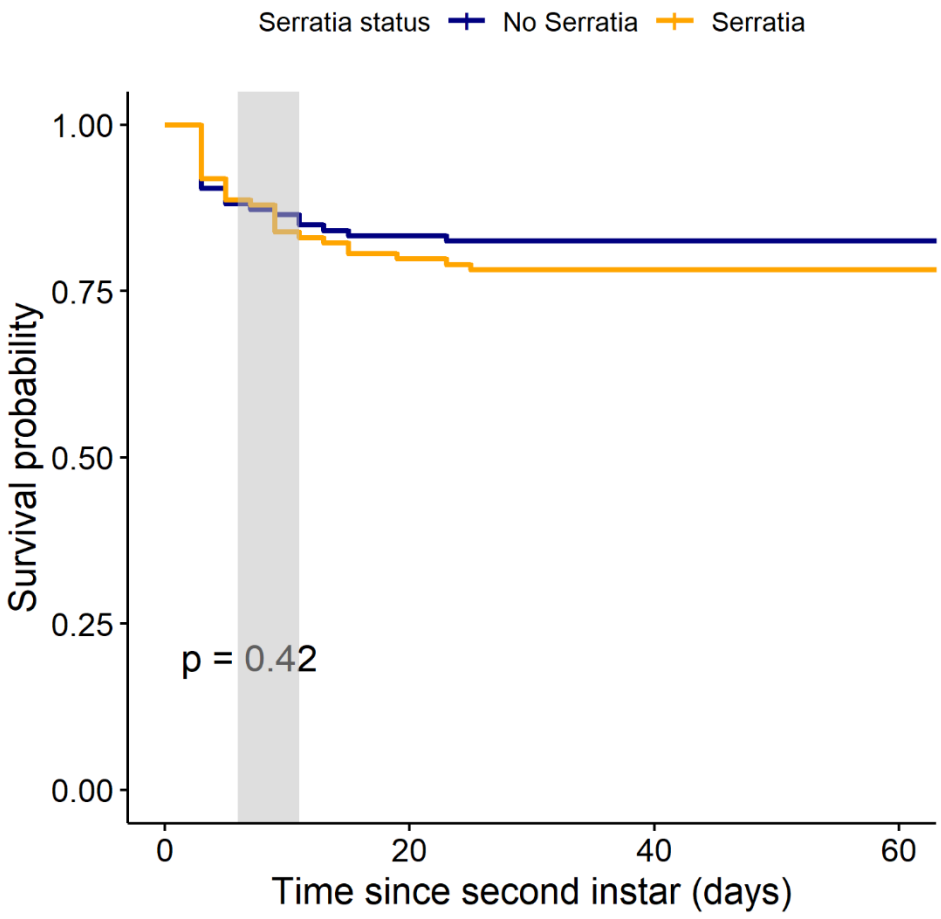
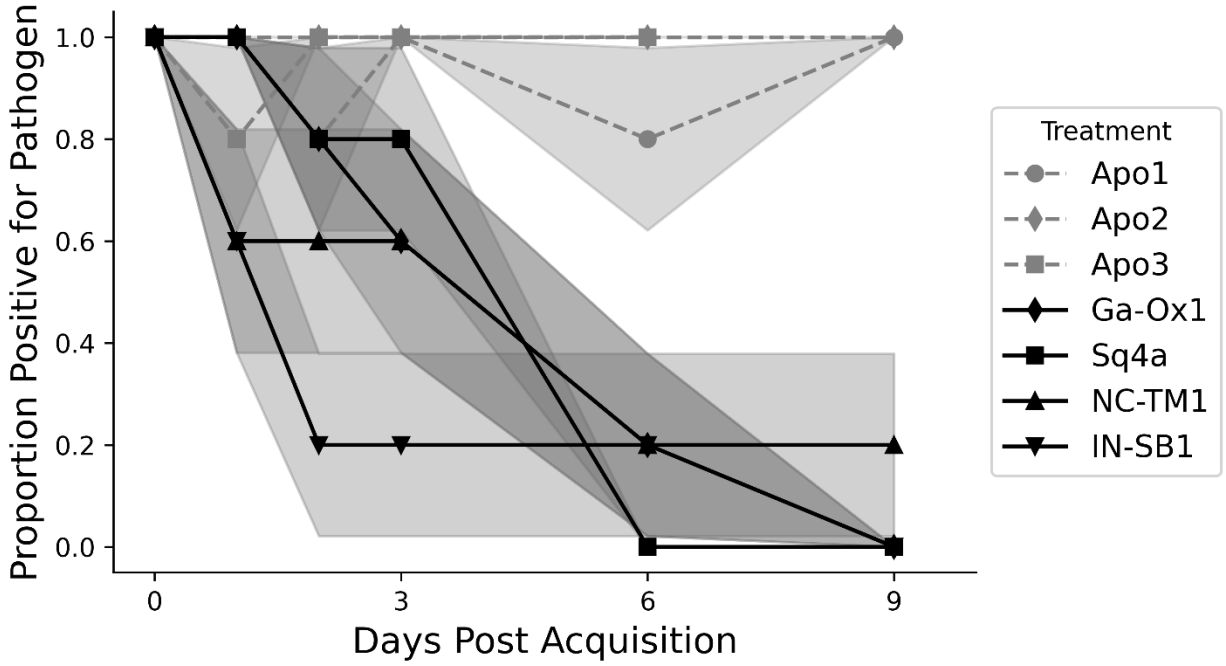


Figure S3.2

(a) The proportion of individual bugs that tested positive for *S. marcescens* at a given time point across aposymbiotic and symbiont positive treatments. Each point represents 5 individual bugs. Shading represents standard error estimates. (b) *S. marcescens* titer recovered from infected squash bugs over time. Only non-zero values were included in this analysis. Points represent up to 5 individuals in each treatment. Shading represents 95% confidence intervals. Differences between individual symbiont isolates (shown in black) and aposymbiotic batches (shown in gray) were negligible compared to differences between symbiont positive and aposymbiotic treatments. Note that confidence intervals are not calculated in (b) when only one positive individual was observed in a treatment (e.g., for observations > 3 days post acquisition).

a)



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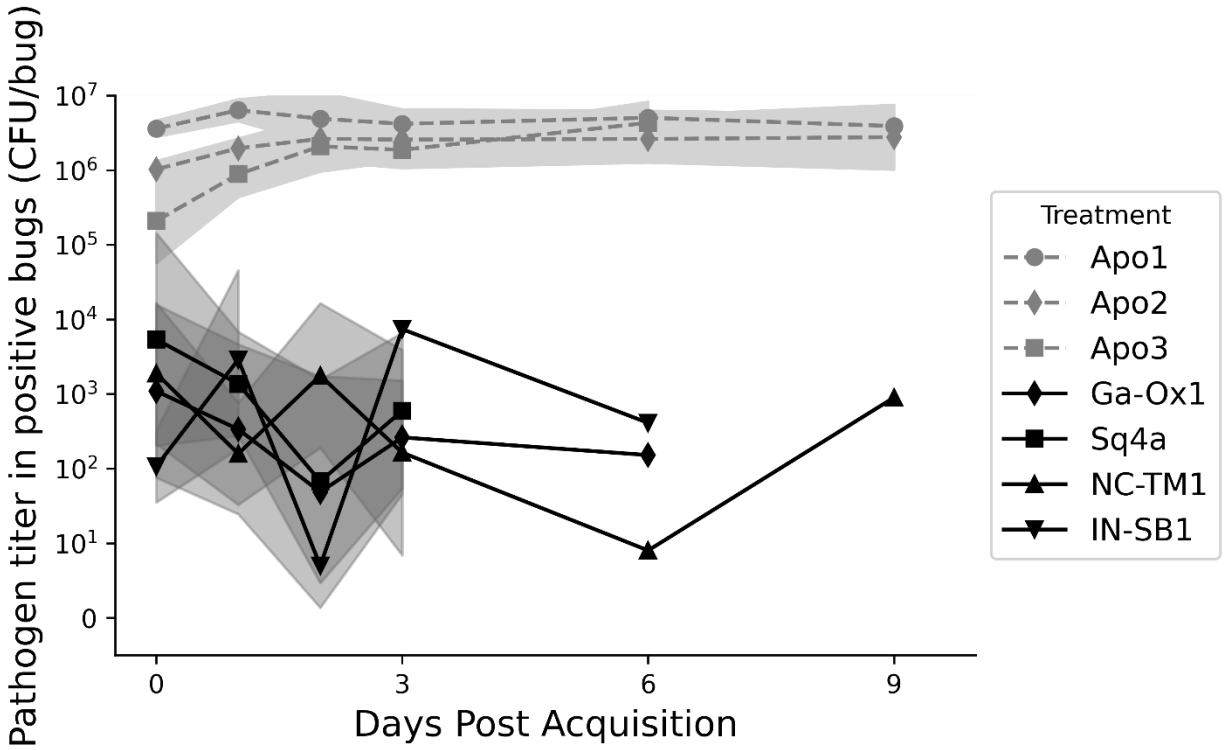
Pathogen titer in positive bugs (CFU/bug)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
0
3
6
9
Days Post Acquisition
Treatment
Apo1
Apo2
Apo3
Ga-Ox1
Sq4a
NC-TM1
IN-SB1

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Chapter IV: Ecological modeling evaluates the potential impacts of symbionts on plant pathogen vectoring in field populations

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Abstract

Within the last decade, research into the use of insect microbial symbionts as a means of controlling populations of insect vectors and the pathogens they transmit has advanced substantially. Many microbes have been identified that affect important epidemiological traits of vectors or pathogens in the laboratory, yet few have been tested in the field. Consequently, it remains unknown which effects of symbionts drive successful control. We investigated the relative importance of simultaneous effects caused by one such microbe, *Caballeronia* spp., on the potential of its squash bug host to vector phytopathogenic *Serratia marcescens*. Infection with *Caballeronia*, a beneficial symbiont of squash bugs, leads to reduced pathogen titers and rapid clearance of *S. marcescens* in bugs, reducing the vectoring potential of a significant pest in squash agriculture. Using simulation modeling and sensitivity analysis, we determined the relative impact that reducing the vector potential of symbiont-free (aposymbiotic) bugs and increasing population-level symbiont coverage would have on overall pathogen transmission in a field setting. In this system, we show that aposymbiotic insects contribute significantly to pathogen outbreaks even when they comprise a small portion of the population. While reducing the transmission rate of aposymbiotic insects shows promise in disease mitigation, maximizing symbiont prevalence in the vector population is likely to have the most impact on mitigating plant infections. We conclude that for symbiont-mediated interventions where disparities in

transmission between aposymbiotic and symbiotic individuals are already high, ensuring high symbiont uptake in a population is critical for success.

Introduction

The last few decades have seen an uptick in the emergence and resurgence of vector-borne diseases (Kilpatrick and Randolph, 2012), a trend that is unlikely to change in the future given current projections of expanding habitat suitability for vector insects (Manwar and Khan, 2022). Increasingly, researchers are looking for novel ways to disrupt the transmission of vector-borne pathogens. While insecticides continue to play a key role in vector-control, the insect microbiome has emerged as a strong contender to mitigate vector-borne diseases (Chuche et al., 2017; Moreira et al., 2009).

An insect's microbiome can have drastic effects on its vector competence, its ability to acquire and transmit pathogens or parasites (Weiss and Aksoy, 2011), and many such relationships have been demonstrated in the laboratory (Gonella et al., 2018; Moreira et al., 2009; Weiss et al., 2012). To date, most tests of how symbiotic microbes can alter insect vector competence have focused on symbionts' effects on important traits of individual insects. While these studies are essential for identifying novel candidates for disease control, deploying these methods requires a cross-scales approach that also addresses how individual physiological effects play out at the population level, a more relevant scale for disease mitigation (Mendiola et al., 2020).

Despite promising results in the lab, symbiont-mediated vector control measures have not been widely implemented in the field. These large-scale interventions can be unpredictable and risky to undertake without due diligence. Mathematical models provide an avenue in which to investigate the efficacy of these interventions and assess potential risks and logistical hurdles to their implementation prior to field release. Such models have been instrumental in the

advancement of one of the foremost examples of field-deployed symbiont-mediated vector control: the development and release of *Wolbachia*-infected *Aedes aegypti* for the control of dengue (Dorigatti et al., 2018). Rearing mosquitoes *en masse* for release into the environment is expensive, risky, and potentially controversial. In the case of *Wolbachia*, mathematical models facilitated the execution of large-scale experiments at the population level and identified a range of potential outcomes prior to intervention deployment. This was particularly useful for evaluating parameters that could not be measured easily in individual insects but were key to the success of *Wolbachia*-based interventions such as symbiont prevalence and persistence within a population (Turelli and Barton, 2017).

Here, we apply this approach to an agricultural pathosystem—*Serratia marcescens*, causative agent of Cucurbit Yellow Vine Disease (CYVD), its insect vector the squash bug, *Anasa tristis*, and squash—with an emphasis on how symbiotic microbes within the insect vector can alter the dynamics of plant infections. Within individual insect vectors, the symbiotic microbe *Caballeronia* has strong documented effects, reducing the duration and intensity of phytopathogen (*S. marcescens*) infection in symbiont-colonized individuals relative to their symbiont-free (aposymbiotic) counterparts (Mendiola et al. 2022). Given the prolonged infections and increased pathogen titers seen in aposymbiotic individuals in the laboratory, we hypothesized that they could play an important role in *S. marcescens* transmission in the field. Thus, effective disease management could be achieved by reducing aposymbiotic bugs' vectoring potential or minimizing their presence in the population by employing symbiont-mediated vector control.

Despite promising results in the laboratory, the success of symbiont-mediated vector control may not translate to agricultural gains. For example, extreme or even complete

prevalence of the symbiont might be needed to achieve beneficial management outcomes, analogous to vaccination campaigns that fail to reduce, or may even exacerbate, human disease burden at moderate coverage but succeed at high coverage (Anderson and Grenfell, 1986; Plans-Rubió, 2012; van Boven et al., 2010). We address this concern by parameterizing a population-level model of this pathosystem with individual-level experimental data from the symbiont-vector-pathogen triad and conducting simulations and sensitivity analyses. Specifically, we explored the relative importance of aposymbiotic squash bugs in overall pathogen transmission as well as the impact of reducing the length and intensity of their pathogen infections to reduce their ability to transmit *S. marcescens*. Our results show that aposymbiotic individuals indeed have an outsized impact on pathogen spread, contributing significantly to outbreaks even when they comprise a small portion of the squash bug population. Furthermore, we found that increasing the proportion of individuals that acquire symbiotic bacteria, boosting overall symbiont prevalence, is an effective way to reduce plant infections. Our findings indicate that, for symbiont-mediated interventions where disparities in transmission between aposymbiotic and symbiotic individuals are already high, ensuring high symbiont uptake in a population is critical for success.

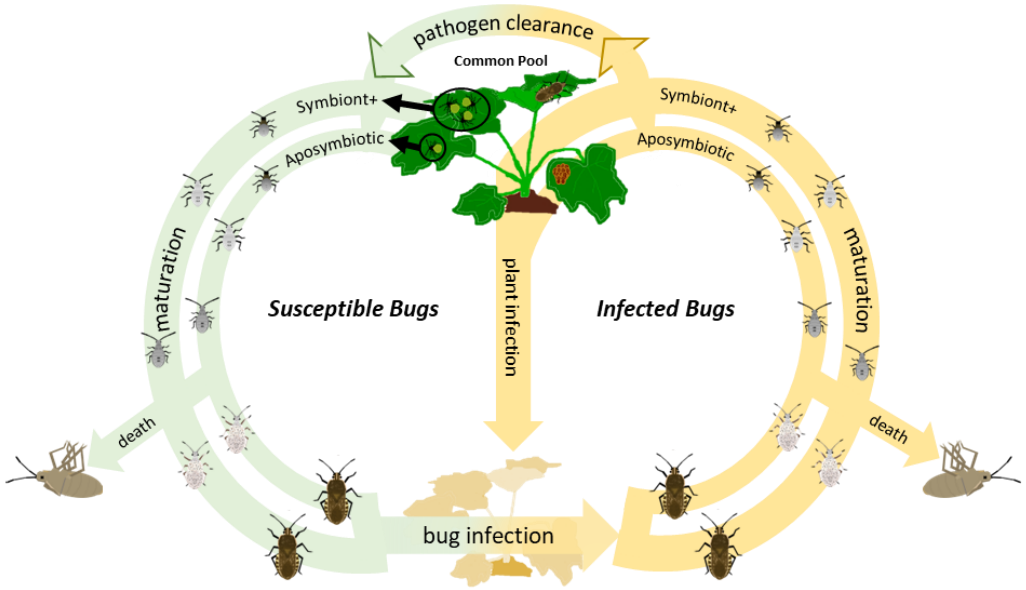
Methods

Natural History

The squash bug, *Anasa tristis*, is a major agricultural pest of squash and other cucurbit crops. In addition to feeding damage caused by high infestations, squash bugs also vector phytopathogenic *Serratia marcescens*, causative agent of Cucurbit Yellow Vine Disease (CYVD) (Bextine et al., 2001). CYVD is characterized by general and rapid yellowing of leaves and gradual vine decline that often worsens before fruit set (Bruton et al., 1998). Squash and other

cucurbit plants may become infected when fed upon by infected bugs. Plants are most susceptible to infection when exposed early in the season (Bruton et al., 2003), and pre-harvest

infections contribute significantly to yield losses as plants die before producing fruit (Bruton et al., 1998).



Overwintering Adults

$$\frac{dO_A}{dt} = -(d_A + l)O_A + cO_{AI}$$

$$\frac{dO_{AI}}{dt} = -(d_A + l + c)O_A$$

Common Bug Pool

$$\frac{dE}{dt} = \frac{1}{2}b(O_A + O_{AI})\left(1 - \frac{E}{K_E}\right) - (d_E + m_E)E$$

$$\frac{dS_1}{dt} = m_E E - (d_1 + m_1)S_1$$

Uninfected Squash Bugs

$$\frac{dS_{2\pm}}{dt} = p_{\pm}m_1 S_1 - (d_{2\pm} + m_{2\pm})S_{2\pm} + \gamma c_+ I_{2\pm} - \beta_p S_{2\pm} P_I$$

$$\frac{dS_{i\pm}}{dt} = m_{(i-1)\pm}S_{i\pm} - (d_{i\pm} + m_{i\pm})S_{i\pm} + \gamma c_+ I_{i\pm} - \beta_p S_{i\pm} P_I$$

$$\frac{dS_{A\pm}}{dt} = m_{5\pm}S_{5\pm} - d_{A\pm}S_{A\pm} + \gamma c_+ I_{A\pm} - \beta_p S_{A\pm} P_I$$

Infected Squash Bugs

$$\frac{dI_{2\pm}}{dt} = \beta_p S_{2\pm} P_I - (d_{2\pm} + m_{2\pm} + \gamma c_+)I_{2\pm}$$

$$\frac{dI_{i\pm}}{dt} = m_{(i-1)\pm}I_{(i-1)\pm} + \beta_p S_{i\pm} P_I - (d_{i\pm} + m_{i\pm} + \gamma c_+)I_{i\pm}$$

$$\frac{dI_{A\pm}}{dt} = m_{5\pm}I_{5\pm} + \beta_p S_{A\pm} P_I - (d_{A\pm} + \gamma c_+)I_{A\pm}$$

Infected Plants

$$\frac{dP_I}{dt} = (\beta_+\left(\sum_{i=2}^{5} I_{i+} + I_{A+} + O_{AI}\right) + \beta_+\lambda\left(\sum_{i=2}^{5} I_{i-} + I_{A-}\right))(P_0 - P_I)$$

Figure 4.1. Model diagram depicting squash bug population dynamics and pathogen transmission. Simulations begin with overwintering adults that seed both the squash bug population and plant infections. Infected and uninfected overwintering adults lay eggs that contribute to the common pool of individuals. These eggs hatch into symbiont-free first instars that move into the symbiotic or aposymbiotic categories at second instar based on some probability of symbiont acquisition. Individuals cannot move freely between symbiotic and aposymbiotic categories. Within these categories, individuals mature, passing through four more life stages before becoming adults. All individuals experience a background death rate. Any life stage of bug may feed on an infected plant and become infected, except for overwintering adults which can clear infection, but do not become reinfected. Infected individuals continue to mature and die at the same rate as uninfected individuals, but they may clear the pathogen and rejoin the uninfected class. Plants become infected when fed on by infected individuals and do not clear infection. In the model equations, processes that move infected bugs to the uninfected class are color-coded in green. Processes that move uninfected bugs to the infected class are color-coded in yellow.

Table 4.1. State Variables and Model Parameters

Variable	Description	Starting Values	
O_A	Overwintering Adults	9	
O_{AI}	Infected Overwintering Adults	1	
E	Eggs	0	
$S_{i\pm}$, $S_{A\pm}$	Susceptible life stages	0	
$I_{i\pm}$, $I_{A\pm}$	Infected life stages	0	
P_I	Infected plants	0	
Parameter	**Description**	**Value (units)**	**Source**
P_0	initial proportion of uninfected plants	1	
l	loss rate of overwintering adults	0.05 day^{-1}	(Eiben 2004)
b	birth rate	10 eggs female^{-1} day^{-1}	(Villa et al 2021)
K_E	egg carrying capacity	250 eggs plant^{-1}	
d_E, $d_{i\pm}$	survival probability eggs and immature life stages	$\frac{1}{2}$ life stage^{-1}	(Fielding 1988)
$d_{A\pm}$, $d_{AI\pm}$	background death rate adults	$\frac{1}{60}$ day^{-1}	(Fielding 1988)
m_E	maturation rate, eggs	$\frac{1}{7}$ day^{-1}	(Acevedo et al 2021)
$m_{i\pm}$	maturation rates, immatures	$\begin{matrix} & + & - \\ m_1 & 1/4 & 1/4 \\ m_2 & 1/4 & 1/4 \\ m_3 & 1/4 & 1/7 \\ m_4 & 1/4 & 1/13 \\ m_5 & 1/16 & 1/17 \end{matrix}$ day^{-1}	(Acevedo et al 2021)
c	pathogen clearance time, symbiotic bugs	$\frac{1}{3}$ day^{-1}	(Mendiola et al 2022)
β_p	pathogen acquisition rate from plants to bugs	0.025 plant^{-1} bug^{-1}	
β_+	pathogen transmission rate, symbiotic bugs to plants	low: 0.01 bug^{-1} plant^{-1} high: 0.10 bug^{-1} plant^{-1}	
$p_\pm$	Probability of 2nd instars either: acquiring symbiont(p_+), remaining aposymbiotic (p_- = 1 – p_+)	p_+ = 0.9 – 1	
γ	scaling factor for aposymbiotic clearance rate	0.1 – 1	
λ	scaling factor for aposymbiotic transmission rate	10 – 100	

i denotes nymphal life stages (1-5), ± denotes symbiont status (positive or negative),
Shading indicates parameters that were varied for sensitivity analysis

Squash bugs undergo five life stages, or instars, before reaching adulthood and reproductive maturity (Beard, 1940). Squash bugs form symbiotic relationships with bacteria in the genus *Caballeronia*, formerly *Burkholderia* (Dobritsa and Samadpour, 2019). *Caballeronia* symbionts are horizontally transmitted, meaning that individuals must acquire them *de novo* from the environment at every generation, most commonly in the second instar stage (Acevedo et al., 2021). Juveniles may acquire symbionts by probing the feces of adult squash bugs (Villa et al., 2023), however, since there is no strict transmission from parent to offspring, the symbiosis is not considered vertical. Within their insect hosts, *Caballeronia* symbionts are extracellular and localize to a specialized area of the posterior midgut known as the crypts. *Caballeronia* symbionts are culturable and can be isolated from host insects readily on standard laboratory media. These qualities have made the *A. tristis*-*Caballeronia* system tractable for the study of insect-microbe symbiosis. Experimentation in the lab has already shown that *Caballeronia* ymbionts speed the development and decrease the mortality of their insect hosts (Acevedo et al., 2021). Based on data of field-collected *A. tristis* from (Acevedo et al., 2021) we estimate the prevalence of *Caballeronia* symbionts to be approximately 94% in the field. Our field derived estimates rely primarily on sampling of adult bugs indicating that 6% of adults do not acquire *Caballeronia*. This is in line with findings in *Riptortus pedestris*, another hemipteran with environmentally-acquired *Caballeronia* symbionts, where approximately 93% of individuals sampled from the field are symbiotic (Jung and Lee, 2019).

Squash bugs can acquire *S. marcescens* at any stage after reaching second instar. Transmission from insects to plants can happen rather quickly after exposure (Bextine, 2001). Although the pathogen persists through transstadial molts (Wayadande et al., 2005), it is not vertically transmitted to offspring. *S. marcescens* infection is not known to cause detrimental

effects to its squash bug hosts (Mendiola et al. 2022). Adult bugs may overwinter with *S. marcescens* infections that remain viable and capable of infecting plants when bugs become active again in the spring (Pair et al., 2004). Previous work has established that *Caballeronia* symbionts drastically alter infection outcomes in individual insects. Squash bugs harboring *Caballeronia* symbionts have *S. marcescens* infections of ~1000-fold reduced intensity and clear infections ~10-fold faster than symbiont-free counterparts (Mendiola et al. 2022).

Model Specifications

We used a set of Ordinary Differential Equations (ODEs) to model *S. marcescens* transmission in a squash bug population occupying a single field (**Figure 4.1**, **Table 4.1**). Each simulation began with all plants initially uninfected ($P_0 = 0$) and ran over the course of one field season, roughly three months. To quantify disease impact, we assumed a fixed density of plants and tracked the proportion of infected plants (P_I) over the course of the field season. We calculated the area under the plant prevalence vs. time curve for every simulation.

The squash bug population was founded by overwintering adults (O_A), ten percent of which were infected with *S. marcescens* (O_{AI}) and thus capable of seeding infections; this is consistent with findings in the field (Pair et al., 2004). All overwintering adults were assumed to be symbiotic and thus shared parameters with symbiotic adults (Fielding, 1988) with an additional loss rate (l) to account for migration out of the field (Eiben, 2004).

Simulations spanned 90 days, typical of squash growing season in Illinois and Oklahoma, from where our field data is derived (Eiben, 2004; Fielding, 1988). Given this time frame, our squash bug population was effectively univoltine and only completed one generation of development before the end of the field season. Thus, reproduction was restricted to

overwintering adult females. Reproductive rates for adults were obtained from (Villa et al., 2021) and did not differ between *Serratia*-infected and uninfected individuals. Eggs and first instars were part of a common pool of individuals and, consistent with previous findings (Acevedo et al., 2021), were assumed to be symbiont negative. Maturation rates for each life stage were derived from laboratory data and differed by symbiont status (Acevedo et al., 2021). Survival rates in the field were roughly estimated to be ~50% at each immature life stage (Fielding, 1988) and did not differ by symbiont status. Estimates of adult longevity were also derived from (Fielding, 1988).

Beginning with the second instar stage, the squash bug population was divided into four categories that varied by their symbiont and *S. marcescens* infection status: 1) symbiotic susceptible (S_{i+}), 2) symbiotic infected (I_{i+}), 3) aposymbiotic susceptible (S_{i-}), and 4) aposymbiotic infected bugs (I_{i-}). All individuals were initially sorted into either uninfected symbiotic or aposymbiotic categories based on probability of symbiont acquisition (p) but could become infected with *S. marcescens* by feeding on an infected plant at any point afterwards. Symbiont acquisition was permanent and occurred only at the second instar stage, which is consistent with observations in *A. tristis* and other *Caballeronia*-associated insect species (Acevedo et al., 2021; Ohbayashi et al., 2022, 2019). Thereafter, squash bugs could not move freely between aposymbiotic and symbiotic categories. Transmission rates from infected plants to bugs (β_p) did not differ by symbiont status or life stage. Infected squash bugs continued to mature and die at the same rates as their uninfected counterparts, consistent with the minimal effect of *S. marcescens* infection on mortality and development. Infected individuals at any life stage could revert to uninfected status based on *S. marcescens* clearance rates (**Table 4.1**) and could be re-infected when feeding on infected plants. Though overwintering adults cleared *S.*

marcescens infection at the same rate as symbiotic adults, our model did not allow for them to become reinfected and continue contributing to transmission.

Previous work identified symbiont status as the most important determinant of *S. marcescens* infection in squash bugs (Mendiola et al., 2022). This finding, coupled with the difficulty in obtaining life-stage specific pathogen acquisition and transmission parameters, prompted us to simplify our model by not varying pathogen acquisition and transmission rates by life stage, focusing instead on the difference resulting from symbiont status. We identified two vector traits that could be influenced by symbiont status: pathogen infection intensity and clearance rate (Mendiola et al., 2022). For this model, we use pathogen infection intensity as a proxy for transmission rate, with the assumption that higher pathogen loads translate to increased probability of transmission. Because high clearance and low transmission rates in symbiotic bugs already resulted in low vector competence, we focused on how reducing the vectoring potential of aposymbiotic bugs and decreasing the aposymbiotic portion of the population would affect pathogen spread. Because wild populations consist primarily of symbiotic bugs, we treated the pathogen clearance and transmission rates of symbiotic bugs as the baseline. Symbiotic individuals could transmit *S. marcescens* to plants at a rate (β_+) that did not differ by life stage. Transmission rates for aposymbiotic bugs were set up as a factor λ of the transmission rate for symbiotic individuals (i.e., $\lambda*\beta_+$). Clearance rates were similarly incorporated with symbiotic bugs clearing infection at a rate c and aposymbiotic individuals clearing infection at rates $\gamma*c$. This allowed us to investigate how pathogen spread might change if the vectoring potential of aposymbiotic bugs was reduced.

Sensitivity Analysis

We used the R package "sensobol" (v 1.1.1) (Puy et al., 2022) to conduct sensitivity analyses for three parameters of interest in high and low transmission scenarios: 1) probability of symbiont acquisition in the squash bug population; 2) aposymbiotic pathogen clearance rate; and 3) aposymbiotic pathogen transmission rate. For sensitivity analyses, our outcome of interest was the area under the infection prevalence vs. time curve. We chose this measure over infection prevalence alone because it better captures the impact of plant infections that occur earlier in the season, where they have the most impact on crop productivity. We used the trapz function in the caTools package to compute the area under our simulated infection prevalence curves using trapezoid rule integration. Since we set symbiotic individuals as the baseline for transmission, our sensitivity analyses look specifically at the effect of reducing the vectoring potential and prevalence of aposymbiotic bugs. We further tested how symbiont coverage in the population affects plant infections. Because this system lacks concrete estimations of transmission rates, we conducted separate sensitivity analyses for plausible scenarios of high and low baseline pathogen transmission. In both scenarios, we set the size of our base sample matrix at 8192, used Azzini estimators, computed up to second-order effects, and bootstrapped the indices 10^3 times as recommended in the sensobol package documentation (Puy et al. 2022). We varied the values of the three parameters of interest over the ranges included in Table 1. All analyses were run in R version 4.2.2.

Results

Model performance

Squash bug population dynamics generated from our simulations were validated against field data (Eiben, 2004). Our model was a qualitative match to the field data, successfully reproducing the characteristic, offset peaks that declined in magnitude with every subsequent

squash bug life stage (**Figure 4.2**). Our simulated population dynamics further capture the depletion of immature life stages by the end of the season, leaving only adults that can overwinter and seed future generations. In our simulations, population peaks were shifted to slightly earlier in the season. This could be due to allowing overwintering adults to begin laying eggs as soon as they emerged from diapause. While this may not be the case in the real world, there are insufficient data available for us to incorporate this reproductive lag into our model.

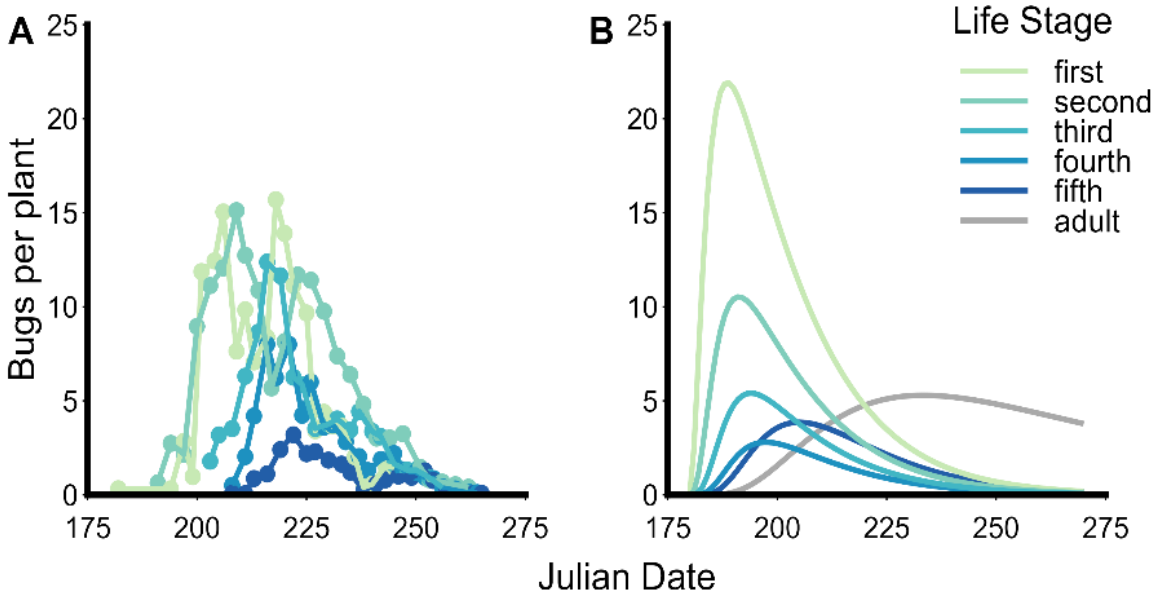


Figure 4.2. Qualitative trends observed in field data are recaptured by simulation output. Egg counts were omitted to achieve better resolution on the curves for the other life stages, but were on a similar scale for field and simulation data. (A) Squash bug counts collected from the field (reproduced from Fielding, 1988). Note, field data did not include counts for adult bugs. (B) Characteristic simulation output. Adult counts do not include overwintering adults, only adults that developed during the current field season. The simulation output captures the characteristic, offset peaks which become smaller in magnitude as bugs mature, leaving only adult bugs at the end of the season as all other life stages diminish.

Sensitivity analyses

Plant infections decline with reduction in aposymbiotic bugs' vectoring potential

Our sensitivity analysis allowed us to determine the effect of reducing the vectoring potential of aposymbiotic bugs by simultaneously decreasing their transmission rates and increasing their pathogen clearance rates. For the transmission rate, we varied the factor by

which aposymbiotic bugs transmitted *S. marcescens* to plants from 10 to 100 times the value of the baseline transmission rate of symbiotic bugs. This trend held true under both the low and high transmission scenarios tested (**Figure 4.3A & C**). We ranged the aposymbiotic bugs' clearance rate from being equal to the symbiotic clearance rate (the baseline rate) to being 10 times slower. For simplicity, we present this as the fold change in clearance time—the inverse of the clearance rate—in aposymbiotic bugs in **Figure 4.3**. Thus, $\gamma^{-1} = 1$ is an equal clearance time to symbiotic bugs and $\gamma^{-1} = 10$ is a 10-fold increase in aposymbiotic pathogen clearance time relative to symbiotic bugs. Plant infections were at their lowest when the clearance rates for

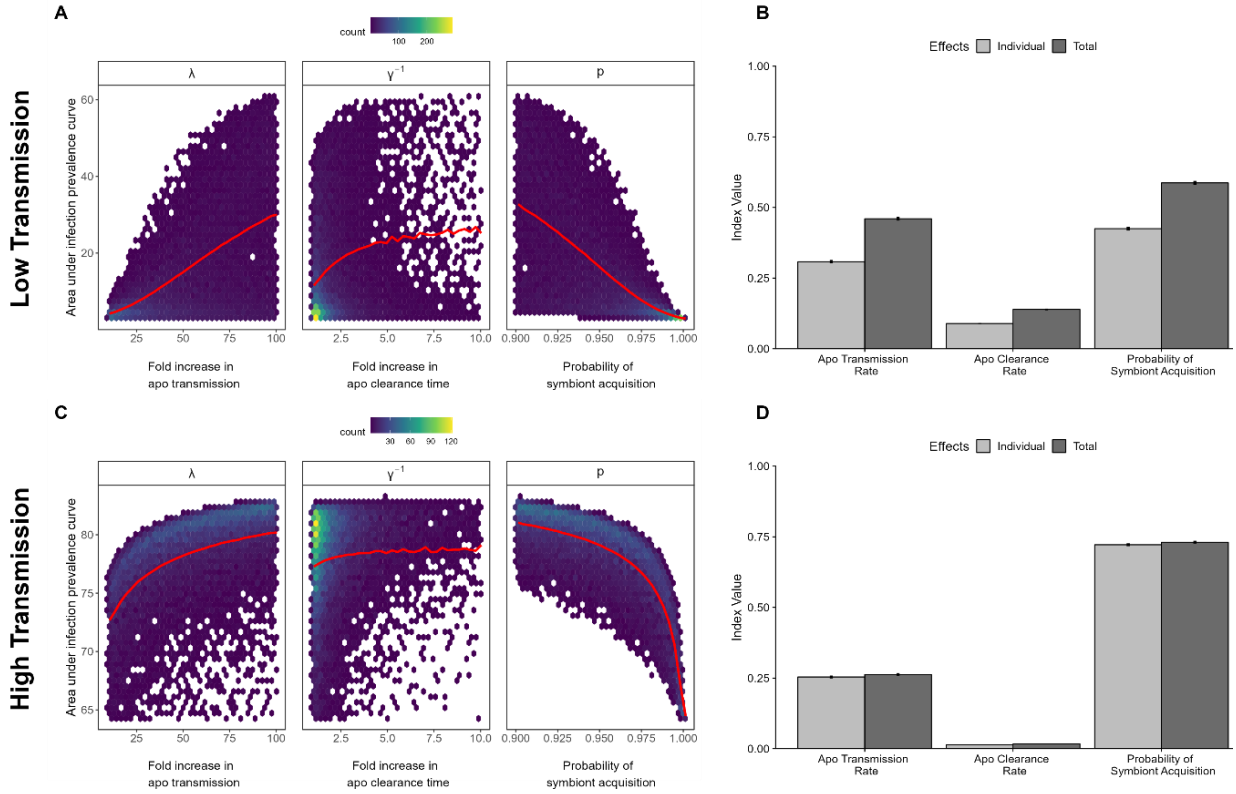


Figure 4.3. Sensitivity analyses show relative importance of each parameter examined. Panels A & B correspond to our low transmission scenario, and panels C & D correspond to high transmission. Panels A & C show heatmaps of plant infection results from 8000 simulations, with red lines depicting the mean outcome across all simulations. Each variable of interest was varied over a different range of parameters, specified on the x-axis. For clarity, we took the inverse of the parameter γ, to show the fold increase in clearance time rather than the fold reduction in clearance rate. Panels B & D show the Sobol indices calculated from our sensitivity analyses under both transmission scenarios. We calculated the individual effect of each parameter tested as well as the total effect which encompasses the main effect of each parameter and all its interaction effects with other parameters tested.

aposymbiotic and symbiotic bugs were equal ($\gamma^{-1} = 1$) regardless of whether baseline transmission was low or high.

Symbiont population prevalence is the main determinant of plant infections

We accounted for high symbiont prevalence in wild squash bug populations by only varying the probability of symbiont acquisition from 90 to 100% of the squash bug population for our sensitivity analyses. This 10% increase in symbiont prevalence resulted in sharp declines in plant infections. The drop was particularly notable when baseline transmission was high (**Figure 4.3C**), where a precipitous drop in the area under the infection prevalence curve was observed when symbiont prevalence increased from 97.5 to 100%.

Sobol indices calculated from our sensitivity analyses revealed that the relative importance of each parameter on plant infections differed based on whether baseline transmission was low or high (**Figure 4.3B & D**). However, in both scenarios, symbiont prevalence was the most significant driver of plant infections, individually explaining 42.5% of output variance when baseline transmission was low and 72.2% when baseline transmission was high. In our low transmission scenario, the percentage of variance explained by symbiont prevalence jumped to 58.7% when we looked at the total effect indices, which account for interaction effects among our three parameters of interest. The total effect index for symbiont prevalence did not increase significantly from the individual effect index under high transmission, jumping only to 73.1% of variance explained. Generally, we noticed a drastic decline in the relative importance of interaction effects for all parameters when baseline transmission was high.

After symbiont prevalence, the scaling factor determining the pathogen transmission rate for aposymbiotic bugs was the second most important driver of plant infections in our model,

explaining 30.8% of variance in plant infections individually (46.0% after accounting for interaction effects) when baseline transmission was low and 25.4% individually (26.3% with interaction effects) when transmission was high. In both transmission scenarios, the scaling factor determining aposymbiotic pathogen clearance rate had the least impact on plant infections, with little impact in our low transmission scenario (individual effect 8.9%, total effect 13.9%) and almost no impact on model outcomes under our high transmission scenario (individual effect 1.4%, total effect 1.7%).

Aposymbiotic bugs contribute disproportionately to plant infections

We tracked plant infections attributable to aposymbiotic and symbiotic bugs separately to determine their relative contributions to plant infections. We further broke down the symbiotic category into overwintering adults and symbiotic bugs from the newly founded generation. As before, we ran simulations under both low and high baseline transmission scenarios. We kept the probability of symbiont acquisition by the F1 generation fixed at 90% of the population, set the pathogen clearance rate of aposymbiotic bugs as 0.1 the rate of symbiotic bugs and made the aposymbiotic transmission rate 10 times that of symbiotic bugs. Under these conditions, our infection prevalence curves show that, although aposymbiotic bugs only accounted for 10% of the total squash bug population, they were responsible for more plant infections than symbiotic bugs in low transmission settings and performed on par with symbiotic bugs in high transmission settings (**Figure 4.4A & B**). **Figure 4.5** shows a breakdown, by life stage, of the aposymbiotic squash bug population over the course of the field season when the probability of symbiont acquisition is set to 90%. Generally, infections attributable to aposymbiotic bugs lagged temporally behind those attributable to symbiotic bugs. Early in the season, infected overwintering adults contributed the most plant infections, while the newly founded generation

slowly grew. This is most obvious in **Figure 4.4B**, where a burst of infections attributable to overwintering adults can be seen prior to any infections by either symbiotic or aposymbiotic F1s

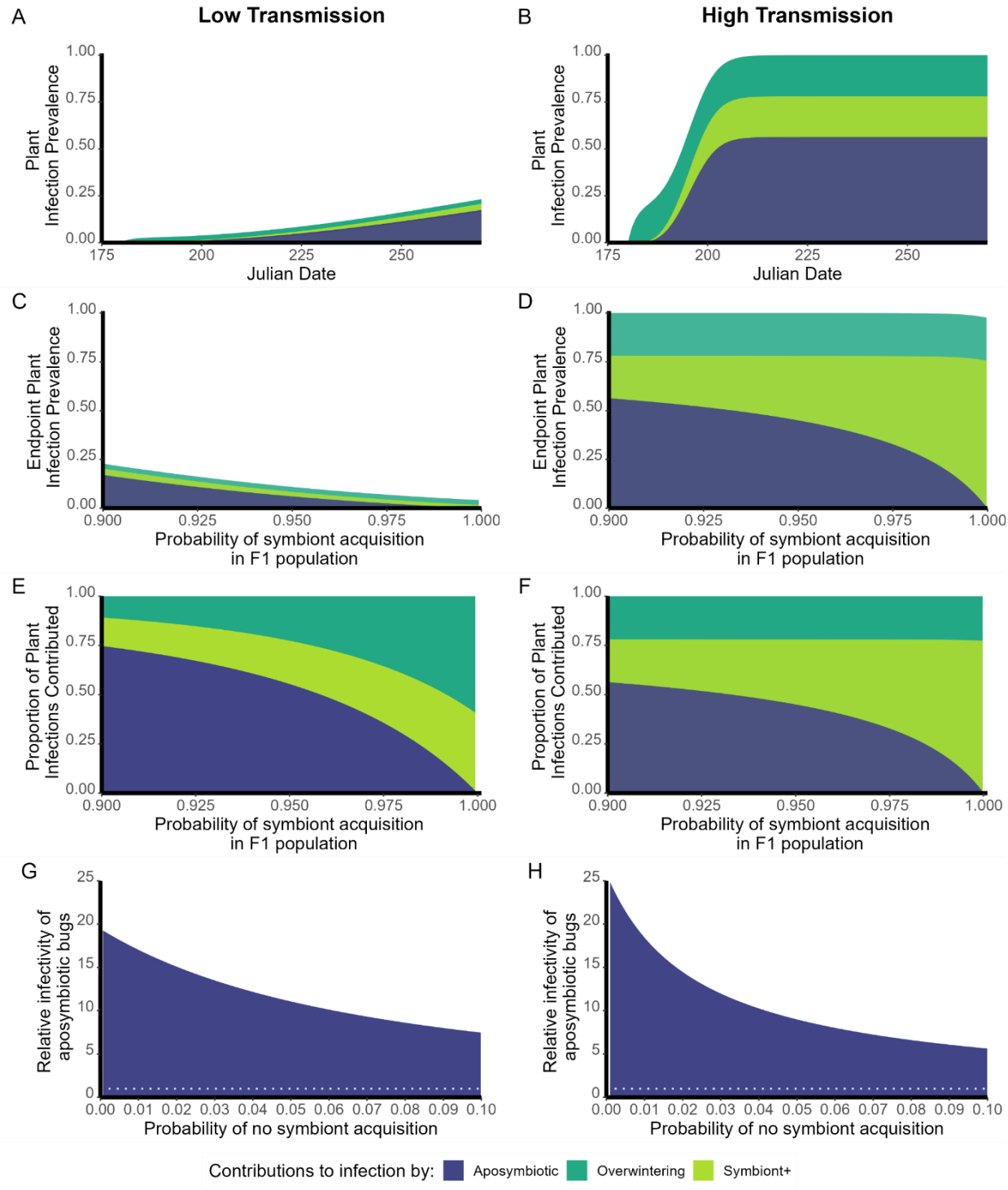


Figure 4.4. Aposymbiotic individuals contribute disproportionately to pathogen transmission. Under both scenarios of low (A, C, E, G) and high (B, D, F, H) baseline pathogen transmission, having even a small proportion ($\leq$ 10%) of aposymbiotic bugs in the population can dramatically increase total plant infections. Plots A and B show the relative contributions of the different bug categories for both the founding generation (overwintering adults) and the new generation (aposymbiotic and symbiotic) to plant infections over the course of a field season. For these plots, the probability of symbiont acquisition (p) in the newly founded generation (F1) is maintained at 90%, λ at 10, and γ at 0.1. All other parameters are as specified in Table 1. For plots C through F, we varied p between .9 and 1, but kept the same λ and γ values. Plots C and D show the relative contributions of different categories of bugs, to the endpoint infection prevalence. Plots E and F, further break down this data to show the proportion of total infections attributable to each bug category. Plots D and F look similar since, at high baseline transmission, all plants are infected at the end of the season. Overwintering adults, though treated as symbiotic in our model, are independent of p and thus present and able to contribute to plant infections even when p = 1. Plots G and H summarize the results above, showing the relative infectivity of aposymbiotic bugs across the range of their relative prevalence in the population (0 to 10%). The dotted line in each plot indicates an infectivity of 1, where infections contributed by aposymbiotic bugs equal our expectations based on their prevalence in the population. All infections over this line represent infections in excess of what is expected based on their relative abundance.

appearing. Despite this lag, aposymbiotic bugs still contributed significantly to total plant infections by the end of the season.

We further examined the impact of aposymbiotic bugs on plant infections by varying the probability of symbiont acquisition in the F1 generation while tracking their relative contributions to endpoint infection prevalence (**Figure 4.4C & D**). We ran simulations at low and high baseline transmission with the transmission and clearance rates for aposymbiotic bugs fixed as before, this time varying the probability of symbiont acquisition from .9 to 1. Unsurprisingly, a higher probability of symbiont acquisition led to less infections overall when baseline transmission was low. Scenarios of high baseline transmission universally resulted in almost all plants being infected at the end of the season, regardless of the probability of symbiont acquisition in our chosen range. **Figures 4.4E** and **4F** show results from the same simulations as **4.4C** and **4.4D**, respectively, but this time as a proportion of plant infections contributed. This makes it more obvious that, even when aposymbiotic bugs comprised a relatively low proportion of the population—such as under a high probability of symbiont acquisition—they contributed

heavily to plant infections. Overwintering adults seem to contribute a steady number of infections, most likely originating from early in the season, under scenarios of high baseline transmission. They have the most effect on pathogen transmission when baseline transmission rates are low and aposymbiotic bugs are not a part of the population.

Lastly, we quantified whether aposymbiotic bugs were disproportionately contributing to plant infections. For this we calculated a new metric to determine whether the probability that infections contributed by aposymbiotic bugs were equal to the probability of bugs in the population being aposymbiotic. We called this metric the relative infectivity of aposymbiotic bugs since it gave us an idea of how much more or less likely aposymbiotic bugs were to infect plants relative to their representation in the population. We plotted this metric against the probability that an individual in the F1 generation would remain aposymbiotic (i.e., the

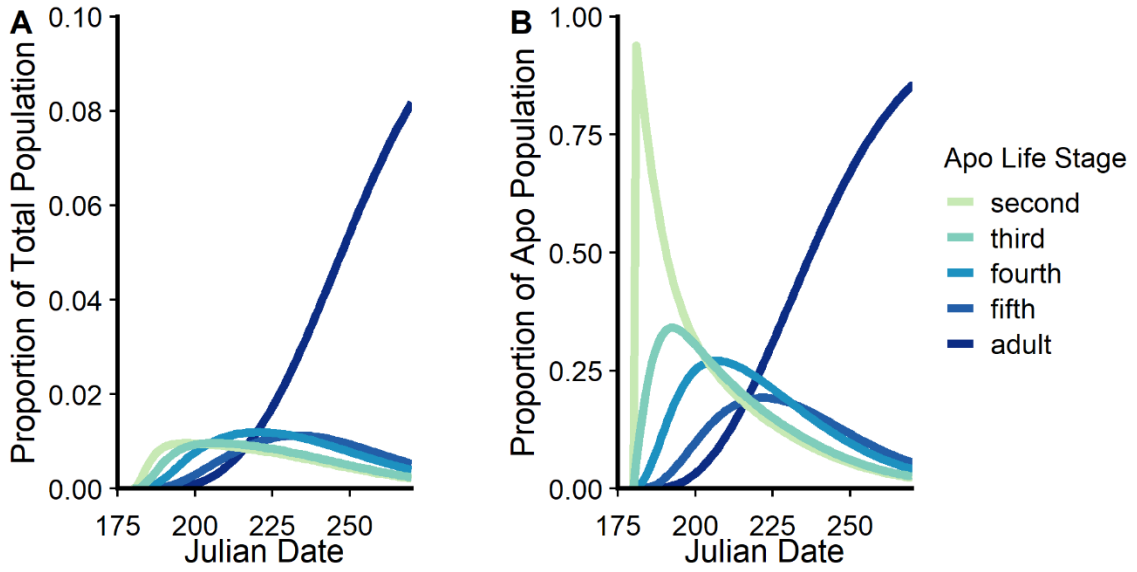


Figure 4.5. Composition of the aposymbiotic squash bug population varies over the course of the field season. When the probability of symbiont acquisition is set to 90%, aposymbiotic bugs account for roughly 10% of the population at the end of the season. Plot A shows the proportion of the total population made up by aposymbiotic bugs in each life stage. Plot B further determines the proportion of the aposymbiotic population made up by each life stage over the course of the field season. As expected, early instars dominate the first half of the season, but are eventually replaced by adult bugs.

probability of no symbiont acquisition). As seen in **Figure 4.4G** and **4.4H**, the relative infectivity of aposymbiotic bugs always exceeded one, the number denoting infection contributions equal to population representation, meaning that aposymbiotic bugs across all scenarios we tested always contribute disproportionately to pathogen transmission.

Discussion

Mathematical models have a long history of use in agricultural pathosystems as key tools to identify potential targets for interventions and assess the feasibility and cost-effectiveness of interventions in the field (Chapwanya and Dumont, 2021; Fishman et al., 1983; Holt et al., 1997; Kendall et al., 1992). As the use of symbiotic microbes to control insect vector populations has become increasingly viable (Arora and Douglas, 2017; Chuche et al., 2017; Darby, 2009), there has been a rise in models that incorporate the effects of symbiotic microbes on vector populations and their ability to transmit pathogens of consequence to human health (Dorigatti et al., 2018; Gilbert et al., 2016). Using similar principles, we built a model that incorporates the impact of symbiotic microbes on individual insect's vectoring capabilities to assess whether symbiont-mediated effects in the vector population could alter transmission of a plant pathogen in the field.

In this system, symbiotic bacteria in the genus *Caballeronia* drastically shorten the duration and reduce the intensity of phytopathogenic *S. marcescens* infections within the insect vector, *A*. *tristis*, relative to aposymbiotic individuals (Mendiola et al., 2022). This prompted us to investigate the potential impact of aposymbiotic individuals in transmitting *S. marcescens* in the field. We leveraged sensitivity analysis to parse the relative importance of pathogen clearance time and transmission rate (as a proxy for infection intensity within the vector) of aposymbiotic bugs in determining overall plant infections. We further determined the impact of overall symbiont prevalence in the vector population on plant infections in the field. In other vector-

borne agricultural pathosystems, similar approaches have successfully employed sensitivity analysis to identify model parameters that are responsive to perturbation, and thus, likely candidates for intervention (Taylor et al., 2016).

Results from simulation modeling can elucidate how the effects of symbiotic microbes on individuals' vector competence manifest at a broader scale. Often, these effects are context-dependent, manifesting differently under scenarios of high versus low transmission or small versus large population sizes. Because researchers have yet to determine the transmission rate for *A. tristis* vectoring *S. marcescens*, we chose to run our model simulating feasible scenarios of both low and high baseline pathogen transmission. Subsequent sensitivity analysis of these scenarios allowed us to determine whether the relative importance of our parameters was robust to different conditions or contingent on the baseline transmission rate. Though plant infection dynamics differed based on the intensity of baseline transmission, our sensitivity analysis revealed the same general patterns for both cases.

Unsurprisingly, we found that the parameter controlling the pathogen transmission rate of aposymbiotic individuals was also an important driver of plant infections. Generally, under scenarios of both low and high baseline transmission by symbiotic bugs, we observed that reducing the transmission rate of aposymbiotic bugs led to fewer overall plant infections. While this result seemed to be robust to changes in baseline transmission, the relative importance of the pathogen transmission rate merits further investigation to more accurately reflect field dynamics.

Although *Caballeronia* exerted strong physiological effects on both pathogen clearance time and transmission rate in the laboratory, in our model, the pathogen clearance rate of aposymbiotic bugs was not an important driver of plant infections, particularly when baseline pathogen transmission was high. Though the duration of pathogen infection in aposymbiotic

bugs could be up to ten times as long as infection in symbiotic bugs in the lab, it had little effect on our simulations of plant infections in the field. This result is not entirely unexpected. While strong physiological effects observed in the laboratory are promising avenues for vector control, they can face unforeseen challenges in the field (Oliveira et al., 2017). For example, mosquito population models determined that even small fitness effects of *Wolbachia* on larvae observed in the lab (Gavotte et al., 2010) could significantly inhibit the potential for *Wolbachia* to invade a wildtype population (Crain et al., 2011). In the case of aposymbiotic squash bugs, we believe the much shorter life spans observed in wild individuals relative to their lab counterparts effectively renders long infection times irrelevant, as infected individuals die before making use of a full infectious period.

The importance of aposymbiotic bugs in driving pathogen transmission was a recurring theme in our findings. Despite the small range it was varied over, the probability of symbiont acquisition—the parameter we used to determine the relative proportion of symbiotic and aposymbiotic individuals in the population—emerged as the single most important driver of plant infections for our model. Further evidence of this result can be seen when we track the contributions to plant infections by the three main categories of individuals in our model (overwintering adults, symbiotic F1s, and aposymbiotic F1s). As expected, overwintering adults are the primary contributors to infection early in the season. However, once aposymbiotic bugs appear on the scene, they quickly become the dominant contributors to infection. Even when they make up a relatively small portion of the newly founded squash bug population ($\leq$ 10%) and even less of the total population, aposymbiotic bugs consistently contribute more than half of plant infections under scenarios of both high and low baseline transmission. Furthermore, our results indicate that drastic reductions in plant infections are not seen until the aposymbiotic

population is depleted to less than 2.5% of the population. Contributions to plant infections by aposymbiotic bugs far exceed what we would expect given their relative representation in the population.

Our models indicate that aposymbiotic bugs are likely important drivers of pathogen transmission in this system. However, their contributions to plant infections could be hindered by their viability in the field, particularly for later instars. Based on our findings, we believe aposymbiotic insects could still contribute to plant infections in the field. In scenarios where the pathogen transmission rate for aposymbiotic insects is high, we saw that the majority of plant infections occur in the first half of the season. During this time, the aposymbiotic population is primarily composed of early second and third instars, where viability has not yet diminished significantly from that of symbiotic insects (Acevedo et al., 2021). Thus, early aposymbiotic instars could drive pathogen transmission if their transmission rates are high. In cases where the transmission rate of aposymbiotic insects is low, on the other hand, aposymbiotic insects do not contribute significantly to transmission until later in the season, likely due to their relatively low abundance. At this time, the aposymbiotic population is dominated by later instars. Previous work characterizing the *A. tristis* microbiome showed that about six percent of adults sampled had not acquired *Caballeronia*, and had been colonized by other bacteria instead (Acevedo et al., 2021). Though these individuals are not, strictly speaking, aposymbiotic they are able to survive to adulthood without acquiring *Caballeronia*. Whether or not these other bacteria affect *S. marcescens* infection in squash bugs is unknown. If transmission rates for insects colonized with non-*Caballeronia* bacteria allow for any pathogen transmission higher than that of symbiotic individuals, they could contribute to plant infections in the latter half of the season.

We show that we can mitigate plant CYVD infections by reducing the transmission potential of aposymbiotic bugs or by minimizing their presence in the population. In our system, these two things go hand in hand. Because *Caballeronia* symbionts already reduce the vectoring potential of *A. tristis*, increasing the proportion of symbiotic individuals will simultaneously deplete the pool of aposymbiotic bugs and decrease the vectoring potential of all bugs in the population. Though symbiont prevalence in wildtype *A. tristis* populations is already high, our results indicate that near complete symbiont coverage is needed to effectively control plant infections; this is due to the expected extremely high vectoring potential of aposymbiotic bugs relative to symbiotic bugs. The importance of aposymbiotic bugs in CYVD infections makes a case for further work specifically investigating their abundance and distribution in the field. The persistence of aposymbiotic bugs into later life stages could have important implications for late season plant infections, particularly if real-world transmission rates are low. Furthermore, there is a need to evaluate how non-*Caballeronia* bacteria that allow insects to reach adulthood affect squash bug vectoring potential. In the field, these insects could be acting as aposymbiotic insects do in our model, having enough viability to survive to adulthood and still transmit *S. marcescens*.

Work in the bean bug, a related agricultural pest with *Caballeronia* symbionts, has shown that insects have a limited window for symbiont acquisition (Kikuchi et al., 2011). Our work further emphasizes the need to intervene early to curb the high transmission potential of early instar aposymbiotic bugs which would be the most prevalent when plants are young and most susceptible to disease. Symbiont-mediated vector control could provide one such intervention, particularly for organic farms which have a limited arsenal against these pests (Doughty et al., 2016). Given that *A. tristis* acquire their symbionts from the environment, high symbiont prevalence in the population could potentially be achieved by flooding crop fields with beneficial

Caballeronia symbionts as second instars begin seeking symbionts. Previous work has established that different strains of *Caballeronia* have similar effects on *A. tristis*' vectoring potential (Mendiola et al., 2022), making for a broad pool from which to draw candidates for symbiont-mediated control. Future work will need to address which strains are the most likely to successfully establish in a population and whether they can remain in the environment without displacement by other strains. If such a strain is found, further models will be needed to address the timing of potential *Caballeronia* releases to maximize symbiont acquisition across squash bug cohorts that are continuously hatching and maturing in the field.

A major caveat in this system is that, at high population sizes, *A. tristis* can cause severe feeding damage to plants even if *S. marcescens* transmission is negligible (Bruton et al., 2003). By ensuring symbionts acquisition, a symbiont-mediated strategy to lessen pathogen vectoring could have the unintended consequence of increasing bug fitness and, in turn, increasing feeding damage. Thus, though our results indicate that a symbiont-mediated approach is theoretically possible to disrupt CYVD in the field, future modeling efforts should expand on this model to include feeding damage and reductions in crop yield to more accurately determine when and whether symbiont-mediated interventions are appropriate.

Though the biological details of vector borne crop pathogens can differ greatly between systems, the general framework we used here can be useful in initial assessments of population-level symbiont-mediated effects on pathogen vectoring. As we discovered, not every physiological effect observed in the laboratory will scale to the population level to produce meaningful effects on pathogen transmission. For symbiotic microbes that already drastically reduce the likelihood of transmission in insect vectors, maximizing symbiont coverage in a population can have a more beneficial effect on disease mitigation than further optimizing the

effect of the symbiont on transmission in individual insects. Using sensitivity analyses on simple models that capture the basic dynamics and effects of a system can help identify important parameters for disrupting pathogen transmission and allow for the testing of population-level parameters for determining the potential of symbiont-mediated vector control.

Acknowledgments

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Chapter V: Differential gene expression in the insect vector *Anasa tristis* in response to symbiont colonization but not infection with the phytopathogen *Serratia marcescens*

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Abstract

Many insects selectively associate with specific microbes in long-term, symbiotic relationships. Maintaining these associations can be vital for the insect hosts' development, but insects must also contend with potential coinfections from other microbes in the environment. Fending off microbial threats while maintaining mutualistic microbes has resulted in many insects developing specialized symbiotic organs to house beneficial microbes. Though locally concentrated in these organs, symbiont establishment can have global consequences for the insect, including influence over the success of coinfecting microbes in colonizing the insect host. We use a transcriptomic approach to examine how the mutualistic symbiosis between the agricultural pest *Anasa tristis* and bacteria in the genus *Caballeronia* affects insect gene expression locally within the symbiotic organs and in the insect host at large. We simultaneously determine whether *Caballeronia* colonization impacts insect host responses to infection with the plant pathogen *Serratia marcescens,* which it vectors to cucurbit plants. Our results indicate that symbiotic and nonsymbiotic tissues in *A. tristis* differ greatly in their gene expression, particularly following successful symbiont colonization. Though symbiont colonization altered host gene expression individuals, no significant differential gene expression was elicited by infection with *S. marcescens,* indicating that *S. marcescens* may subvert host immunity and lead to few physiological changes in the host. This was a surprising finding given previous work indicating that symbiotic *A. tristis* clear *S. marcescens* infection rapidly compared to aposymbiotic individuals and suggests a commensal relationship between *A. tristis* and phytopathogenic *S. marcescens*.

Introduction

Herbivorous insects contend with continuous exposure to a variety of microorganisms as part of their daily lives. These interactions span the continuum of symbiosis and include beneficial microbes as well as potentially life-threatening pathogens. The threat of infection by potentially detrimental microorganisms has complicated the acquisition and accommodation of mutualistic microbes, many of which are necessary to supplement the nutritionally poor diets of their hosts (Gündüz and Douglas 2008, Sudakaran et al. 2015). This poses a dilemma, particularly for insects that acquire mutualistic microbes from the environment. In order for an insect to form persistent associations with mutualistic symbionts, it must distinguish them from their detrimental counterparts (Douglas 2014, Ohbayashi et al. 2020, Wang et al. 2023).

In addition to adaptations to the insect immune system by the symbiotic microbes themselves (Russell and Castillo 2020, Ganesan et al. 2022), many insects have overcome this challenge by confining their mutualists to specialized symbiotic organs (bacteriomes) or cells (bacteriocytes) (Douglas 2020). Conditions within these specialized organs are generally favorable for symbionts (Ferrarini et al. 2022), but hosts can still regulate the microbial population within these tissues (Kim et al. 2013, Whittle et al. 2021). Because of their specialized function, these tissues can differ drastically from the rest of the insect host body (Heddi et al. 2005). Gene expression within bacteriomes and bacteriocytes often reflects the crosstalk between host and symbiont (Nakabachi et al. 2005, Price et al. 2011, Smith and Moran 2020) as both partners balance their needs and concessions to maintain a beneficial relationship.

Inside their host, beneficial microbes can influence the outcomes of subsequent microbial infections. Some mutualists, known as defensive symbionts, protect their insect hosts from pathogens, parasitoids, and other natural enemies (Brownlie and Johnson 2009, Oliver et al.

2012, Oliver and Perlman 2020). Protective mechanisms vary from direct interaction with the microbial intruder to indirect effects modulated by the insect immune system (Gerardo and Parker 2014). These microbial interactions within the insect host are of particular interest in herbivorous insects that vector plant pathogens. Work in various systems has already shown that symbiotic microbes can alter the ability of insects to successfully vector pathogens (Moreira et al. 2009, Weiss and Aksoy 2011, Gonella et al. 2018).

Here, we use transcriptomics to investigate how a mutualistic microbe alters gene expression locally within symbiotic tissues and globally within its insect host. We simultaneously investigate whether these differences contribute to the disparity in the ability of the insect host to maintain coinfection with a vectored pathogen. For this study, we use the squash bug *Anasa tristis,* which has an established beneficial symbiosis with bacteria in the genus *Caballeronia* (Acevedo et al. 2021). *A. tristis* acquires *Caballeronia* from the environment *de novo* at each generation, usually early on in its development. *Caballeronia* symbionts then colonize a specialized region of *A. tristis*' posterior midgut, known as the crypts. Here, they grow with little interference from other microbes. In addition to its mutualistic partner, *A. tristis* can also harbor the phytopathogen *Serratia marcescens*, which it vectors to commercially important cucurbit plants (i.e., squash, pumpkin, watermelon) causing Cucurbit Yellow Vine Disease (Bruton et al. 1998, Bextine et al. 2001). Previous work has established that *A. tristis* harboring *Caballeronia* symbionts have low titers of *S. marcescens* and clear infection rapidly (Mendiola et al. 2022). In aposymbiotic individuals, on the other hand, *S. marcescens* titers can be up to 1000-fold higher and infections can last 10 times longer than in symbiotic individuals.

Given the stark differences between symbiotic and aposymbiotic insects (Acevedo et al. 2021), we expected to see differences in transcription based on symbiont status as well as

between symbiotic and nonsymbiotic tissues. We further expected to see differential transcriptional responses that could explain why *S. marcescens* infection outcomes differ so drastically between symbiotic and aposymbiotic individuals. Though we found strong evidence of differential transcription between symbiotic and nonsymbiotic tissues as well as between symbiotic and aposymbiotic bugs, strikingly, we found no evidence for differential transcription between *S. marcescens* infected and uninfected insects. We conclude that differential regulation of symbiotic organs is essential for the successful establishment and persistence of the *A. tristis*-*Caballeronia* symbiosis. The successful establishment of the symbiosis leads to further transcriptional changes in the symbiotic organs that have global consequences for the growth and development of *A. tristis*. Furthermore, our results support a role for the vectored pathogen *S. marcescens* as a commensal microbe of *A. tristis,* which has implications for its long-term transmission potential.

Methods

Insect Rearing

All insects were reared in an environmental chamber held at constant temperature (27°C) under a long day light cycle (16 hours light, 8 hours dark). We surface sterilized eggs collected from our existing *Anasa tristis* colony by alternately washing them in 70% ethanol and 10% bleach for one minute each and then rinsing with 70% ethanol for 10 seconds. Once hatched, we transferred first instar nymphs to a sterile container and fed them on slices of organic zucchini (*Cucurbita pepo*) fruit, surface sterilized with 70% ethanol and thinly wrapped in parafilm. We maintained insects in sterile containers with regular fruit changes until they molted to second instars.

Treatment administration

We collected one to two day old second instars (n=180) hatched from eggs collected on the same day into sterile rearing boxes for administration of treatments. Aposymbiotic bugs (n = 100) were fed on a liquid diet consisting of five mLs of sterile water mixed with 100 uL of blue dye. Symbiont-positive bugs (n = 80) were fed on five mL liquid diets with *Caballeronia* strain GAOX1 standardized to a concentration of $2x10^7$ CFUs per mL using sterile water and 100 uL of blue dye. All groups were given access to liquid diets for 24 hours before being placed back on organic zucchini slices. See Mendiola et al. (2022) for more detailed methods on feeding protocol.

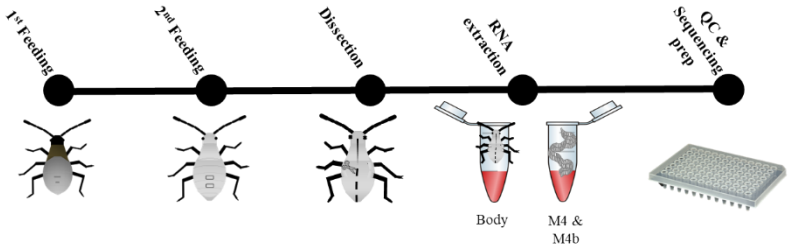
At the third instar stage, insects were further separated into four different treatments. Only individuals that molted within one day of each other were used, dwindling the initial sample size somewhat. Sixty insects were allocated to two aposymbiotic treatments (n = 30 for each). Forty insects were allocated to two symbiont-positive treatments (n = 20 for each).

One group from the aposymbiotic and one group from the symbiotic treatment were fed Z01 (a GFP-labeled strain of *Serratia marcescens*) via vacuum-infused zucchini cubes (Bextine 2001). The remaining group in each treatment was similarly fed sterile water. Insects had access to infused-zucchini for 24 hours. Dissections and sample harvesting were done immediately following this feeding period. Experimental details are laid out schematically in **Figure 5.1**.

A

Treatment	1st Feeding	2nd Feeding
Symbiotic Control	+*Caballeronia* GaOx1	+H_2O
Aposymbiotic Control	+H_2O	+H_2O
Symbiotic *Serratia* Infected	+*Caballeronia* GaOx1	+*S. marcescens* Z01
Aposymbiotic *Serratia* Infected	+H_2O	+*S. marcescens* Z01

B



C

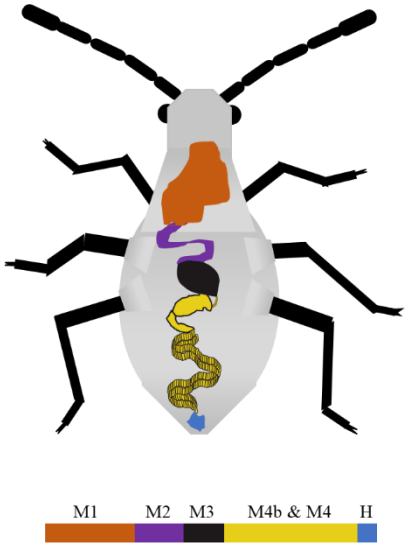


Figure 5.1. Schematic of experimental methods. (A) Table summarizing diets administered to each treatment group. (B) Relative timeline of experimental workflow. (C) Diagram of the squash bug gut. Regions of the gut are labeled as follows: M1 midgut section one, M2 midgut section two, M3 midgut section three, M4b and M4 are the symbiotic organs, the pre-crypt bulb and the crypts, respectively, H hindgut. For our experiment, the M4b and M4 were dissected out and are collectively referred to as the crypts. The remainder of the body, including other sections of the gut are referred to simply as body.

Sample Preparation

All squash bug dissections took place on the same day, and all individuals were still in the third instar stage. Only five bugs from each treatment underwent full dissection. An additional five bugs were flash frozen on dry ice and stored in RNALater at -80C as back-ups. We also sacrificed five random bugs from each treatment to ensure that control bugs were not Z01 positive and that Z01 fed bugs were Z01 positive. For those bugs that underwent dissection: we flash froze bugs individually on dry ice before surface sterilizing them in 70% ethanol for five minutes. Bugs were then dissected in ~200 uL of RNALater. The symbiotic organs (the crypts and the M4b, heretofore referred to as the crypts) and the remaining carcass of the bug were harvested separately and placed directly into RNA lysis buffer from the Promega Total RNA Extraction kit. Once in the lysis buffer, samples were crushed immediately using sterile,

nuclease-free micropestles. Fully homogenized samples were then frozen on dry ice before being stored at -80C until RNA extraction.

RNA extraction & Sequencing

All samples were processed within two days of dissection. Total RNA was extracted from all samples using the Promega Total RNA Extraction kit following manufacturer's instructions, including the three-minute incubation period at 70C. RNA was eluted into 100 uL of nuclease-free water and split into three ~30 uL aliquots, which were all stored at -80C. We tested the quality of all extracted RNA using an Agilent 4200 TapeStation System. All samples exceeded the RNA integrity score of seven recommended for proceeding with RNAseq.

Total RNA was submitted to the Georgia Genomics and Bioinformatics Core for library prep and sequencing. Two samples per individual (symbiotic organ, rest of body) for each of five individuals in our treatments were submitted for sequencing insect host RNA. QIAseq FastSelect kits were used for removal of unwanted RNA. Libraries were prepared using Kapa Biosystems RNA Hyperprep kit. Quality control using the Agilent BioAnalyzer eliminated a handful of samples with low RNA concentrations, but not multiple samples from the same treatment. Samples that passed QC were sequenced. All samples were sequenced on the Illumina NextSeq 2000 platform using the paired-end 100 bp technology. Eukaryotic RNA was sequenced in the same run using a high-output, P3 flow cell.

Data Processing & Analysis

We obtained a total of ~949 million sequences across all samples. We used Trimmomatic v0.39 set to TruSeq3 universal Illumina adapters to trim adapters from reads before further filtering out any eukaryotic and prokaryotic ribosomal RNA reads using existing rRNA databases

in sortmeRNA v4.3.6. Following adapter trimming and rRNA filtering, we had ~904 million sequences across all samples, with an average of ~26.5 million sequences per sample.

Processed samples were used to assemble the squash bug transcriptome. We used reads obtained from all samples across treatments to assemble one reference *A. tristis* transcriptome *de novo* with Trinity v2.8.5. We then assessed the quality of the transcriptome assembly with BUSCO v.5.4.2 for completeness. Our assembly was missing only two out of 255 BUSCO groups with a completeness score of 99.2%. We further verified read alignment from all our samples to the assembled transcriptome using bowtie2. Over 90% of reads for all samples mapped to the assembled reference transcriptome. Once the quality of the assembly was verified, we proceeded to conduct differential gene expression analyses.

We first performed transcript quantification using Trinity's align_and_estimate_abundance.pl script with the pseudoaligner Kallisto. Initial data exploration was conducted in R version 4.3.0 using the "DESeq2" package. Briefly, gene count matrices were standardized using a variance stabilizing transformation for generation of heatmaps and principal component analysis. After data exploration, the raw gene count matrix generated in Trinity was used to conduct differential gene expression analyses using the run_DE_analysis.pl Trinity script employing the "voom" method within the "limma" package. Results were used to generate volcano plots of relevant contrasts. Genes were labeled as significantly differentially expressed if they had $\log_2$ fold change greater than two and adjusted p-values less than 0.05.

Subsets of significantly differentially expressed genes (DEGs) were used in Gene Ontology (GO) enrichment analysis using the enrichGO function in the "clusterProfiler" package in R. Using the assembled transcriptome generated with Trinity, we used Transdecoder v5.7.0 to identify candidate protein-coding regions within our transcriptome. Because an annotated

genome is not currently publicly available for *A. tristis*, we then blasted our candidate genes against the genome of a more well-annotated insect and applied any matching annotations to our candidate genes. We initially attempted to run the GO analysis with the most closely related insect we could find annotations for, the brown marmorated stinkbug *Halyomorpha halys*. Unfortunately, the annotations were insufficient to carry out our analyses. For our final analysis, we settled on a more extensively annotated insect, the pea aphid *Acyrthosiphon pisum*. We applied the annotations from *A. pisum* to our assembly to carry out the GO analysis using the organismal database obtained from the "AnnotationHub" package for *A. pisum*. We used the default parameters for the enrichGO function: p-value cutoff of 0.05 with the Bejamini-Hochberg "BH" correction for multiple comparisons, q-value cutoff of 0.2, minimum gene size of 10, maximum gene size of 500. Our analyses included all three ontology groups: biological processes (BP), cellular components (CC), and molecular function (MF).

In addition to GO enrichment analysis, we also looked for evidence of canonical insect innate immune gene expression in our subset of DEGs. We used the Interactive Database for Insect Innate Immunity (http://bf2i300.insa-lyon.fr:443/home) to obtain a list of 434 known innate immune genes in *A. pisum* and 391 genes for *Drosophila melanogaster* for a total of 825 known insect innate immune genes. We matched the AphidBase and FlyBase identifiers in the database back to NCBI's Gene IDs using GenBank's index of gene data. We then took our candidate gene hits, obtained from blasting against the *A. pisum* and *D. melanogaster* genomes, and looked for immune-specific matches. Hits that mapped to both the list of immune genes and our identified squash bug genes were then used to create a database of canonical immune genes in the squash bug. We then used this database to identify differentially expressed innate immune genes amongst our treatment groups.

Results

No effect of *Serratia marcescens* infection status on sample clustering.

Initial data exploration via principal component analysis revealed samples clustering primarily by tissue type, with crypts and bodies tending to cluster on opposite sides of the PC1 axis (**Figure 5.2**). Samples were further stratified by symbiont status along PC2. In our PCA, symbiotic and aposymbiotic crypts exhibited more differentiation than symbiotic and aposymbiotic bodies. Most notably, we observed no differential clustering by *Serratia* infection status, though *Serratia* infected and uninfected insects clustered by their other treatment conditions (symbiont status and tissue type). A few of our samples did not cluster with their respective groups, possibly due to imperfect dissection of tissues or imperfect symbiont colonization.

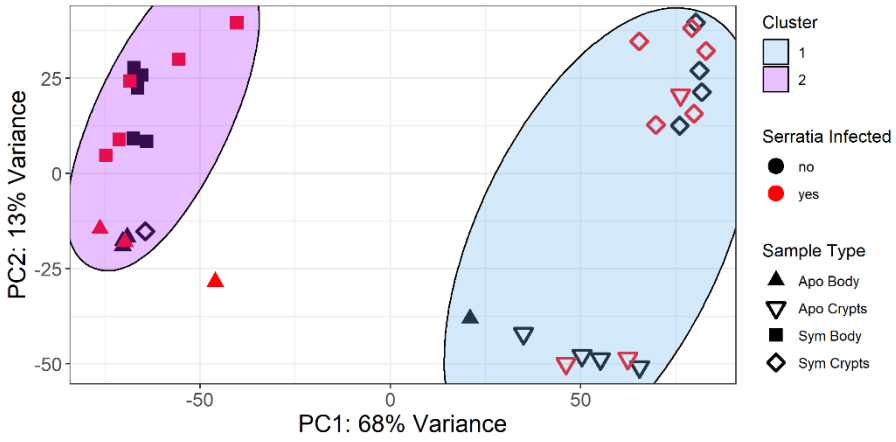


Figure 5.2. PCA plot of variance stabilized gene counts for all samples. Samples clustered primarily by tissue type (PC1, body vs crypts) and symbiont status (PC2, aposymbiotic = apo and sym = symbiotic). Kmeans clustering of gene counts revealed two primary sample clusters: Cluster 1 corresponds to crypt tissues and Cluster 2 corresponds to body tissues. *Serratia* infection status had little influence on sample clustering. *Serratia* infected and uninfected samples clustered together based on tissue type and symbiont status, with no distinct cluster correlated with infection status. Notably, a few samples clustered with different groups, possibly due to imperfect symbiont colonization or imperfect dissection of tissue types.

Visualization of the top 100 occurring genes in the variance stabilized gene count matrix as a heatmap further corroborated patterns observed in the principal component analysis. Samples primarily clustered by tissue type and symbiont status, but no obvious pattern emerged based on infection status (**Figure 5.3**). Based on the lack of differential clustering by *Serratia* infection

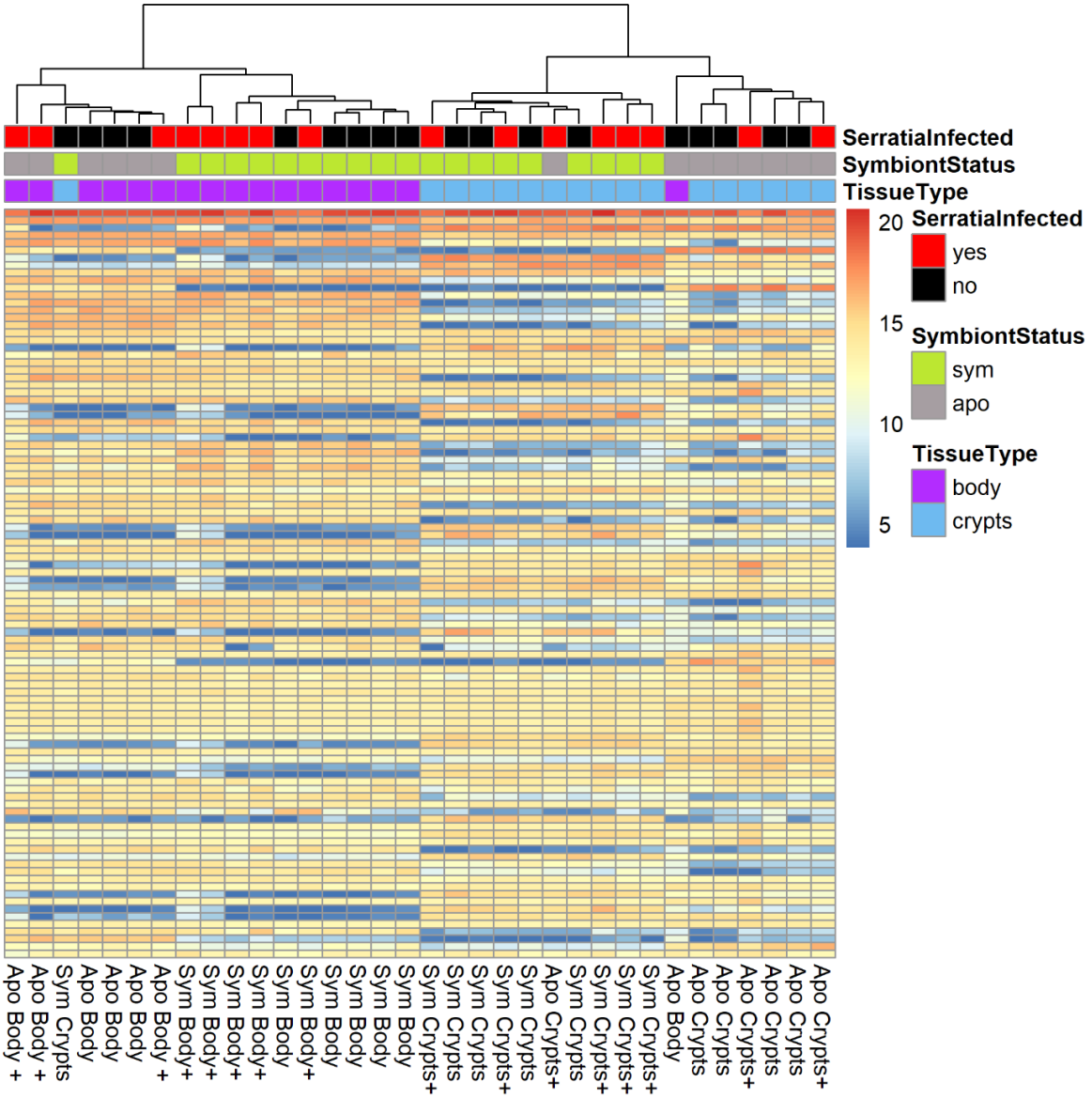


Figure 5.3. Expression heatmap of 100 genes in the variance stabilized gene count matrix. Here, we show only the 100 genes with the most transcript counts. Samples clustered by tissue type and symbiont status, but the distribution of infected samples was haphazard. Differential patterns in gene expression can be seen in the heatmap for both tissue type and symbiont status conditions, but no obvious patterns emerge for clusters of infected or uninfected samples. Column names denote the treatment groups of each sample. The "+" indicates positive *Serratia* infection.

status, we decided to pool *Serratia* infected and uninfected samples into treatments based on tissue type and symbiont status for further analysis.

Symbiont status and host tissue type significantly affect gene expression.

We looked for DEGs among four treatment groups stratified by symbiont status and tissue type: symbiotic body, symbiotic crypts, aposymbiotic body, and aposymbiotic crypts. **Table 5.1** provides a summary of DEGs that met our cutoff criteria (adjusted p value ≤ 0.05 and $\log_2$ fold change > 2). These results are also visualized in volcano plots in **Figure 5.4**. We compared the same host tissue types across symbiont status (**Figure 5.4A & 5.4B**) and different host tissue types within symbiont status (**Figure 5.4C & 5.4D**). All four comparisons yielded DEGs, though the most DEGs were observed when comparing the bodies and crypts of symbiotic bugs. Generally, we noted that contrasting by host tissue type produced more DEGs than contrasting by host symbiont status.

Table 5.1. Summary of DEGs across treatment groups.

	Comparison	Upregulated*	Downregulated*	Unique Immune Genes	Total
Symbiont Status	Sym Body v Apo Body	174	131	0	**305**
	Sym Crypts v Apo Crypts	391	585	5	**976**
Tissue Type	Sym Body v Sym Crypts	8609	4881	32	**13490**
	Apo Body v Apo Crypts	4196	733	12	**4929**

**Direction of regulation is for the first sample listed in the comparison column relative to the second sample*

Differential regulation of metabolic and oxidative stress pathways across symbiont status and host tissue types

We performed GO enrichment analysis on DEGs from each contrast to gain insight into their functions. We found upregulation of metabolic pathways in symbiotic bodies compared to aposymbiotic bodies. When comparing tissue types across symbiont status, we found a general pattern of downregulation for pathways related to oxidative stress in symbiotic insects relative to aposymbiotic insects. These included: oxidoreductase activity, response to oxidative stress, peroxidase activity, and antioxidant activity (**Figure 5.5**). Pathways related to oxidative stress were also prominent in GO analysis of different host tissue types within insects of the same symbiont status (**Figure 5.6**). We found a general trend of upregulation of these pathways in body tissues relative to crypt tissues. Additionally, we noticed many of the categories within the

Biological Processes ontology group were similarly regulated across host tissue types for both symbiotic and aposymbiotic insects (**Figure 5.6**).

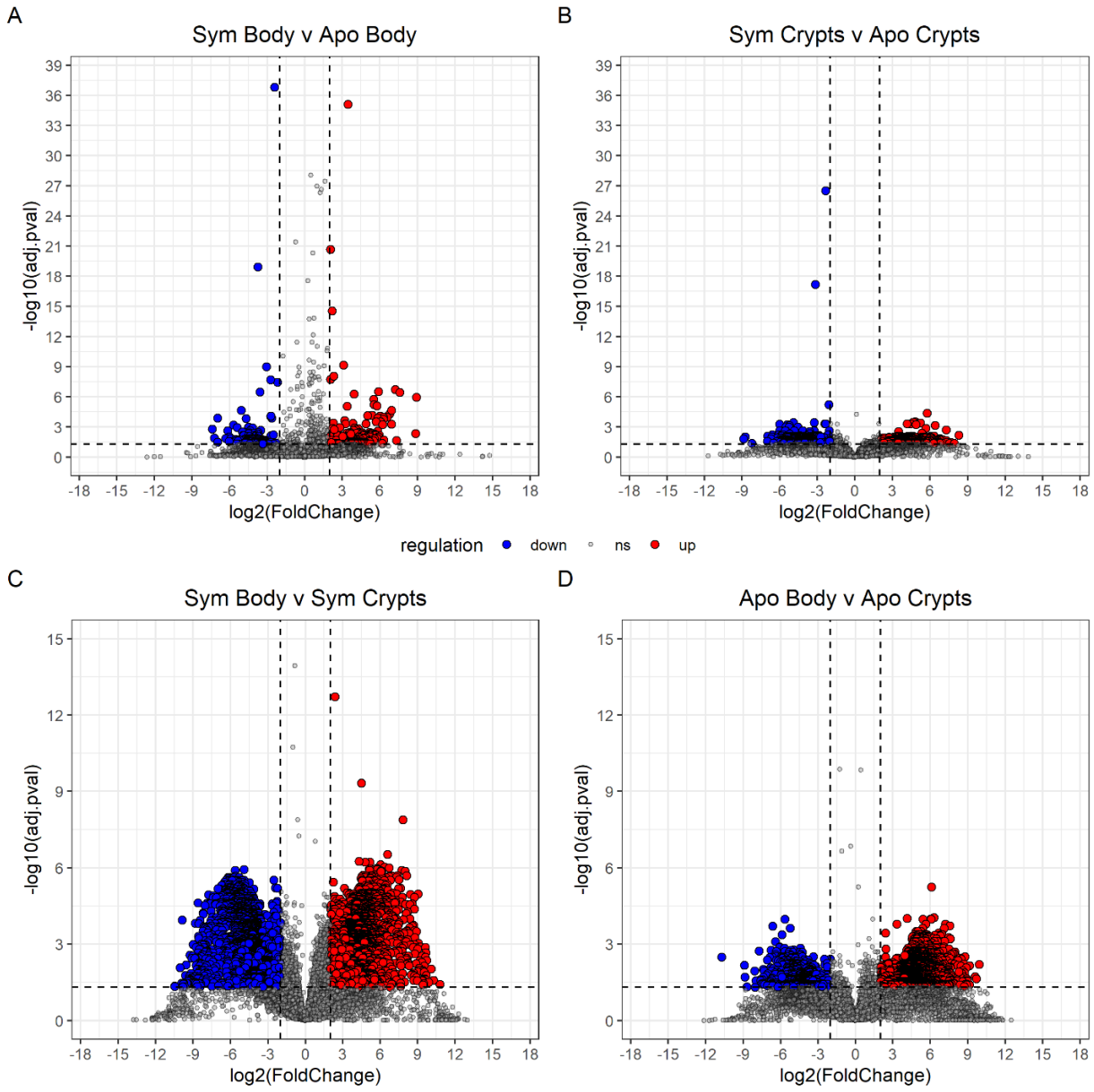


Figure 5.4. Volcano plots showing differentially expressed genes across treatment groups. Plots A and B show contrasts for the same tissue type across symbiont status. Plots C and D show contrasts across tissue types within the same symbiont status. Dashed vertical lines show the cut off for $\log_2$ fold changes greater than two while the horizontal dashed line corresponds to an adjusted p value of 0.05. All significantly differentially expressed genes are colored either red for upregulated or blue for downregulated. The direction of regulation is always for the first sample listed in the contrast relative to the second sample. Two nonsignificantly regulated points from plot C and one point from plot D have been omitted for visual clarity.

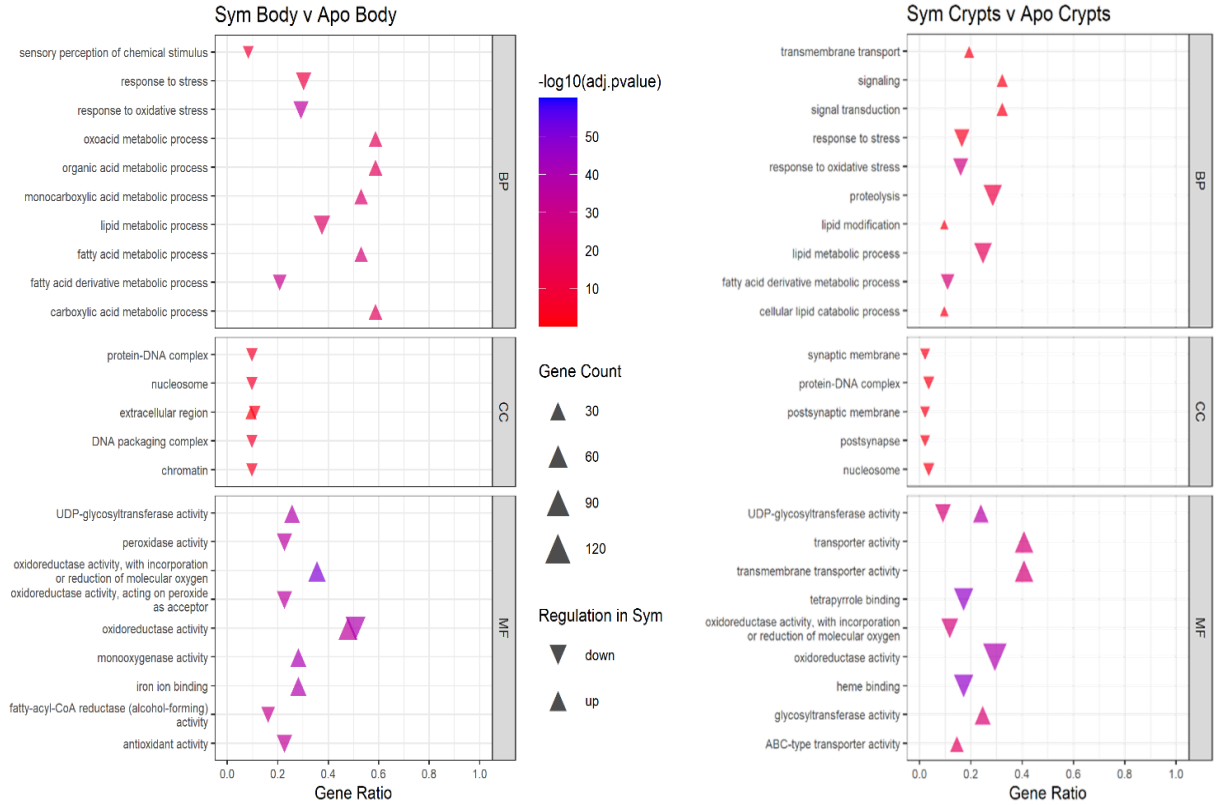


Figure 5.5. GO enrichment analysis for contrasts across symbiont status. Plot A shows the contrast of body tissue across symbiont status. Plot B shows the contrast for crypt tissue across symbiont status. Only the top five up and downregulated categories in each ontology group were plotted. Ontology groups were abbreviated: Biological Process (BP), Cellular Component (CC), and Molecular Function). Not all categories had unidirectional gene regulation, resulting in categories with both up and down regulation. Note that adjusted p-values have been $\log_{10}$ transformed for better visualization of results. Gene ratio was calculated as the percentage of total DEGs identified in the given GO term (restricted to only genes we could annotate).

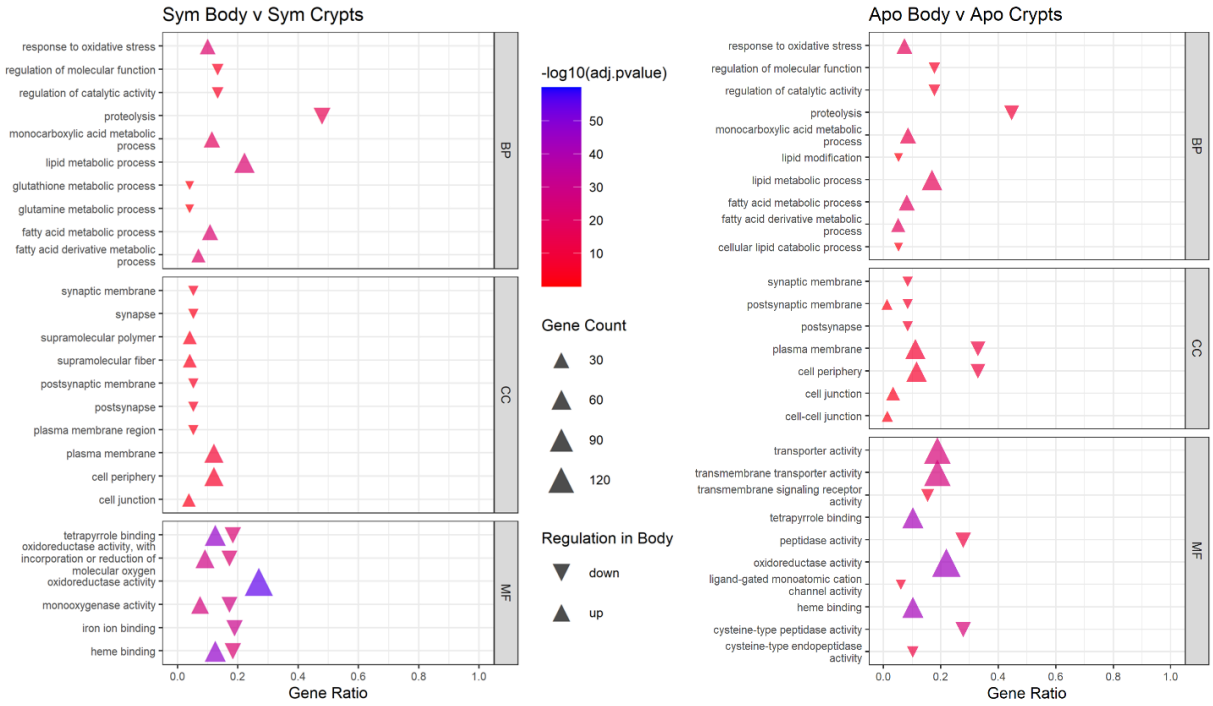


Figure 5.6. GO enrichment analysis for contrasts across tissue types. Plot A shows the contrast of tissue types in symbiotic insects. Plot B shows the same contrast in aposymbiotic insects. Only the top five up and downregulated categories in each ontology group were plotted. Ontology groups were abbreviated: Biological Process (BP), Cellular Component (CC), and Molecular Function). Not all categories had unidirectional gene regulation, resulting in categories with both up and down regulation. Note that adjusted p values have been $\log_{10}$ transformed for better visualization of results. Gene ratio was calculated as the percentage of total DEGs identified in the given GO term (restricted to only genes we could annotate).

Known insect immune genes are upregulated in body tissues relative to crypts, regardless of symbiont status.

We further examined subsets of DEGs for evidence of immune gene activity. No differentially expressed immune genes were found when comparing the body tissues of symbiotic bugs to their aposymbiotic counterparts (**Table 5.1**). Using our database of canonical insect immune genes, we were able to detect immune genes among significant DEGs for all other comparisons. Immune gene hits for each comparison are summarized in Tables 2 – 4. Some squash bug genes mapped to multiple known immune genes in *D. melanogaster* and *A. pisum*.

We filtered out immune genes that duplicated Gene ID, role, pathway and name to present only unique immune gene hits here.

We identified five immune DEGs when comparing symbiotic to aposymbiotic crypts. All five genes were downregulated in symbiotic crypts (**Table 5.2**). Differences in immune gene expression were more apparent between insect host tissue types. We identified the most immune DEGs when comparing body tissue to crypt tissue in symbiotic bugs (**Table 5.3**). The vast majority of these genes were upregulated in the body relative to the crypts. Several upregulated genes were involved in Reactive Oxygen Species (ROS) pathways. This result is consistent with our GO enrichment analysis, where we observed upregulation in oxidative stress in insect bodies relative to crypts. A similar pattern emerged when comparing aposymbiotic host tissues, in which all identified immune DEGs were upregulated in the body relative to the crypts (**Table 5.4**). The immune genes upregulated in the aposymbiotic body were primarily in microbial recognition pathways.

Table 5.2. Summary of immune genes differentially expressed in symbiotic crypts vs aposymbiotic crypts

Gene_ID	Role	Pathway	Name	Species	log_2FC	Adjusted p value
TRINITY_DN10848_c0_g2	Recognition	Microbial recognition	Peptidoglycan recognition protein	*D. melanogaster*	-3.37	0.0312
	Recognition	TOLL pathway	Peptidoglycan recognition protein SA	*D. melanogaster*		
TRINITY_DN4635_c2_g1	Effectors	ROS pathway		*D. melanogaster*	-2.33	0.0102
TRINITY_DN971_c1_g1	Signaling	IMD pathway	Niemann-Pick type C-2	*D. melanogaster*	-2.10	0.0301
	Recognition	Microbial recognition	Ecdysteroid-regulated 16 kDa protein	*D. melanogaster*		

Table 5.3. Summary of immune genes differentially expressed in the symbiotic body vs symbiotic crypts

Gene_ID	Role	Pathway	Name	Species	log_2FC	Adjusted p value
TRINITY_DN36_c0_g1		Apoptosis/ Autophagy (autophagy)	autophagy-related protein 9A	*D. melanogaster*	-2.37	0.0009
TRINITY_DN264_c1_g1	Signaling	Serine Proteases/Serpins		*D. melanogaster*	-2.24	0.0003
TRINITY_DN331_c0_g1	Effectors	ROS pathway	superoxide dismutase	*D. melanogaster*	2.02	0.0332
TRINITY_DN31190_c0_g1	Signaling	JAK/STAT pathway	hopscotch	*D. melanogaster*	2.16	0.0155
TRINITY_DN1794_c0_g4	Transcriptional regulator	TOLL pathway	GATA-binding factor A	*A. pisum*	2.16	0.0339
TRINITY_DN715_c1_g1		JAK/STAT pathway	Zn finger homeodomain 1	*A. pisum*	2.21	0.0001
TRINITY_DN9539_c0_g3		MAKP-JNK-p38 pathways	Anaplastic lymphoma kinase	*D. melanogaster*	2.38	0.0186
TRINITY_DN1535_c2_g1	Effectors	Coagulation	Annexin IX	*A. pisum*	2.41	0.0001
TRINITY_DN5559_c0_g1	Effectors	IMD pathway	Dual oxidase	*D. melanogaster*	2.60	0.0343
TRINITY_DN3085_c0_g1	Signaling	TOLL pathway	Spaetzle	*D. melanogaster*	2.78	0.0004
TRINITY_DN2607_c0_g1	Recognition	Microbial recognition	Peptidoglycan recognition protein	*D. melanogaster*	2.91	0.0136
	Recognition	TOLL pathway	Peptidoglycan recognition protein SA	*D. melanogaster*		
TRINITY_DN3085_c0_g2	Signaling	TOLL pathway	Spaetzle	*D. melanogaster*	3.06	0.0004
TRINITY_DN6790_c0_g1	Effectors	ROS pathway	Peroxidase	*A. pisum*	3.21	0.0039
	Effectors	ROS pathway	cardinal	*A. pisum*		
	Effectors	ROS pathway	heme peroxidase	*D. melanogaster*		
	Effectors	ROS pathway	Peroxinectin-like	*D. melanogaster*		
TRINITY_DN15275_c1_g1	Recognition	Microbial recognition	scavenger receptor class B	*A. pisum*	3.22	0.04
	Recognition, phagocytosis	Microbial recognition	Similar to scavenger receptor class B or Croquemort	*A. pisum*		
TRINITY_DN14003_c0_g1	Signaling	IMD pathway	Niemann-Pick type C-2	*D. melanogaster*	3.25	0.0145
	Recognition	Microbial recognition	Ecdysteroid-regulated 16 kDa protein	*D. melanogaster*		
TRINITY_DN17805_c0_g1		MAKP-JNK-p38 pathways	Anaplastic lymphoma kinase	*D. melanogaster*	3.30	0.0013
TRINITY_DN49030_c0_g2	Recognition	Microbial recognition		*D. melanogaster*	4.05	0.0036
TRINITY_DN10848_c0_g2	Recognition	Microbial recognition	Peptidoglycan recognition protein	*D. melanogaster*	4.20	0.0106
	Recognition	TOLL pathway	Peptidoglycan recognition protein SA	*D. melanogaster*		
TRINITY_DN5218_c3_g1	Effectors	ROS pathway	cardinal	*D. melanogaster*	5.06	0.0133
	Effectors	ROS pathway	Peroxidase	*A. pisum*		
	Effectors	ROS pathway	heme peroxidase	*A. pisum*		
	Effectors	ROS pathway	Peroxinectin-like	*D. melanogaster*		
TRINITY_DN5082_c0_g1	Signaling	Serine Proteases/Serpins		*D. melanogaster*	7.66	0.00002

Table 5.4. Summary of immune genes differentially expressed in aposymbiotic bodies vs aposymbiotic crypts

Gene_ID	Role	Pathway	Name	Species	log_2FC	Adjusted p value
TRINITY_DN2771_c0_g3	Modulation	Serine Proteases/Serpins	Serpin 27A	*A. pisum*	2.14	0.0074
TRINITY_DN1723_c0_g1	Effector	Protease	Chitinase	*D. melanogaster*	3.02	0.0292
TRINITY_DN6812_c0_g1	Effectors	Coagulation	Transglutaminase	*D. melanogaster*	3.32	0.0229
TRINITY_DN4265_c0_g1	Signaling	Serine Proteases/Serpins		*D. melanogaster*	3.38	0.0224
TRINITY_DN21704_c0_g1	Effectors	Other Humoral response	Dopa decarboxylase	*A. pisum*	3.84	0.0062
TRINITY_DN43740_c0_g1	Recognition	Microbial recognition	scavenger receptor class B	*A. pisum*	3.98	0.0214
	Recognition, phagocytosis	Microbial recognition	Similar to scavenger receptor class B or Croquemort	*A. pisum*		
TRINITY_DN49030_c0_g2	Recognition	Microbial recognition		*A. pisum*	4.05	0.0446
TRINITY_DN5559_c0_g1	Effectors	IMD pathway	Dual oxidase	*A. pisum*	4.44	0.0097
TRINITY_DN11792_c0_g1	Recognition	Microbial recognition	scavenger receptor class B	*A. pisum*	4.82	0.0035
	Recognition, phagocytosis	Microbial recognition	Similar to scavenger receptor class B or Croquemort	*A. pisum*		
TRINITY_DN58456_c0_g2	Signaling	Serine Proteases/Serpins		*A. pisum*	5.35	0.0352

Discussion

S. marcescens is a cosmopolitan microbe that occupies diverse ecological niches. Phytopathogenic strains of *S. marcescens* differ substantially from strains isolated from other environmental niches and consistently cluster together in *S. marcescens* strain phylogenies (Rascoe et al. 2003, Zhang et al. 2005). The loss of several canonical *Serratia* metabolic functions in phytopathogenic *S. marcescens* is hypothesized to be a result of its adaptation to life within cucurbit plants (Rascoe et al. 2003), but reliance on an insect vector for transmission has also undoubtedly shaped the evolution of these strains. Several non-phytopathogenic *S. marcescens* strains are recognized as virulent insect pathogens (Omoya and Kelly 2014, Wang and Rozen 2018), yet phytopathogenic *S. marcescens* are able to persistently colonize *A. tristis* (Pair et al. 2004, Wayadande et al. 2005, Mendiola et al. 2022). Differential clustering by *S. marcescens* infection status was not apparent in any of our analyses. Gene expression in *S. marcescens* infected and uninfected samples was not distinct for each other for either symbiotic

or aposymbiotic individuals (**Figure S5.1**), indicating a lack of response, immune or otherwise, from *A. tristis*.

The complete lack of transcriptional differences between *S. marcescens* infected and uninfected insects that we observed suggests that the insect does not recognize the bacteria as a threat. Even in aposymbiotic insects, where *S. marcescens* infections can reach extremely high titers (Mendiola et al. 2022), we observed no transcriptional response to mitigate *S. marcescens* infection. Mounting an immune response can be energetically costly for the insect host (Ardia et al. 2012). In the case of vectored pathogens, evolution toward reduced antagonism would favor longer host infection times and could contribute to higher transmission success. While individual interactions between vectors and the pathogens they transmit span the entire mutualistic-parasitic continuum, there is broad evidence for a net neutral effect of vectored pathogens on their vectors (Santiago et al. 2023). We hypothesize that the rapid clearance of *S. marcescens* observed in symbiotic individuals could be a byproduct of other changes in the host elicited by the symbiosis rather than *A. tristis* mounting an immune response upon *S. marcescens* infection.

Not surprisingly, we found evidence of transcriptional differentiation between the symbiotic organs and the rest of the insect host body in *Anasa tristis*. Overall, our findings indicate that the same tissues across aposymbiotic and symbiotic individuals are more similar than symbiotic and nonsymbiotic tissues within insects of the same symbiont status. We identified the fewest differentially expressed genes (DEGs) between aposymbiotic and symbiotic bodies, but differences were more pronounced when comparing the crypts of symbiotic and aposymbiotic individuals. This increase in differential gene expression coincides with our observations of morphological differences in the crypts of symbiotic and aposymbiotic individuals (**Figure S5.2**). At this stage in their development, symbiotic *A. tristis* show robust,

dense crypts while the crypts in aposymbiotic bugs look atrophied in comparison. Furthermore, our results are consistent with previous work in the bean bug-*Caballeronia* symbiosis, which shows that symbiont colonization triggers morphological and transcriptional changes in crypt tissues (Kikuchi et al. 2020, Jang et al. 2023).

In addition to differences in tissues across symbiont status, we observed DEGs between symbiotic and nonsymbiotic tissues within both symbiotic and aposymbiotic individuals. This suggests that the symbiotic organs may be primed for microbial occupation prior to the initiation of host colonization by *Caballeronia* in a way that the remainder of the insect body is not. Differential gene expression observed across the crypts and bodies of aposymbiotic bugs pales in comparison to those DEGs observed between the bodies and crypts of symbiotic insects. Thus, it seems that symbiont acquisition leads to further transcriptional differentiation between the crypts and the rest of the host body, which is again consistent with morphological observations as well as data from other *Caballeronia* symbiotic interactions (Jang et al. 2023).

Functional analysis of our DEGs across symbiont status showed that, in general, metabolic processes were upregulated in the symbiotic body relative to the bodies of aposymbiotic insects. This relative increase in metabolism reflects the fact that symbiotic *A. tristis* dedicate more time to feeding than aposymbiotic insects (Villa et al. 2023) and grow more rapidly. We further found that membrane transport was upregulated in the symbiotic crypts relative to the aposymbiotic crypts. Upregulation of transporters has also been shown in aphid bacteriocytes, where they play a role in the exchange of various metabolites and substrates between host and symbiont (Nakabachi et al. 2005). As a symbiotic organ, the crypts in *A. tristis* are at the interface of host-symbiont interactions. In symbiotic individuals, transporters are necessary to facilitate the exchange of nutrients and metabolites between host and symbiont, but

such a need would be greatly reduced in aposymbiotic individuals. Lastly, we found that stress responses and responses to oxidative stress were downregulated in symbiotic tissues relative to aposymbiotic ones. This is not entirely surprising given that aposymbiotic *A. tristis* are generally not as hearty as symbiotic insects, suffering from slower development and increased mortality (Acevedo et al. 2021).

When examining the DEGs between insect tissues from bugs of the same symbiont status, we found that, generally, body and crypt tissues differed in similar ways in aposymbiotic and symbiotic insects. Notably, we saw that oxidative stress was upregulated in the bodies of both symbiotic and aposymbiotic bugs relative to the crypts. Though reactive oxygen species (ROS) have traditionally been viewed as a first line antimicrobial defense for insects (Wong et al. 2015), research in various systems has demonstrated that ROS can play vital roles in various physiological processes (D'Autréaux and Toledano 2007, Puppo et al. 2013, Blackstone 2022). In the bean bug-*Caballeronia* symbiosis, recent work has shown that dual oxidase, an ROS producing enzyme, plays a critical role in the development of trachea and oxygenation to the entire bean bug gut, including the crypts, and that the enhanced ROS levels observed did not play an antimicrobial role against an entomopathogenic strain of *S. marcescens* (Jang et al. 2021). Similarly, we found an insect dual oxidase gene upregulated in both symbiotic and aposymbiotic bodies relative to crypts. Oxidative stress may serve a similar role in *A. tristis* as in the bean bug, contributing to tracheal formation throughout the gut, but not exhibiting antimicrobial activity against our strain of *S. marcescens*.

Our findings do differ from Jang and colleagues, however, in that we found upregulation of oxidative stress in aposymbiotic tissues relative to symbiotic ones. This could be due to differences between our insect species or the level of our analysis. While we present data on

transcriptional responses in our insects, Jang and colleagues measured ROS directly. Furthermore, our functional analyses only allowed us to determine pathway-level regulation. It is possible that genes within the identified pathways could be up or downregulating oxidative stress. When we looked for canonical insect immune gene activity across our groups, we did note that symbiotic individuals had more genes in the ROS pathway differentially expressed than aposymbiotic individuals.

In general, we found that most immune genes we could identify were downregulated in the symbiotic organs relative to the insect body. Consistent with our findings of total DEGs, we also found that more immune genes were differentially expressed across tissues in symbiotic insects than in aposymbiotic individuals. Though few immune genes were differentially expressed between symbiotic and aposymbiotic crypts, those we did find were downregulated in the symbiotic crypts. Our findings support the local downregulation of insect immunity within symbiotic organs. Though this seems to occur in both symbiotic and aposymbiotic *A. tristis*, *Caballeronia* colonization seems to trigger further immune suppression within the crypts.

Our work lends further support to the growing body of evidence that shows differentiation among symbiotic and nonsymbiotic tissues across eukaryotic organisms (Nakabachi et al. 2005, Moriano-Gutierrez et al. 2019, Tang et al. 2021). In horizontally transmitted mutualisms, these differences are further exacerbated upon successful symbiont colonization (Jang et al. 2023) and most likely enable the long-term persistence of the microbial population within the insect host. Though mutualistic microbes have been known to alter the outcome of host infection with vectored pathogens, we saw no evidence that *A. tristis* individuals mounted a differential response to *S. marcescens* infection based on symbiont status. Rapid clearance of *S. marcescens* in symbiotic *A. tristis* could then be a byproduct of changes

undergone by *A. tristis* as part of *Caballeronia* establishment with no need for an additional host response. Finally, our work indicates that phytopathogenic *S. marcescens* acts largely as a commensal microbe of its insect vector, a possible adaptation to its reliance on *A. tristis* for successful transmission.

Acknowledgments

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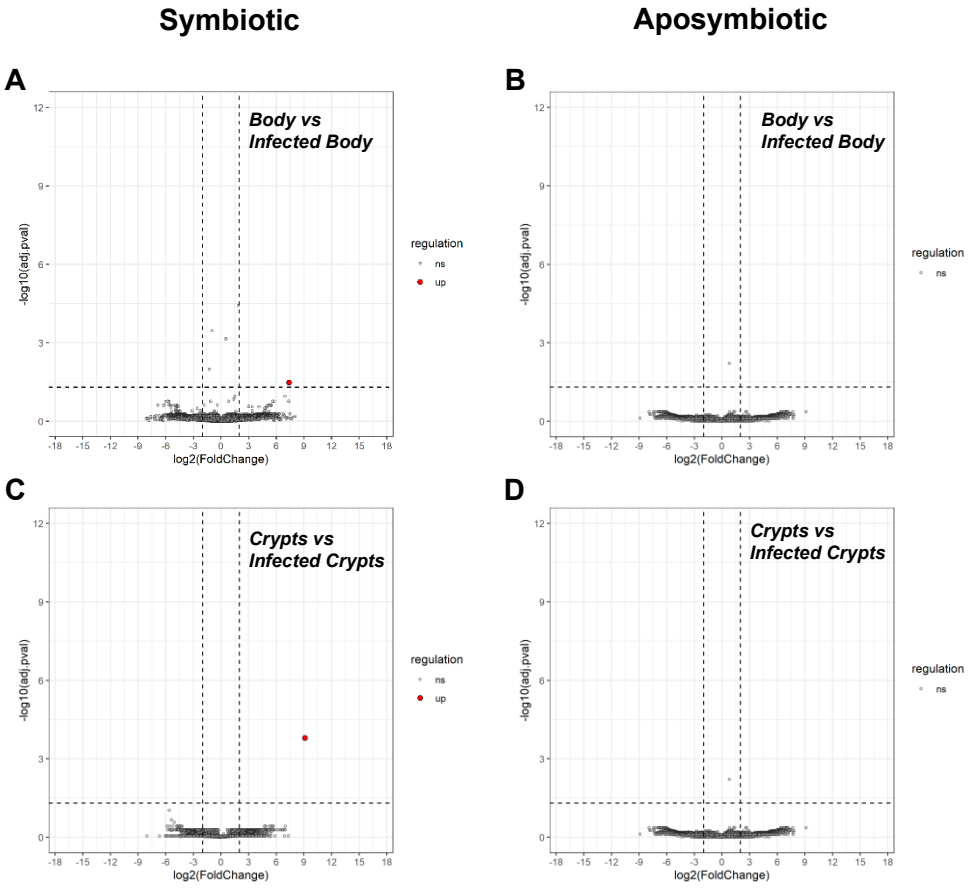
Supplementary Material

Supplementary Figure S5.1

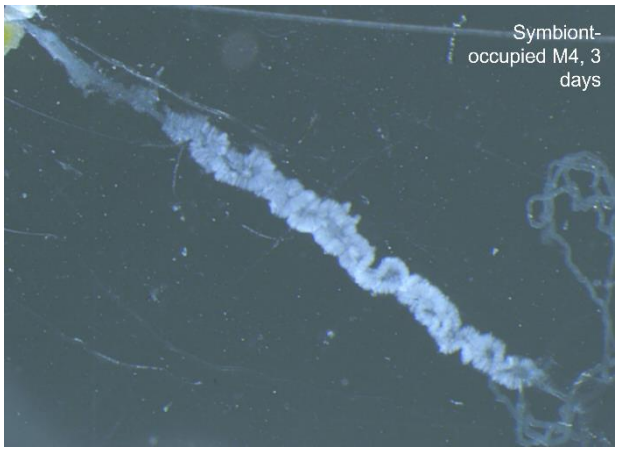
Volcano plots depicting differential gene expression for the same tissue types across *Serratia* infection status. Plots A and C show comparisons between the bodies (A) and crypts (C) of *Serratia* infected and uninfected symbiotic bugs. Plots B and D show the same comparisons, but for aposymbiotic bugs. Neither of the two upregulated genes in plots A and C mapped to genes of known function.



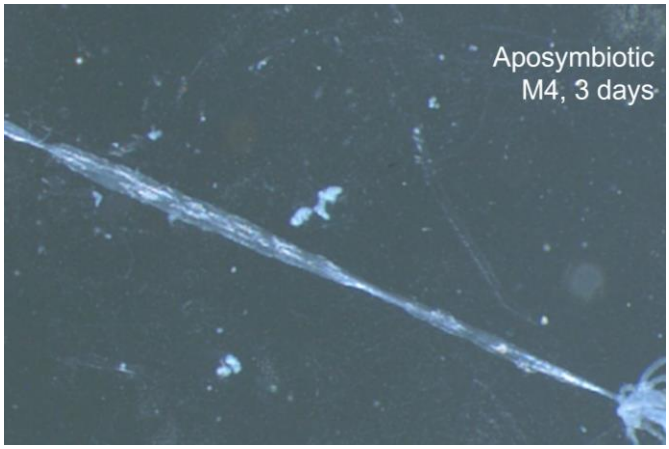
Supplementary Figure S5.2

Morphological differences in the crypt tissues of symbiotic and aposymbiotic insects. (A) Picture of crypt tissues in a symbiotic second instar squash bug three days after symbiont establishment. (B) Picture of crypt tissues in an aposymbiotic second instar squash bug three days after being fed sham diet.

A



B



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Chapter VI: Conclusion

Summary and discussion of previous chapters

Beneficial symbiotic microbes play fundamental roles in the ecology and physiology of their insect hosts (Feldhaar 2011, Cornwallis et al. 2023). Interactions between beneficial symbionts and coinfecting microbes can also have important implications for infection as symbionts often contribute to host immunity and defense (Brownlie and Johnson 2009, Welchman et al. 2009). Interactions where symbiotic microbes can hinder host infection with pathogens are of special interest as they can be leveraged to mitigate the threat of vector-borne diseases. Leveraging insect-microbe symbioses against vectored pathogens, however, requires an intimate understanding of how symbiotic microbes interact with both their insect host and coinfecting pathogens. This dissertation dissects these interactions across scales, from the molecular to the population level, using the squash bug *Anasa tristis*.

Symbiont colonization limits infection with the phytopathogen Serratia marcescens in its insect vector

Symbiotic microbes can interact with an invading pathogen or parasite either directly or indirectly, often making them important players in driving host infection outcomes (Gerardo and Parker 2014). Studies transplanting one or a few key symbiont species between hosts have successfully created pathogen resistant phenotypes in previously susceptible insects (Moreira et al. 2009, Koch and Schmid-Hempel 2011, Gonella et al. 2018). In fact, the insect microbiota has emerged as a strong determinant of vector competence, an insect's ability to harbor and transmit pathogens, in several systems (Cirimotich et al. 2011, Weiss and Aksoy 2011).

To test whether this was the case in *A. tristis*, I reared insects with and without their *Caballeronia* symbionts. I then infected them with the phytopathogen they vector, *S. marcescens*,

and tracked their pathogen infection status as well as their pathogen load over time. Not only were *S. marcescens* titers in symbiotic insects vastly reduced (~1000 fold lower) compared to their symbiont free (aposymbiotic) counterparts, but symbiotic insects also cleared *S. marcescens* infection as much as 10 times faster than aposymbiotic insects. These patterns held true even when I exposed insects to *S. marcescens* before symbiont colonization. In other words, I found no priority effects in this interaction.

In *A. tristis*, *Caballeronia* symbionts have a marked effect on infection with the vectored pathogen *S. marcescens*. Traits like pathogen load and infectious period are important determinants of an insect's ability to successfully transmit a pathogen. These results showed that symbiotic vectors are much less likely to transmit *S. marcescens* than aposymbiotic vectors, yet they were at odds with our observations of the scarcity of aposymbiotic *A. tristis* in the field.

Maximizing symbiont coverage at the population level can mitigate S. marcescens transmission

Observing pathogen inhibition in the laboratory is a promising first step in the development of novel symbiont-mediated vector control techniques, but patterns observed in individual insects do not always scale to the population level. For this reason, mathematical models have played a key role in predicting the outcomes of symbiont-mediated vector control on disease dynamics and identifying potential pitfalls (Dorigatti et al. 2018).

To determine the potential role of symbiotic and aposymbiotic insects in driving *S. marcescens* outbreaks, I constructed a mathematical model of *S. marcescens* transmission. My model tracked four distinct populations of *A. tristis* that varied by symbiont status (symbiotic or aposymbiotic) and *S. marcescens* infection status (susceptible or infected). I parameterized the model using existing data on *A. tristis* natural history. I used data collected from my previous

experiment to determine the infectious period and calculate relative pathogen transmission parameters for symbiotic and aposymbiotic populations. I conducted sensitivity analyses in which I varied three parameters of interest: pathogen transmission rate, pathogen clearance rate, and probability of symbiont acquisition. As I suspected that aposymbiotic insects were driving *S. marcescens* transmission, I kept the symbiotic parameters fixed while varying the parameters of aposymbiotic insects to test the impact of varying pathogen clearance and transmission rate. Furthermore, I varied the probability of symbiont acquisition between 90 and 100 percent to mimic the high rate of successful symbiont acquisition in the field.

As expected, the transmission rate of aposymbiotic bugs was also important. Surprisingly, pathogen clearance rate, despite being an important physiological distinction between symbiotic and aposymbiotic bugs, was of little consequence at the population level. This could be because longer infectious periods observed in the lab are irrelevant in the field, where insect survival is greatly reduced. My analyses showed that the probability of symbiont acquisition was the strongest determinant in overall plant infections. Given this, it is not surprising that aposymbiotic individuals contribute disproportionately to *S. marcescens* transmission in the field. Even when they comprise a small portion of the population, aposymbiotic individuals contribute the majority of plant infections. This suggests that near complete symbiont coverage may be necessary in this system to mitigate plant infections.

Symbiont colonization, but not S. marcescens infection, alters gene expression in A. tristis

The disparity in *S. marcescens* infection outcomes between aposymbiotic and symbiotic insects and its implications for pathogen transmission fueled my curiosity in identifying the potential mechanism underlying these differences. I used transcriptomics to search for evidence of differential gene expression among symbiotic and aposymbiotic insects that differed in *S.*

marcescens infection status. I sampled the symbiotic organs and the remainder of the insect body separately to determine whether symbionts elicited a localized or global transcriptional response to infection.

Surprisingly, I found no evidence of differential gene expression between *S. marcescens* infected and uninfected tissues. Although aposymbiotic and symbiotic insects showed differences in gene expression, neither group responded to *S. marcescens* infection. Thus, the rapid clearance of *S. marcescens* from symbiotic individuals seems to be a byproduct of other changes in the host elicited by symbiont colonization, rather than a direct response to phytopathogen infection.

I found that aposymbiotic and symbiotic insects differed significantly in stress responses, with less stress responses apparent in symbiotic insects. Furthermore, I found that symbiotic insects experienced upregulation of various metabolic pathways which is consistent with their increased feeding patterns and rapid development relative to aposymbiotic insects. Not surprisingly, I found that the symbiotic organs differed significantly from the rest of the insect body, with symbiont colonization driving even further differentiation between the crypts and body. The upregulation of transporters in the symbiotic crypts suggests a need for the exchange of nutrients and other substrates between host and symbiont. I also found evidence supporting overall downregulation of genes related to insect innate immunity within the crypts.

These results are consistent with a growing body of work showing differentiation between symbiotic and nonsymbiotic tissues. Many insects house their beneficial microbes in specialized cells or organs to avoid constant conflict with their innate immune systems (Douglas 2020). Conditions within these specialized organs are generally favorable for symbionts (Ferrarini et al. 2022), but hosts can still regulate the microbial population within these tissues

(Kim et al. 2013, Whittle et al. 2021). Because of their specialized function, these tissues can differ drastically from the rest of the insect host body (Heddi et al. 2005) in ways that reflect the crosstalk between host and symbiont as both partners balance their needs and concessions to maintain a beneficial relationship.

Future directions

Calculating pathogen transmission parameters

Despite several valiant efforts to directly calculate *S. marcescens* transmission rates from insects to squash plants, I was unable to reliably infect plants in the laboratory. This led me to use data I could obtain (i.e., *S. marcescens* titers in insects) as a proxy for calculating relative pathogen transmission rates. However, accurately measuring transmission parameters is desirable for future applications. In some insect vectors, a threshold of pathogen titer is sufficient to drastically increase the chances of successful pathogen transmission (Eschbaumer et al. 2012). Should symbiotic *A. tristis* exceed this threshold, they could serve as competent vectors and contribute substantially to pathogen transmission despite their relatively low *S. marcescens* titers.

Successful calculation of pathogen transmission rates could expand the utility of existing *S. marcescens* transmission models. In Chapter IV, my model uses the simplifying assumption that transmission rates are equal across all feeding life stages of *A. tristis*. However, this is unlikely to be the case, particularly when comparing earlier instars to adults. Incorporating life stage specific transmission rates into the model could allow us to further investigate which life stages are driving plant infections and more effectively time interventions.

Probing the relationship between S. marcescens and A. tristis

Again, despite my efforts, much remains to be known about how *S. marcescens* interacts within *A. tristis*. For example, although we know that *S. marcescens* does not colocalize with

Caballeronia in the insect crypts, its exact whereabouts within its host are unknown. Pathogen localization has important implications for transmission, with exact localization having implications for transmission routes and latent periods. Determining where *S. marcescens* localizes within the insect vector and how this localization varies over time should be a priority in future endeavors.

S. marcescens' potential commensal relationship with *A. tristis* also merits further investigation. In Chapter V, I show that infection with *S. marcescens* does not elicit a differential response in gene expression in either symbiotic or aposymbiotic insects. It is possible that *A. tristis* has adapted to *S. marcescens* and thus does not mount an immune response when infected, but it is also possible that *S. marcescens* is actively subverting *A. tristis*' immune response. Either of these scenarios would allow for *S. marcescens* to infect *A. tristis* for longer periods, increasing its chances of transmission. Though the majority of symbiotic insects clear *S. marcescens* infection rapidly (Mendiola et al. 2022) there is evidence of long term infections in some insects (Pair et al. 2004, Wayadande et al. 2005). Determining whether and how *S. marcescens* avoids a detrimental response from its insect vector is an intriguing next step in the investigation of this interaction.

Furthermore, I determined that the competitive exclusion of *S. marcescens* by *Caballeronia* is unlikely to be mediated by an immune response in the host insect. However, the possibility of exploitative competition or interference competition between microbes remains. Given that *Serratia* does not colonize the host crypts, where *Caballeronia* is most abundant, I think exploitative competition more likely. Determining whether this is the case will require more knowledge on the nutritional requirements of both bacteria and their efficacy in obtaining those nutrients within and outside of the host insect.

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