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The influence of *Burkholderia* endosymbionts on squash bugs' (*Anasa tristis*) responses to *Serratia marcescens*, the plant pathogen that they vector

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

The influence of *Burkholderia* endosymbionts on squash bugsʾ (*Anasa tristis*) responses to *Serratia marcescens*, the plant pathogen that they vector

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Many animals form and maintain long-term, symbiotic relationships with microbes. Symbiotic microbes can be acquired vertically, passing down from parents to their offspring. Alternatively, hosts can acquire symbionts horizontally from the environment. Primary, or obligate, symbionts usually play pivotal roles in increasing host fitness, and thus are beneficial most of the time. In contrast, secondary, or facultative, symbionts can benefit, harm, or have no influence upon a host. Symbiotic microbes can influence many aspects of host biology, including developmental time, nutrient acquisition, and defense. In terms of defense, while many symbiotic microbes enhances host resistance and tolerance towards pathogens and parasites, symbionts can also weaken a host's defenses by down-regulating host immunity. Because a host's response towards foreign microbes can be influenced by the presence of symbionts, a insect's vectoring ability might also be influenced by its symbiotic microbes. Because of this, there is increasing interest in developing symbiont-mediated biocontrol strategies.

The squash bug (*Anasa tristis*) is a vector of *Serratia marcescens*, the plant pathogen that causes cucurbit yellow vine disease (CYVD). Previous studies in our lab have shown that *A.*

tristis partners with bacteria in the genus *Burkholderia* spp., forming a symbiotic relationship. It is of particular interest to study symbiosis in *A. tristis* given its potential to be applied to create novel biocontrol strategies. In my investigations, I examined whether the presence of symbiotic *Burkholderia* influences the establishment of *S. marcescens* in *A. tristis*. I found that symbiont-containing *A. tristis* had lower *S. marcescens* establishment than symbiont-lacking *A. tristis* after being orally inoculated with *S. marcescens*. It was also found that symbiont-containing *A. tristis* had higher survivorship than symbiont-lacking *A. tristis* after a subsequent introduction of *S. marcescens* through injection. This indicates that symbiont-containing *A. tristis* may have a stronger general immune response towards *S. marcescens* infection.

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Abstract

Many animals form and maintain long-term, symbiotic relationships with microbes. Symbiotic microbes can be acquired vertically, passed down from parents to their offspring. Alternatively, hosts can acquire symbionts horizontally from the environment. Primary, or obligate, symbionts usually play pivotal roles in increasing host fitness, and thus are beneficial most of the time. In contrast, secondary, or facultative, symbionts can benefit, harm, or have no influence upon a host. Symbiotic microbes can influence many aspects of host biology, including developmental time, nutrient acquisition, and defense. In terms of defense, while many symbiotic microbes enhances host resistance and tolerance towards pathogens and parasites, symbionts can also weaken a host's defenses by down-regulating host immunity. Because a host's response towards foreign microbes can be influenced by the presence of symbionts, an insect's ability to vector pathogens might also be influenced by its symbiotic microbes. Because of this, there is increasing interest in developing symbiont-mediated biocontrol strategies.

The squash bug (*Anasa tristis)* is a vector of *Serratia marcescens*, the plant pathogen that causes cucurbit yellow vine disease (CYVD). Previous studies in our lab have shown that *A. tristis* partners with bacteria in the genus *Burkholderia*, forming a symbiotic relationship. It is of particular interest to study symbiosis in *A. tristis* given its potential to be applied to create novel biocontrol strategies. In my investigations, I examined whether the presence of symbiotic *Burkholderia* influences the establishment of *S. marcescens* in *A. tristis*. I found that symbiont-containing *A. tristis* had lower *S. marcescens* establishment than symbiont-lacking *A. tristis* after being orally inoculated with *S. marcescens*. I also found that symbiont-containing *A. tristis* had higher survivorship than symbiont-lacking *A. tristis* after a subsequent introduction of *S. marcescens* through injection. These results indicate that symbiont-containing *A. tristis* may have a stronger general immune response towards *S. marcescens* infection, which has implications for considering biocontrol strategies.

Introduction

Symbiosis arises when two organisms from different species form long-term relationships (Moran 2006). In many symbiotic relationships, one organism, the symbiont, resides within the body or cells of the other organism, its host. Symbionts can be acquired through vertical transmission or horizontal transmission. Vertical transmission occurs when the symbionts are passed down from parent to offspring. Examples of vertical transmission of symbionts include several bacterial symbionts of aphids (Oliver et al. 2010), the bacterial symbiont *Wolbachia*, which is associated with many arthropod hosts (Hilgenboecker et al. 2008), and cyanobacterial symbionts and their marine sponge host, *Chondrilla australiensis* (Usher et al. 2001). Vertical transmission guarantees the establishment of the symbiotic relationship. It often occurs when the symbiotic relationship is obligate for the host, the symbiont, or both in order to survive. In horizontal transmission, hosts are born aposymbiotically and acquire symbiotic bacteria from the environment. Symbiosis thus needs to be re-established every generation. Horizontal transmission of symbionts is seen in several host-symbionts pairings, including the broad-head bug *Riptortus clavatus* and its *Burkholderia sp.* symbiont (Yoshitomo et al. 2005), the white fly *Bemisia tabaci* and its symbiotic bacteria in the genus *Rickettsia* (Caspi-Fluger et al. 2012), and the sepiolid squid *Euprymna scolopes* and its luminous bacterium *Vibrio fischeri* (Nyholm & McFall-Ngai 2010).

Endosymbiotic microbes can have significant effects on host fitness. Obligate, or primary, symbionts are indispensable partners of host organisms. They are usually transmitted vertically and are hosted in specialized cells and tissues including bacteriocytes, bacteriomes, and midgut crypts (Kim et al. 2013). Obligate symbionts play essential roles in their hosts' survivorship and development. Examples include the pea aphid *Acyrthosiphon pisum* and its primary symbiont *Buchnera aphidicol*a, which provisions aphids with essential amino acids (Douglas 1998), and the tsetse fly, *Glossina morsitans*, which requires the presence of the symbiont *Wigglesworthia glossinidia* for its immune system to reach maturity (Weiss

et al. 2011). In contrast, facultative, or secondary, symbionts can either negatively or positively influence hosts' fitness. They may, for example, manipulate hosts' reproduction for their own use or defend hosts against parasites and pathogens (Oliver et al. 2003; Hosokawa et al. 2010; Werren et al. 2008; Haine 2007).

Enhancing host defense against foreign microbes is one of the most important roles that can be played by both obligate and facultative endosymbionts. There are mainly three mechanisms through which symbionts defend their hosts against parasites and pathogens (Supp. Fig. 9). First, resource competition between the symbionts and foreign bacteria can prevent the proliferation of pathogens or parasites and protect the host from infection. For example, endosymbiotic *Wolbachia* help defend their host *Drosophila melanogaster* against *Drosophila C virus* by competing for cholesterol (Caragata et al. 2013). Cholesterol is needed by both *Wolbachia* and *Drosophila C virus* for replication. *Wolbachia* exploiting the essential resources of *Drosophila C virus* prevents the virus from proliferating within *D. melanogaster* (Caragata et al. 2013). Second, symbiotic microbes can defend their hosts by directly inhibiting foreign microbes from entering the hosts' system. For example, symbiotic *Enterobacter sp.* can produce reactive oxygen species and inhibit the growth of the malaria parasite, *Plasmodium falciparum*, inside of mosquitoes (Cirimotich 2011), whereas *Spiroplasma* bacteria protects its host, *Drosophila* flies, from parasitic wasps and nematodes by producing a ribosome-inactivating protein (Ballinger 2017). Third, endosymbiotic microbes can

change hosts' immune responses to increase hosts' resistance to parasites and pathogens. The symbiotic *Wolbachia* strain wMelPop, for example, can up-regulate genes within *Aedes aegypti*'s innate immune system, in turn enhancing its resistance towards filarial nematodes (Kambris et al. 2009). However, host resistance towards invading microbes could also be weakened if the host immune response is down-regulated as a cost for tolerating beneficial symbionts (McFall-Ngai 2010).

The squash bug *Anasa tristis* is a true bug (Hemiptera). It is a pest that both withers squash plants by removing substantial amounts of phloem sap as it feeds and that serves as the vector of a bacterial plant pathogen, *Serratia marcescens*, that causes *Cucurbit Yellow Vine Disease* (CYVD) (Pair 2004). Cucurbit Yellow Vine Disease is characterized by rapid yellowing and wilting of leaves followed by discoloration and death of the phloem of cucurbit plants (Besler et al. 2015). Current strategies for controlling *A. tristis* colonization on cucurbit crops include: insecticides, row cover, squash trap plants and attracticidal baits (Pair 1997), habitat modification (Fair et al. 2017), varied planting dates (Fair et al. 2017), and parasitoid release (Olson et al. 1996).

Burkholderia bacteria are symbionts of many heteropteran insects, including stink bugs, broad-headed bugs, rice bugs, and bean bugs (Hosokawa et al. 2010; Kikuchi et al. 2005; Kikuchi et al. 2010; Kim et al. 2015). The *Burkholderia* symbionts are stored in heteropteran bugs' terminal midgut structures, known as the midgut crypts (Olivier-Espejel et al. 2011; Itoh 2014). In previous studies, our laboratory has determined that the dominant bacteria that occupy *A. tristis*'s midgut crypts belong to the genus *Burkholderia*. *Burkholderia* has important impacts on host fitness: *Burkholderia* symbionts significantly increase *A. tristis*'s survival into adulthood and decrease *A. tristis*'s developmental time (Acevedo et al., in prep). This suggests that *Burkholderia* is a beneficial symbiont of this insect.

While many insects acquire obligate symbionts through vertical transmission, *A. tristis* are born aposymbiotically (without symbionts). Every generation, they acquire symbiotic *Burkholderia* from the environment after they molt into second instars (Acevedo et al., in prep). This symbiont-acquiring mechanism provides advantages as a system to study symbiosis and its effects because we can culture the host and symbionts separately and introduce symbionts in a controlled fashion. Moreover, unlike many

uncultivatable symbiotic bacteria, *Burkholderia* can be easily maintained on Luria-Bertani (LB) media and genetically-modified (*e.g.,* by Green Fluorescence Protein (GFP) labeling). The symbiotic relationship between *A. tristis* and *Burkholderia* bacteria make squash bugs an ideal model for studying how one specific symbiont influences the host. Examining how and in what way the symbiotic microbe *Burkholderia* influences *A. tristis*'s response towards foreign bacteria entering its system will allow us to understand how symbiosis affects *A.tristis*'s ability to vector *S. marcescens*. Such insights may allow us to develop novel strategies to prevent *A. tristis* from vectoring this plant pathogen.

This thesis is composed of two data chapters. In Chapter 1, I elaborate on my investigation into whether the presence of symbiotic *Burkholderia* influences the establishment of *S. marcescens* plant pathogens within *A. tristis* upon oral acquisition of the pathogen, the likely natural route of acquisition. In Chapter 2, to shed further light into the influence of *Burkholderia* on *A. tristis* defense, I investigate whether the presence of symbiotic *Burkholderia* influences *A. tristis*'s responses towards subsequent introduction of *S. marcescens* through injection. In the conclusion, I briefly summarize my findings and highlight important future directions.

Chapter 1:The Influence of Symbiotic *Burkholderia* on *Anasa tristis*'s Response Towards Subsequent Introduction of *Serratia marcescens* through Oral Feeding

Abstract

Endosymbiotic microbes can alter a host's response towards foreign microbes. Symbiotic microbes can suppress the proliferation of pathogens and parasites through various mechanisms, such as outcompeting foreign microbes, releasing toxins, or up-regulating host immune systems. Symbiont-mediated host defense is particularly interesting in vectors, because the presence of symbionts may influence an insect's vectoring capacity. Previous studies have found that while some symbiotic microbes suppress within host establishment of pathogens, others facilitate establishment, and both outcomes can influence transmission. By orally introducing *S. marcescens* bacteria to fourth instar *A. tristis* , I quantified and compared *S. marcescens* establishment in *Burkholderia* symbiont-containing and symbiont-lacking *A. tristis*. Symbiont-containing *A. tristis* had lower *S. marcescens* establishment than symbiont-lacking *A. tristis*. A zone of inhibition assay showed that the *Burkholderia* bacteria present in these bugs during the oral feeding assay do not directly inhibit the growth of *S. marcescens.* Instead, *Burkholderia* symbionts may suppress *S. marcescens* establishment through other mechanisms, such as up-regulating the host immune system or competing over a common resource.

Introduction

Endosymbiotic microbes have significant influences over their hosts, such as providing essential nutrients, facilitating host development, promoting immune system maturity, and defending hosts against parasites and pathogens (Weiss, Wang & Aksoy 2011; Moran 2006; Oliver et al. 2014). Symbiont-conferred protection against pathogens and parasites can play an essential role in host fitness. Mechanisms of symbiont-conferred protection vary across systems. Symbionts can enhance host resistance or tolerance towards highly virulent pathogens by enhancing host immunity, or by defending their hosts by directly inhibiting or out-competing foreign microbes invading the host (Oliver et al. 2003; Jaenike et al. 2002; Baldni et al. 2014; Pais et al. 2008).

Because of symbiotic microbes' influences on host responses towards foreign microbes, a vector's ability to temporarily host and then transmit pathogens can also be influenced by symbiotic microbes. Many endosymbionts, especially obligate symbionts, require sustained maintenance within the host (Oliver et al. 2010;Usher et al. 2001). Thus, the symbiotic microbes must hold certain advantages over non-symbiotic microbes in order to be maintained (Zindel et al. 2011) and may repress the establishment and growth of pathogens. For example, symbiotic *Wolbachia* strain *w*MelPop-CLA is found to block the transmission of *dengue serotype 2* viruses by colonizing *Aedes aegypti* mosquito's salivary gland and blocking the establishment of *dengue serotype 2* viruses (Walker et al. 2011). Similarly, *S. marcescens* symbionts can effectively colonize the guts of *Anopheles gambiae* mosquitoes and reduce the mosquitoes' ability to transmit the malaria parasite, *Plasmodium falciparum,* by secreting inhibitory factors (Bahia et al. 2014).

However, symbiotic microbes can also enhance vectoring capacity in some cases. For example, symbiotic *Hamiltonella* bacteria increase the ability of white flies (*Bemisia* tabaci) to transmit *Tomato yellow leaf*

curl virus (TYLCV). The *Hamiltonella* symbiont protects the virus from proteolysis by interacting with the virus' protein coat, increasing its likelihood of being transmitted to tomato plants (Yuval et al. 2010). In pea aphids (*Acyrthosiphon pisum*), proteins produced by its symbiont *Buchnera aphidicola* facilitate the transmission of potato leafroll virus (PLRV) by binding to the virus's protein coat and guiding the virus to *A. pisum*s's salivary gland, where it is secreted into plants (Van den Heuvel et al. 1999).

The fact that endosymbionts play pivotal roles in host fitness and disease transmission suggests symbiosis could be manipulated to control vectoring of pathogens. Biological control of vector-borne diseases via symbiont manipulation is mainly achieved through two strategies. The first strategy involves introducing certain symbiont strains that repress the vectoring insects' proliferation in order to reduce pathogen transmission. Some strains of *Wolbachia* bacteria in mosquitoes, for example, are found to shorten mosquitoes' life spans and have strong inhibitory effects on the transmission of human diseases, including dengue fever, chikungunya and malaria (McMeniman et al. 2009; Moreira et al. 2009; Walker et al. 2011). The second strategy involves genetically modifying symbiotic bacteria to eliminate or prevent the establishment of pathogens within the vectoring insect. For example, the parasitic protozoan *Trypanosoma cruzi*, the causing agent of Chagas disease, is vectored by kissing bugs (*Rhodnius prolixus)*. Researchers have genetically modified the symbiotic bacteria to produce antitrypanosomal products in kissing bug guts, preventing the establishment of *Trypanosoma cruzi* inside of the bugs (Beard et al. 2002).

Exploitation of symbiosis to limit pathogen vectoring could be a feasible strategy for limiting damage caused by the squash bug, *A. tristis*. *A. tristis* is both a pest that feeds on the phloem sap of cucurbit plants, and a vector that transmits *S. marcescens*, the causal agent of Curcurbit Yellow Vine Disease (Pair et al. 2004). Given the damage caused by *A. tristis*, considerable research has been done on control strategies

for *A. tristis*. These strategies include companion planting (Kahn et al. 2017), squash trap plants and attracticidal baits (Pair 1997), habitat modification (Fair et al. 2017), alteration of planting dates (Fair et al. 2017), and parasitoid release (Olson et al. 1996). Despite the importance of symbionts for many insects, no research has focused on how symbiosis could be exploited to lessen *A. tristis* proliferation or *A. tristis*'s vectoring of *S. marcescens*. Recent work indicates that *A. tristis* is associated with bacteria in the genus *Burkholderia,* which are stored at high density in midgut crypts within the insect (Acevedo et al., in prep). The presence of *Burkholderia* significantly increases *A. tristis*'s survivorship into adulthood, and shortens *A. tristis*'s developmental time (Acevedo et al., in review).

Here, I build upon these findings to investigate *Burkholderia*'s influence on *A. tristis*'s vectoring capacity by orally introducing *S. marcescens* into symbiont-containing and symbiont-lacking *A. tristis* and then assessing pathogen load in these hosts. Reduced *S. marcescens* load in symbiont-containing *A. tristis* would suggest that the presence of the *Burkholderia* symbiont reduces establishment and/or proliferation of *S. marcescens* in these insect hosts. Such a difference in *S. marcescens* establishment might be due to direct inhibition of *S. marcescens* by *Burkholderia* through mechanisms such secretion of inhibitory factors or competition over critical resources. Alternatively, *Burkholderia* could up-regulate *A. tristis* immunity, which in turn suppresses *S. marcescens* establishment. As a first attempt to test the possibility that *Burkholderia* directly inhibits the growth of *S. marcescens*, I carried out a bacteria zone of inhibition assays.

Methods and Material

Insects and Bacteria

We collected adult *A. tristis* from organic farms in Georgia. These adults were used to start lineages of laboratory-reared *A. tristis*. The insects are fed on yellow summer squash plants and allowed to mate and lay eggs freely. Eggs are subsequently collected for experiments.

In previous experiments, the our laboratory has established a phylogeny of *Burkholderia* symbiont bacteria isolated from *A. tristis* midgut crypts (Supp. fig. 1). Previous research has shown that, when introduced as a symbiont, *Burkholderia* SQ4A significantly increases *A. tristis*' survivorship and development into later life stages (Supp. fig. 2 &3). Like SQ4A, A33.M4.C was also isolated from *A. tristis* (Supp. fig. 1), and previous research has shown that A33.M4.C symbiont-containing *A. tristis* have shorter developmental time into adulthood than symbiont-lacking *A. tristis* (Supp. fig. 7). A33.M4.C belongs to a clade that is different from SQ4A (Supp. fig. 1). The combination of A33.M4.C and SQ4A thus allowed me to have a relatively comprehensive insight into how *Burkholderia* symbiotic bacteria influence *A. tristis'*s response towards *S. marcescens*.

The cosmopolitan bacterium *S. marcescens* assumes a variety of roles in nature including insect symbionts (Mazano-Marin et al. 2012), plant pathogens (Bruton et al. 2003), nematode pathogens (Schulenburg & Ewbank 2004), and human pathogens (Alexander et al. 1969; Mills et al. 1976; Cohen et al. 1980). Previous research isolated both strain Z01 and P01 in CYVD-infected cucurbit plants (Zhang et al. 2005; Besler et al. 2017). As a result, I selected *S. marcescens* strain Z01 and P01 as the plant pathogen to introduce into *A. tristis.*

Rearing of Aposymbiotic and Symbiotic Nymphs

Aposymbiotic nymphs were reared using previously established protocols (Acevedo et al., in prep). Briefly, *A. tristis* eggs were surface sterilized with 10% bleach and 70% alcohol and maintained at 27℃. After the eggs hatched, first instar nymphs were fed fresh, organic zucchini fruit and raised at 27℃ until they molted into second instars. Since other bug species have a limited developmental window during the second instar stage in which to establish endosymbiotic bacteria (Kikuch et al. 2011), *A. tristis* were fed *Burkholderia* symbionts on the second day after they molted into second instars. To feed *A. tristis Burkholderia* symbiotic bacteria, newly molted second instar *A. tristis* were starved overnight (approximately 12 hours). To rear symbiont positive nymphs, after the overnight starvation period, second instar nymphs were given access to a liquid diet containing molecular grade water and *Burkholderia* bacteria at a concentration of 2 x 10^7 cells/mL. Aposymbiotic nymphs were given water with no bacteria. After 24 hours with full access to the bacteria- or water-control diet, the second instar nymphs were fed zucchini fruit until they molted into fourth instars, the life stage when experiments were carried out. The efficiency of this feeding method was tested and shown to be high. In the efficiency testing assay using GFP-labeled *Burkholderia* strains, 19 out of 20 *A. tristis* had fluorescent bacteria established in their gut. Furthermore, in another experiment using this oral, feeding method, some *A. tristis* were dissected after they developed into adulthood and the number of fluorescent bacteria was quantified (Supp. fig. 5). The percentage of *A. tristis* adults that contained fluorescent symbiotic bacteria was 89%.

Fourth instar *A. tristis* were chosen for my experiments because: 1) many symbiont-lacking *A. tristis* can only survive until the fourth instar stage and die before they molt into fifth instar (Supp. fig. 3); 2) fourth instars have an optimum body size for introducing pathogens through injection; and, 3) fourth instars have not yet developed an exoskeleton, which allows also facilitates injection. The injection assays will be elaborated in chapter 2.

Oral Feeding Assays

While it is known that *A. tristis* transmits *S. marcescens* (Bruton et al. 2003), few studies have been done on how *A. tristis* acquires *S. marcescens.* Considering the fact that *S. marcescens* is a phloem-limited pathogen (Bruton et al. 2003; Bendix et al. 2018) and *A. tristis* is a phloem-feeding pest (Biernacki et al. 2002), it is likely that *A. tristis* acquires *S. marcescens* through sap-feeding. Therefore, I established an oral feeding method intended to mimic how *A. tristis* acquire *S. marcescens* in nature. *A. tristis* were infected with GFP-labeled *S. marcescens* one day after they molted into fourth instar. To introduce *S. marcescens* into these insects, 1 cm^3 pieces of fresh organic zucchini fruits were infused with fresh *S. marcescens* culture by using a vacuum flask. The culture was prepared by growing *S. marcescens* to log phase in Luria Broth; this culture was then standardized to a concentration of 1 x 10^7 cells/mL culture prior to fruit infusion. *A. tristis* were then allowed to feed on *S. marcescens*-infused zucchini fruits for one hour. During this time each *A. tristis* was placed into a container and given access to one piece of *S. marcescens* infused fruit, and the feeding status of each *A. tristis* was checked every 15 minutes. *A. tristis* tend to feed for a long time once they start feeding. Thus, when an *A. tristis* was found dwelling on the piece of fruit at the 15 minute time point, it was counted as having been feeding for the past 15 minutes. When an *A. tristis* was found feeding four times, it was counted as having fed for 1 hour, and which point feeding was terminated by replacing the *S. marcescens* infused fruit with fresh organic zucchini. The consistency of this feeding method was examined through sacrificing *A. tristis* right after feeding (0hr post-feeding). Even though there was variation in *S. marcescens* bacteria intake between the symbiont-containing and symbiont-lacking group (Supp. fig. 4), the variation only decreased the likelihood of observing a significant difference between the estimated number of *S. marcescens* that established inside of symbiont-containing and symbiont-lacking *A. tristis.* After being orally introduced *S. marcescens*, *A. tristis* were sacrificed by surface sterializing each *A. tristis* in 99.5% alcohol for 30 seconds. Whole bug *A. tristis* were crushed up in450 mL liquid LB.The number of *S. marcescens* that established in each *A.*

tristis's was quantified at the 20 hour time point through plating, serial dilution, and counting fluorescent colony forming units (CFUs). Supplemental figure 8 demonstrates *A. tristis* rearing, symbiont establishment, oral feeding, and fluorescent *S. marcescens* quantification.

Bacteria Zone of Inhibition Assay

Different symbiotic *Burkholderia* strains (producer strains) were streaked across an LB plate and incubated overnight at 27℃ (Supp. fig. 5). The next day, *S. marcescens* tester strains were streaked perpendicular to the producer strain. The tester strains were streaked by letting a drop of *S. marcescens* Luria Broth bacteria culture trail from the top of the LB plate until it touches the producer strain. The plates were incubated for 24 hours at 27℃. If any *Burkholderia* strain could directly suppress the growth of *S. marcescens*, there should be a zone of inhibition. *Burkholderia* strains used in this assay were SQ4A and A33. M4.C. The *S. marcescens* strains included Z01, P01, and 2.61a. Bacteria G1-8I and GI-8P were known to exhibit inhibitory effect, and thus were used as controls in the assay. Another bacteria strain, A33.M4.D was also used in this assay. *Bacteria* A33.M4.D is a bacteria isolated from the same individual *A. tristis* as *Burkholderia* A33. M4.C. Previous 16s rDNA sequencing revealed that A33.M4.D is most closely related to *Escherichia coli.*

Data Analysis

Oral Feeding Assay. The number of CFUs per bug was analyzed with a Wilcoxon rank sum test in R as it did not conform to normality. In the experiment with *S. marcescens* P01 and *Burkholderia* SQ4A, human error occurred in the serial dilution of bacteria from two symbiont-lacking *A. tristis*; bacteria load in these individuals was estimated based on counting only the CFUs that could be seen on a plate that was mainly a lawn of bacteria. This is a conservative estimate, which only decreases the likelihood that the number of

S. marcescens that established in symbiont-lacking *A. tristis* would be found to be significantly higher than that in symbiont-containing *A. tristis*.

Zone of Inhibition Assay. Analysis was done by qualitatively assessing the presence of a zone of inhibition.

Results

Oral Feeding Assay

A.tristis were co-infected by *Burkholderia* SQ4A and *S. marcescens* Z01, *Burkholderia* A33.M4.C and *S. marcescens* Z01, and *Burkholderia* SQ4A and *S. marcescens* P01. In all three experiments, the estimated number of *S. marcescens* in symbiont-lacking *A. tristis* was significantly greater than the estimated number of *S. marcescens* in symbiont-containing *A. tristis* 20 hours after orally introducing *S. marcescens* to *A. tristis* (Figure 1.1A-C).

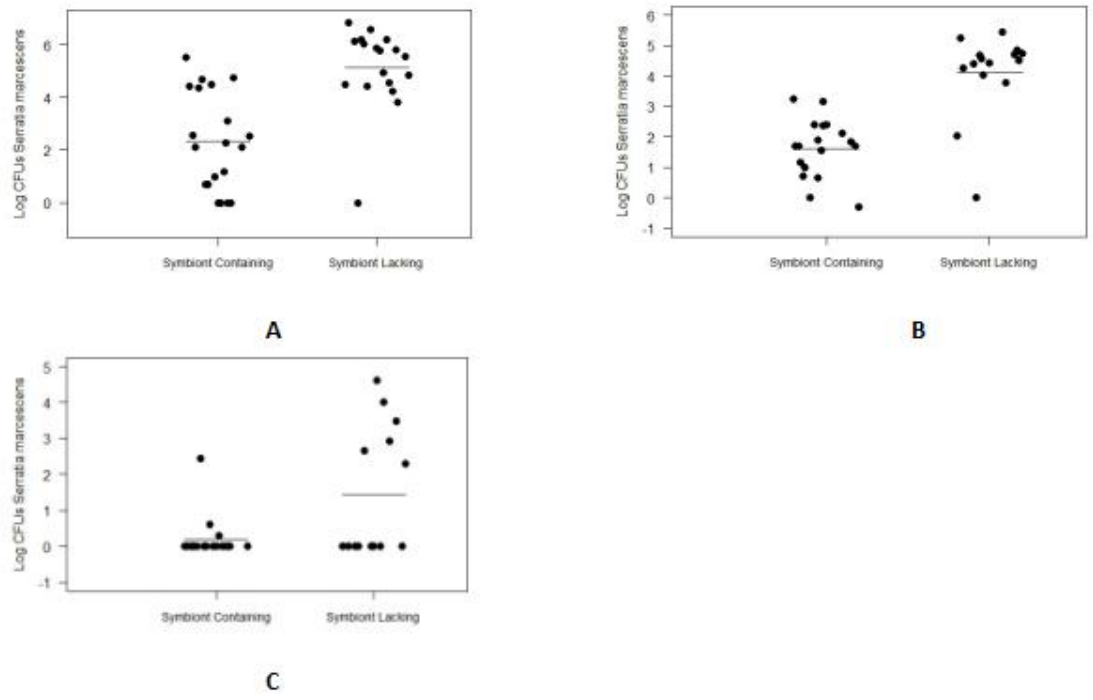


Figure 1.1. Estimated Log CFUs of fluorescent *S. marcescens* that established in *A. tristis* 20 hours after orally introducing *A. tristis* with fluorescent *S. marcescens*. Statistic used was Wilcoxon analysis. A) Strains used: *S. marcescens* ZO1, *Burkholderia* SQ4A ($p = 6.464e\text{-}05$, n (symbiont-lacking) = 18, n (symbiont-containing) = 20. B) Strains used: *S. marcescens* ZO1, *Burkholderia* A33. M4.C $p = 5.981e\text{-}05$, n (symbiont-lacking) = 15, n (symbiont-containing) = 18. C) Strains used: *S. marcescens* PO1, *Burkholderia* SQ4A. $p = 0.035$, n (symbiont-lacking) = 15, n (symbiont-containing) = 17.

Bacteria Zone of Inhibition Assay

A zone of inhibition occurs when the growth of a tester bacteria fades at the point when it reaches the producer bacteria as shown in figure 2.2 C. No zone of inhibition was observed when *Burkholderia* SQ4A and A33. M4.C were streaked as producers, and *S. marcescens* Z01 and P01 were streaked as testers (Figure 1.2 A-B). In contrasts, zone of inhibition were observed when *S. marcescens* 2.61a and G1-8P, two bacteria strains known to inhibit many *Burkholderia* and *S. marcescens* bacteria, were streaked as

producers (Figure 1.2 C-D).

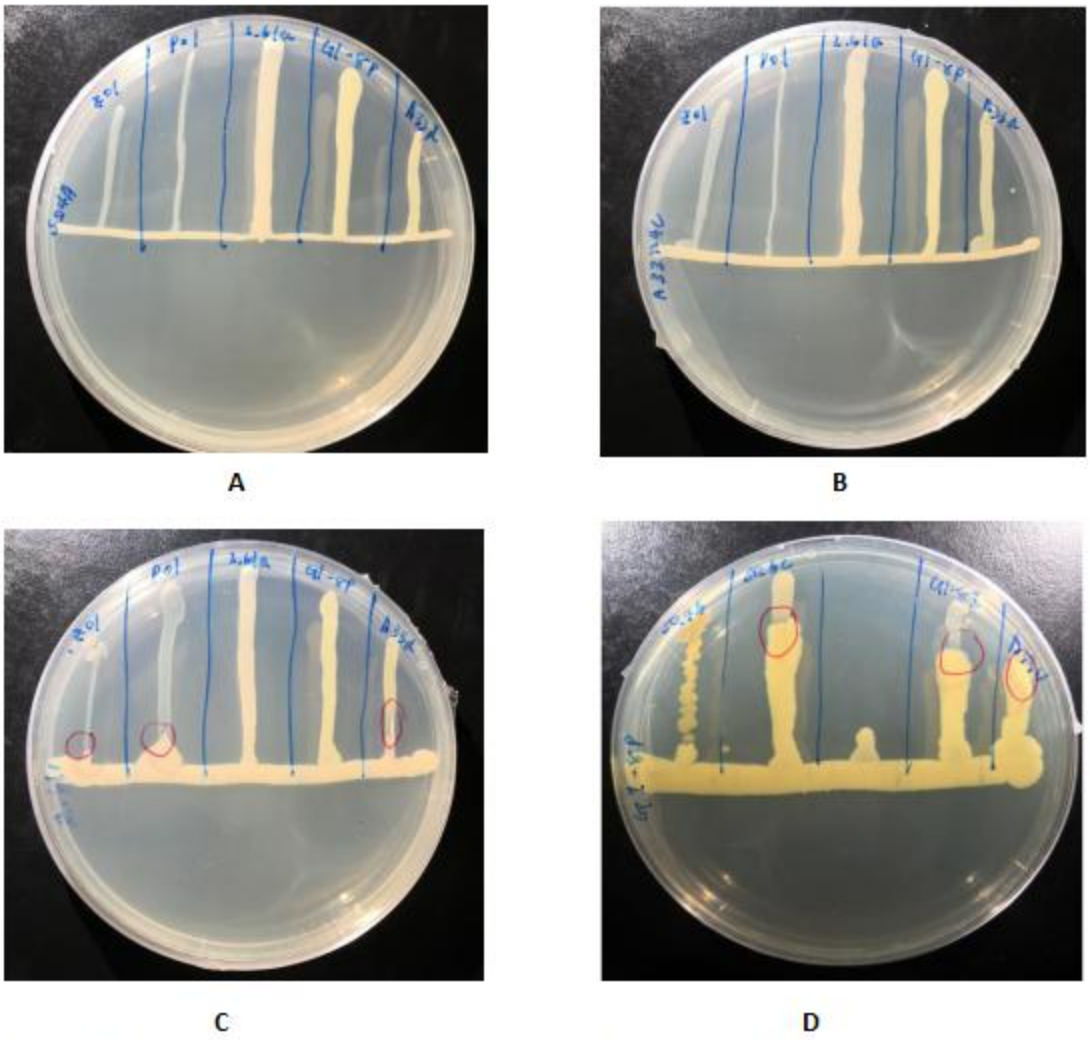


Figure 1.2. Bacteria zone of inhibition assay. A) Producer strain: *Burkholderia* SQ4A. Tester strains (from left to right): *S. marcescens* ZO1, *S. marcescens* PO1, *S. marcescens* 2.61A, *S. marcescens* G1-8P, A33.M4.D; B) Producer strain: *Burkholderia* A33. M4.C. Tester strains (from left to right): *S. marcescens* ZO1, *S. marcescens* PO1, *S. marcescens* 2.61A, *S. marcescens* G1-8P, A33.M4.D; C) Producer strain: *S. marcescens* 2. 61 A. Tester strains (from left to right): *S. marcescens* ZO1, *S. marcescens* PO1, *S. marcescens* 2.61A, G1-8P, A33.M4.D. Three zones of inhibition are indicated by red circles; D) Producer strain: *S. marcescens* G1-8P. Tester strains (from left to right): *Burkholderia* SQ4A (contaminated), *Burkholderia* SQ6C, *Burkholderia* G1-8I, *Burkholderia* A33.M4.C. Three zones of inhibition is indicated by red circles. The *S. marcescens* G1-8P producer strain overgrown and covered part of the tester strains, and thus zones of inhibition appeared at a higher position on the plate. *Burkholderia* A33.M4.C was overgrown by *S. marcescens* G1-8P and the zone of inhibition was covered by *S. marcescens* G1-8P growth.

Discussion

Endosymbiotic bacteria can influence a host vector's vectoring capacity. Endosymbionts, for example, can repress pathogen transmission by colonizing a host's pathogen-hosting gland, or by reducing a host's life span (Walker et al. 2011;Bahia et al. 2014; McMeniman et al. 2009; Moreira et al. 2009). Alternatively, endosymbionts can facilitate pathogen establishment and transmission by protecting a pathogen from the host's immune system or guiding a pathogen to a vector's salivary gland where it will be secreted and transmitted to other organisms (Yuval et al. 2010;Van den Heuvel et al. 1999).

The squash bug *A. tristis* vectors *S. marcescens*, the plant pathogen that causes *Cucurbit Yellow Vine Disease* (Pair et al. 2004). To investigate whether the presence of *A. tristis*'s symbiont, *Burkholderia* sp., influences *A. tristis*'s ability to vector *S. marcescens*, I orally introduced *S. marcescens* bacteria into *A. tristis* and compared the number of *S. marcescens* in symbiont-containing and symbiont-lacking *A. tristis.* The results of my experiments indicate that the presence of *Burkholderia* leads to fewer S. *marcescens* persisting in *A. tristis*. *Burkholderia* colonizing *A. tristis*'s midgut crypt might repress the establishment of *S. marcescens* through secreting inhibitory factors or by competing over common critical resources. It is also possible that the presence of *Burkholderia* up-regulates *A. tristis*'s immune response towards *S. marcescens,* and thus represses the establishment of *S. marcescens*.

S. marcescens strains varied in their ability to establish within the host. In two experiments, one involving *Burkholderia* strain SQ4A and one involving *Burkholderia* strain A33. M4.C, *S. marcescens* Z01 proliferated in both symbiont-containing and symbiont-lacking insects, though it reached a significantly lower titer in symbiont-containing insects. In comparison, in both symbiont-containing and symbiont-lacking *A. tristis, S. marcescens* P01 did not persist in many individuals at 20hrs post feeding, and, in fact, many bugs did not harbor detectable levels of P01 just after the feeding period (the 0hr post feeding time

point) (Supp. fig. 4). There are two possible explanations. First, it is possible that the bugs fed less on P01 infused fruits. At 15 minute intervals, when an *A. tristis* was seen dwelling on the *S. marcescens* infused zucchini, it was counted as having been feeding. It is possible that many *A. tristis* in the *S. marcescens* P01 group only dwelled on fruits but did not actually feed during the 15 minute time period. However, all *A. tristis* were starved 24 hours before orally feeding, making it likely that they would immediately feed. In addition, each *A. tristis* was checked and confirmed to be feeding four times before being removed from the fruit. Alternatively, *S. marcescens* P01 might be less able to colonize *A. tristis* than *S. marcescens* Z01. For example, *A. tristis*'s immune system may clear *S. marcescens* P01 quickly once the bacteria enters *A. tristis*'s system. In order to examine the proposed possible causes, I will carry out further experiments, including replication of the experiment with *S. marcescens* P01 and coupling *S. marcescens* P01 with a different *Burkholderia* strain.

Since *Burkholderia* could directly inhibit the growth of *S. marcescens*, I carried out a zone of inhibition assay to test whether *Burkholderia* can inhibit *S. marcescens* through secreting inhibitory factors. I found no inhibitory effect between the two *S. marcescens* strains (Z01 and A33.M4.C) and the two *Burkholderia* strains (SQ4A and A33.M.4.C) used in the oral feeding experiments. However, this *in vitro* assay only tests whether *Burkholderia* excrete factors that can directly inhibit *S. marcescens*. Even though there is no inhibition effect between the two bacteria *in vitro*, it is still possible that *Burkholderia* can inhibit the growth of *S. marcescens in vivo*, through mechanisms such as its excretions being metabolized into an active intermediate that has an inhibitory effect. In addition, *Burkholderia* may also directly inhibit *S. marcescens in vivo* by exploiting space and common critical resources required by *S. marcescens* establishment. Further experiments need to be done to test the aforementioned possibilities. Notably, it is still not known where *S. marcescens* proliferates within *A. tristis*, making it unclear as to how much direct inference could occur between *Burkholderia* and *Serratia.*

According to current data that shows lower *S. marcescens* establishment in symbiont-containing *A. tristis*, it is possible that the presence of *Burkholderia* symbiont can suppress *A. tristis*'s ability to vector *S. marcescens* . However, more experiments, such as assays testing *S. marcescens* transmission rate to plants when they are fed upon by symbiont-containing and symbiont-lacking *A. tristis*, need to be done in order to determine whether the presence of symbiotic *Burkholderia* influences *A. tristis*'s vectoring capacity.

Chapter 2: The Influence of Symbiotic *Burkholderia* on *Anasa tristis*'s Response Towards Subsequent Introduction of *Serratia marcescens* through Injection

Abstract

Symbiont-conferred host defense plays an essential role in host fitness. Symbiotic microbes can protect a host by up-regulating the host's immune system, a phenomenon observed in organisms such as bean bugs and weevils. Symbionts can influence three known alternative forms of defense: anti-infection resistance, anti-growth resistance, or tolerance. To investigate whether the presence of symbiotic *Burkholderia* influences *A. tristis*' resistance or tolerance, I examined *A. tristis*' post-infection survivorship and pathogen proliferation upon injection of *S. marcescens* into symbiont-containing and symbiont-lacking bugs. Experimental results showed that symbiont-containing *A. tristis* had higher survivorship than symbiont-lacking *A. tristis.* However, it is still unclear whether the higher survivorship is due to an increase in resistance or tolerance.

Introduction

Like vertebrates, invertebrate hosts have an innate immune system that involves three lines of defense. The first line of defense is anti-infection resistance, which prevents pathogens or parasites from entering hosts (Canny et al.2002; Corteel et al. 2009). If the first line of defense is breached, the second line of defense, anti-growth resistance, represses growth of pathogens or parasites through chemical and cellular responses (Frank 2002; Kurtz 2005). The last line of defense is tolerance. If a pathogen or a parasite manages to enter and proliferate in a host, tolerance helps alleviate the harm endured by the host by reducing the severity of consequences of infection without necessarily repressing the growth of the pathogen or parasite (Read et al. 2008; Schneider& Ayres 2008).

While host immune systems defend hosts against foreign microbes, they are also modulated to tolerate and regulate beneficial endosymbionts (symbionts, usually bacteria, that live inside their hosts). A host may down-regulate its immune system in order to maintain beneficial symbionts. In the well-studied squid-vibrio symbiosis, for example, components of the squid's (*Euprymna scolopes*) immune system, such as hemocytes (immune cells), have evolved to tolerate the presence of symbiotic *Vibrio fischeri* (Mcfall-Ngai et al. 2010). In contrast, a host can up-regulate its immune system in order to regulate endosymbiont populations (Kim et al. 2014). Such regulation may be necessary in some systems as, even though the establishment of symbiotic relationships presents the host with various benefits, over-proliferation of endosymbionts can deprive the host of nutrients and become pathogenic to the host (McFall-Ngai 2007).

Endosymbionts alter host immunity to increase defense against pathogens and parasites in a number of systems. In organisms such as bean bugs and weevils, the host insects exhibit more robust antimicrobial activity that target their endosymbionts and keep the symbiont population in check (Kim et al. 2014;

Login et al. 2011). In the bean bug *Riptortus pedestris*, the presence of symbiotic *Burkholderia* enhances the hosts' humoral immunity against bacterial challenges (Kim et al. 2015), and in the tsetse fly, peptidoglycan recognition protein (PGRP-LB), a component of the innate immune system, plays a crucial role in tolerating symbiotic *Wigglesworthia glossinidiae* and conferring host resistance towards trypanosome infection (Wang et al. 2009). It is presumed that these increases in immunity are evolved responses by the hosts to regulate symbiont populations, though it is also possible in some systems that the symbionts have evolved to upregulate host immunity in order to defend their hosts.

In the squash bug, *A. tristis*, symbionts in the genus *Burkholderia* play a crucial role in host development and survival. The bacteria are acquired each generation from the environment and stored in specialized midgut crypts (Acevedo et al., in prep). All wild caught adult *A. tristis* harbor *Burkholderia* (Acevedeo et al., in prep), suggesting that *Burkholderia* are important for these insects. Indeed, harboring *Burkholderia* increases *A. tristis'* survival and decreases *A. tristis'* development time (Acevedo et al., in prep). It is not known if this association impacts other hosts phenotypes.

Building on previous work demonstrating that *Burkholderia* symbionts can alter immunity of other heteropterans, such as the bean bug *Riptortus pedestris* (Kim et al. 2015), here, I explore whether harboring *Burkholderia* influences *A. tristis*'s resistance towards microbial invaders. By comparing the survivorship of *Serratia*-injected individuals to that of control, uninjected individuals, I investigated whether association with *Burkholderia* influences *A. tristis*' survivorship upon *Serratia* infection. Experimental results demonstrate that association with *Burkholderia* increases the insect's survival upon pathogen infection. By injecting a strain of fluorescently tagged *S. marcescens* into individual *A. tristis* with and without *Burkholderia* symbionts, I quantify proliferation of *S. marcescens* in the presence and absence of symbionts. As a comparison, in a separate experiment, I also injected individuals with a

fluorescently-tagged symbiotic *Burkholderia* strain. Results indicate that there is no difference in *Burkholderia* proliferation between symbiont-containing and symbiont-lacking *A. tristis* after injecting them with fluorescent *Burkholderia*. While data suggest that *Burkholderia* influences *S. marcescens* proliferation upon injection, due to limitation of sample size and experimental constraints, it is not yet well-supported. Further experiments need to be done in order to determine whether *Burkholderia* increase *A. tristis* survivorship through enhancing host tolerance or anti-growth resistance towards *S. marcescens*.

Methods and Material

Insects and Bacteria

We collected adult *A. tristis* from organic farms in Georgia. Adults were used to start lineages of laboratory-reared *A. tristis*. The insects are fed on yellow summer squash plants and allowed to mate and lay eggs freely. Eggs are subsequently collected for experiments. *A. tristis* were reared in the same way as mentioned in Chapter 1.

Bacteria strains used in my experiments were *Burkholderia* SQ4A and *S. marcescens* Z01. As mentioned in Chapter 1, *Burkholderia* SQ4A is known to be a beneficial symbiotic bacteria strain for *A. tristis*. *S. marcescens* Z01 is known to be a plant pathogen that causes *Cucurbit Yellow Vine Disease*. Thus, these two bacteria strains were chosen as suitable candidates for examining whether the presence of symbiotic *Burkholderia* influences *A. tristis*' response towards an injection of foreign bacteria. In addition, these two strains of bacteria were used in the oral feeding assay described in Chapter 1. The experiments also serve as a follow-up of the oral feeding assay, examining the hypothesis that the presence of symbiotic *Burkholderia* up-regulates *A. tristis*'s general immunity.

The *S. marcescens* Z01strain used in preliminary assays was fluorescently labeled by introducing a plasmid that contains the GFP-coding gene. This strain was later found to be potentially losing the GFP-containing plasmid and was replaced by a stable fluorescently labeled *S. marcescens* Z01 (the GFP-coding gene was incorporated into the chromosome) in a later experiment.

Survivorship Assay

In preliminary assays, I discovered that injection of *S. marcescens* in to A. trsitis decreases the inscets' survival. In order to establish a time point at which I could assay pathgen load in the insects before most of them had died, I examined *A. tristis*' survivorship after *S. marcescens* infection. Twenty-two *Burkholderia* SQ4A symbiont-containing and 22 symbiont-lacking fourth instar *A. tristis* (one day post molt) were first anesthetized with carbon dioxide. *S. marcescens* Z01 was introduced into *A. tristis* through a minutin pin, the tip of which was dipped in a fresh culture of *S. marcescens* Z01. In order to estimate the number of *S. marcescens* Z01 entering each *A. tristis,* I performed a control serial dilution in which I dipped a minutin pin into the *S. marcescens* Z01 and then dipped the minutin pin into 450 ml liquid LB. According to serial dilution, the approximate number of *S. marcescens* Z01 bacteria injected into *A. tristis* is 1.19×10^2 CFUs. In addition, 5 symbiont-containing and 4 symbiont-lacking *A. tristis* were stabbed with a sterile minutin pin and put into the control group. After injection, symbiont-containing and symbiont-lacking *A. tristis* were raised on fresh, organic zucchini. The survivorship of *A. tristis* was monitored every six hours for approximately four days.

During the experiment, one symbiont-containing *A. tristis* was misplaced into the symbiont-lacking group. However, this error only decreases the likelihood of observing a significant increase in the survivorship of symbiont-containing *A. tristis* compared to symbiont-lacking *A. tristis*. One data point in the symbiont-containing *A. tristis* was removed from the data set in that the *A. tristis* died of starvation instead of *S. marcescens* Z01.

Bacterial Pathogen Proliferation Assay

Burkholderia SQ4A Injection. Before injecting *A. tristis* with *S. marcescens*, I injected *Burkholderia* SQ4A symbiont-containing and symbiont-lacking *A. tristis* with *Burkholderia* SQ4A bacteria. The purpose of injecting *A. tristis* with its own symbiotic bacteria was to see whether the mere action of

injection *A. tristis* with 0.5 µl bacteria at a concentration of approximately 2.0×10^7 cells / ml would cause mortality in *A. tristis* or cause a difference of bacteria proliferation between symbiont-containing and symbiont-lacking *A. tristis*. To inject *A. tristis* with *Burkholderia* SQ4A, 22 symbiont-containing and 21 symbiont-lacking fourth instar *A. tristis* (one day post molt) were anesthetized with carbon dioxide and injected with 0.5 µL GFP-labeled *Burkholderia* SQ4A fresh culture (standardized by diluting a log-phase liquid bacteria culture to optical density (OD) value of 0.8 with liquid LB) using a glass needle. After injection, symbiont-containing *A. tristis* and symbiont-lacking *A. tristis* were divided into different sub-groups for sampling at the 0 hour and 24 hour time points. At the designated time point, each *A. tristis* was surface sterilized in 99.5% ethanol and the whole bug was crushed in 450 ml liquid LB. The bacteria load was quantified through serial dilution, plating, and then counting GFP labeled colony forming units (CFUs). In order to estimate the number of bacteria introduced into each *A. tristis,* I performed a control serial dilution in which I diluted 0.5 µm *Burkholderia* SQ4A in 450 ml liquid LB. According to serial dilution, the number of *Burkholderia* bacteria entering *A. trisitis* through injection is approximately 7.7×10^4 CFUs .

S.marcescens Z01 injection. This experiment was carried out in the same way as the *Burkholderia* SQ4A injection assay. In the survivorship assay, both symbiont-containing and symbiont-lacking *A. tristis* started to die 18 hours after being injected *S. marcescens* Z01 (Figure 2.1). As a result, the 0 hour and 18 hour time point were used in a preliminary *S. marcescens* Z01 injection assay. However, in later experiments, *A. tristis* started to die at earlier time points after injection. Due to the limited available samples, I chose the 6 hour time point instead in order to acquire a bigger sample size. Each *A. tristis* was sacrificed and the number of *S. marcescens* Z01 was quantified using the same method as described in the *Burkholderia* SQ4A injection assay.

The *S. marcescens* Z01 culture used in the preliminary assay was standardized to an OD value of 0.8. An 18-hour time point was used in an earlier experiment. However, as mentioned above, *A. tristis* started to die sooner after injection in later experiments. Therefore, a 6-hour time point was used. According to the control serial dilution, the estimated number of *S. marcescens* bacteria entering *A. tristis*'s system is approximately 7.2×10^4 CFUs (Figure 2.3 A&B) and 1.9×10^4 CFUs (Figure 2.3 C&D). In the experiment with the stable GFP-labeled *S. marcescens* (Figure 2.4), the *S. marcescens* Z01 culture was standardized to an OD value of 0.4 in the hope that a lower *S. marcescens* Z01 concentration would be cleared faster by *A. tristi*s, potentially revealing a significant difference in pathogen proliferation between symbiont-containing and symbiont-lacking *A. tristis* at the 6-hour time point. According to the control serial dilution, the estimated number of *S. marcescens* bacteria entering *A. tristis*' system is approximately 7.3×10^4 CFUs.

Data Analysis

Survivorship Assay. A Cox Proportional Hazard Model in R was used to analyze the survival data.

Pathogen Proliferation Assay. A Wilcoxon analysis in R was used to analyze the pathogen proliferation data.

Results

Survival after challenge with S. marcescens. The presence of symbiotic *Burkholderia* SQ4A has a significant effect on squash bug's survival after being infected with *S. marcescens* Z01 by injection (Cox proportional hazards model, P = 0.034; Figure 2.1). Symbiont-containing squash bugs had higher survivorship than symbiont-lacking squash bugs after infection with *S. marcescens* Z01.

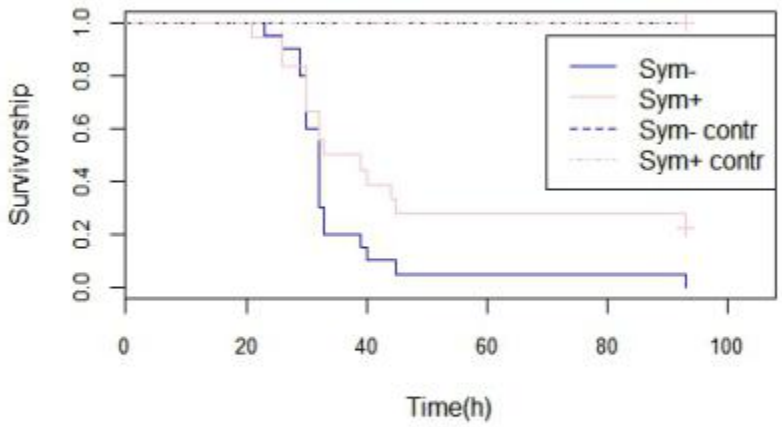


Figure 2.1. Survivorship of *A. tristis* after being infected by *S. marcescens* ZO1 bacteria with a minutin pin. Sym - = symbiont-lacking. Sym + = symbiont-containing. Cox proportional hazards model: p = 0.037, n (symbiont-lacking) = 18, n (symbiont-containing) = 18, n (symbiont-containing control) = 5, n (symbiont-lacking control) = 4.

Burkholderia *proliferation upon injection.*

There was no significant difference between the number of GFP-labeled *Burkholderia* SQ4A in symbiont-containing and symbiont-lacking *A. tristis* 24 hours after injection. (Wilcoxon test, p = 0.84 Figure 2.2 A-B).

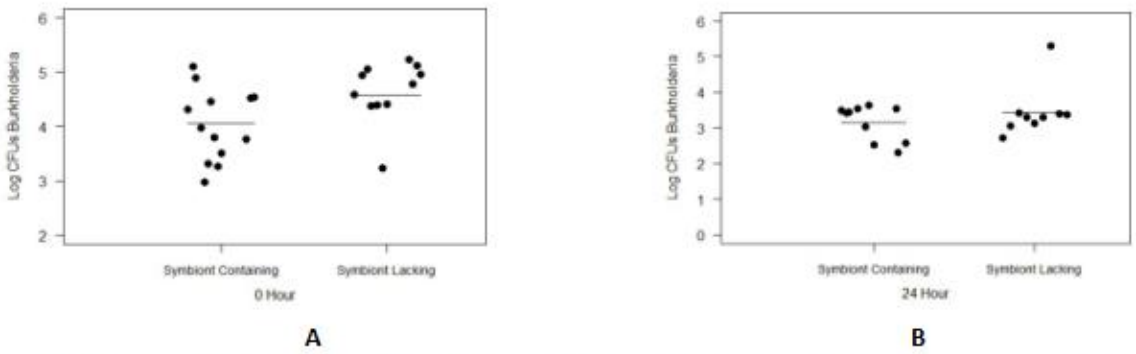


Figure 2.2. A) Estimated Log CFUs of fluorescent *Burkholderia* SQ4A that established in *A. tristis* 0 hours after introducing *A. tristis* with fluorescent *Burkholderia* SQ4A through glass needle injection. Wilcoxon analysis: p = 0.078. n (symbiont-containing) = 12, n (symbiont -lacking) = 12. B) Estimated Log CFU of *Burkholderia* SQ4A remained in *A. tristis* 24 hours after introducing *A. tristis* with *Burkholderia* SQ4A through injection. Wilcoxon analysis: p = 0.84. n (symbiont-containing) = 10, n (symbiont-lacking) = 9.

Serratia *proliferation upon infection.*

In the experiments with the *S. marcescens* Z01 strain in which the GFP-encoding genes were on a plasmid, the number of *S. marcescens Z01* pathogen in symbiont-containing *A. tristis* was significantly lower than the number of *S. marcescens* Z01 pathogen in symbiont-lacking *A. tristis* six hours post-injection (Wilcoxon test, p = 0.022, Figure 2.3 B). At the 18 hour time point, the numbers of *S. marcescens* Z01 in symbiont-containing and symbiont-lacking *A. tristis* did not differ significantly (Wilcoxon analysis, p = 0.057, Figure 2.3 D), however, there is a trend that symbiont-lacking *A. tristis* had more *S. marcescens* Z01 than symbiont-containing *A. tristis*.

In the experiment with the *S. marcescens* Z01 strain in which the GFP-encoding genes were genomically integrated, there was no difference between the number of *S. marcescens* Z01 pathogen in symbiont-containing *A. tristis* and the number of *S. marcescens* Z01 pathogen in symbiont-lacking *A. tristis* six hours post-injection (Wilcoxon test, p = 0.59, Figure 2.4).

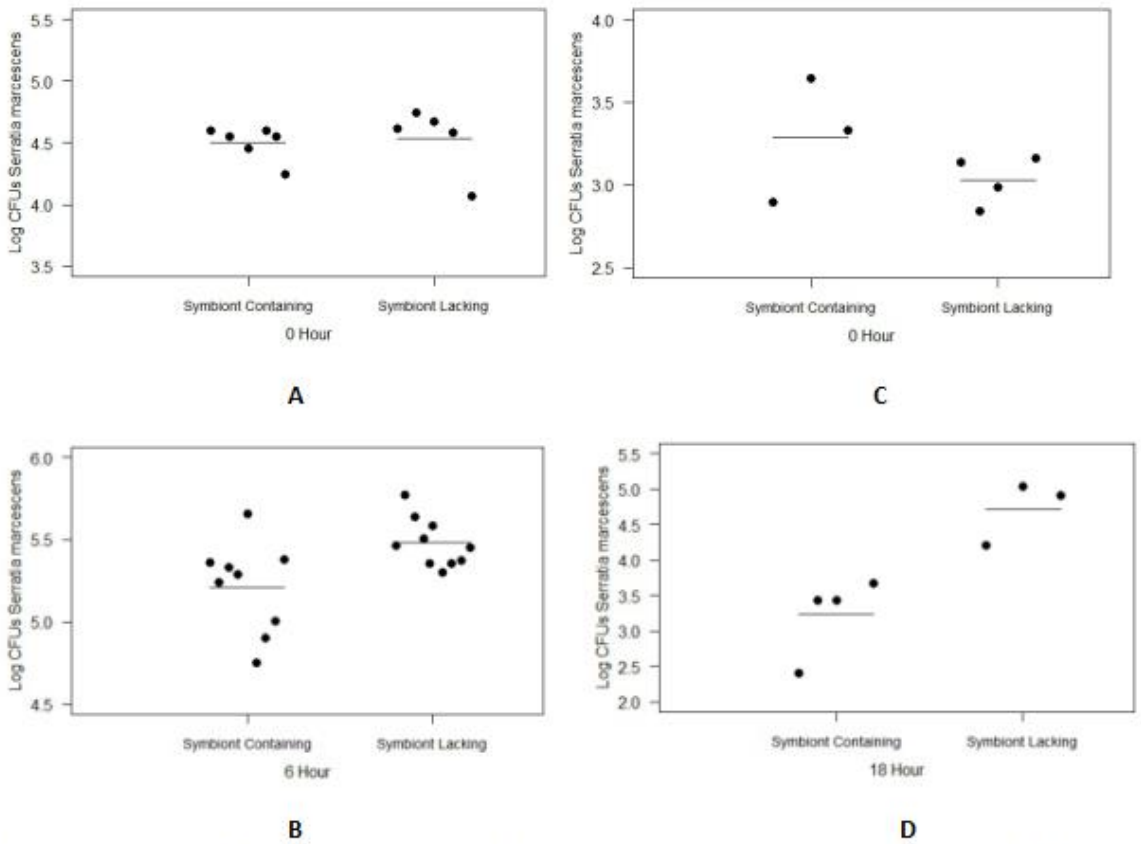


Figure 2.3. Estimated Log CFUs of fluorescent *S.marcescens* ZO1 that established in *A. tristis* after introducing *A. tristis* with fluorescent *S. marcescens* ZO1 through glass needle injection. Data obtained separately from two two experiments. A) Experiment 1: 0 hour after injection. Student t test: p = 0.48. n (symbiont-containing) = 6, n (symbiont-lacking) = 5. B) Experiment 1: 6 hours after injection. Wilcoxon analysis: p= 0. 022. n (symbiont-containing) = 10, n (symbiont-lacking) = 9. C) Experiment 2: 0 hour after injection. Wilcoxon analysist: 0.40, n (symbiont-containing) = 3, n (symbiont-lacking) = 4; D) Experiment 2: 18 hours after injection. Wilcoxon analysis: 0.057, n (symbiont-containing) = 4, n (symbiont-lacking) = 3.

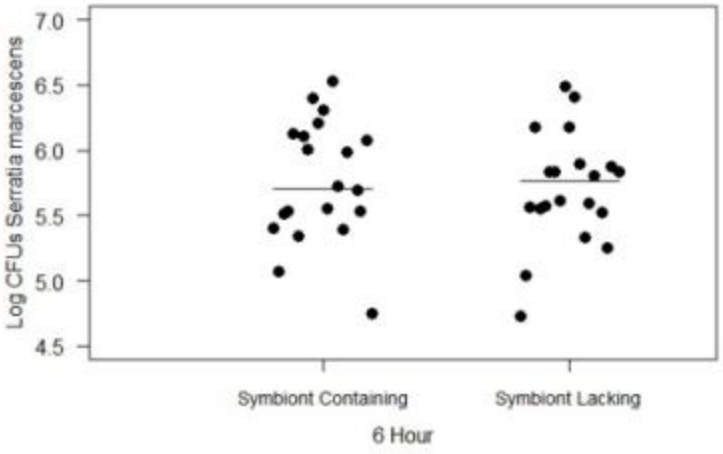


Figure 2.4. Estimated Log CFUs of fluorescent *S. marcescens* Z01 that remained in *A. tristis* 6 hours after introducing *A. tristis* with fluorescent *S. marcescens* Z01 through glass needle injection. Statistic used was Wilcoxon analysis. $p = 0.59$, $n = 20$ per sample.

Discussion

Endosymbionts can alter host immune responses (Kim et al. 2013; Login et al. 2011;Kim et al. 2015;Wang et al. 2009), which in turn can alter their interactions with foreign invaders (Kim et al. 2015). In order to examine whether symbiotic *Burkholderia* alters *A. tristis*'s response to foreign invaders, I introduced *S. marcescens* strain Z01 into *A. tristis* containing symbiotic *Burkholderia* strain SQ4A and *A. tristis* containing no *Burkholderia* symbionts to assays pathogen proliferation in aposymbiotic and symbiotic hosts. Some *S. marcescens* strains, including Z01, are known as plant pathogens that causes cucurbit yellow vine disease (CYVD) (Besler et al. 2017). However, in my experiments, I discovered that *A. tristis* die from *S. marcescens* Z01 infection. This indicates that *S. marcescens* can be pathogenic to its vector *A. tristis,* at least when it gets into the body cavity, which in nature would be most likely through a wound. This pathogenesis allowed me to explore whether aposymbiotic and symbiotic individuals differ in their responses towards pathogen infection.

In the survivorship assay, the post-infection survivorship of symbiont-containing *A. tristis* is significantly higher than the survivorship of symbiont-lacking *A. tristis.* Previous studies in the Gerardo lab have shown that symbiont-containing *A. tritis* are generally healthier than symbiont-lacking *A. tristis* (Acevedo et al., in review). Nutrient depletion may have caused lower survivorship in symbiont-lacking *A. tristis.* The difference in survivorship can also be caused by symbiont-mediated difference in *S. marcescens* anti-growth resistance or tolerance. If the presence of symbiont *Burkholderia* enhances *A. trisitis*'s resistance, symbiont-containing *A. tristis* would exhibit stronger clearance of *S. marcescens* and thus have higher survivorship than symbiont-lacking *A. tristis.* Alternatively, the difference in survivorship might be due to a difference in *A. tristis* tolerance. The presence of symbiotic *Burkholderia* reduces the adverse effects caused by *S. marcescens* infection without repressing the proliferation of *S. marcescens*. Symbiotic *Burkholderia* can influence *A. tristis* through different mechanisms. For example, the presence of

Burkholderia SQ4A facilitates the development and maturity of *A. tristis*, and consequently symbiont-containing *A. tristis* survive better than symbiont-lacking *A. tristis* post infection. Symbiont *Burkholderia* may also secrete certain metabolite that helps reduce the harm of *S. marcescens* infection without repressing *S. marcescens* proliferation. These mechansims are not mutually exclusive and warrant further investigation.

I carried out pathogen proliferation assays to examine whether the presence of symbiont *Burkholderia* enhances *A. tristis*'s anti-growth resistance towards *S. marcescens.* No significant difference in *Burkholderia* proliferation was observed when *A. tristis* was infected by *Burkholderia* SQ4A through injection (Figure 2.2 A-B). There are two possible explanations: 1) *Burkholderia* SQ4A is a symbiont strain to *A. tristis*. The *A. tristis* immune system has already adapted to the presence of *Burkholderia* bacteria. Thus, the introduction of *Burkholderia* SQ4A does not elicit an immune response. 2) The *Burkholderia* SQ4A symbiont does not influence *A. tristis*'s resistance towards foreign microbial invaders, and thus there is no significant difference in the bacteria clearance ability of symbiont-containing and symbiont-lacking *A. tristis*. Whichever mechanism is in effect, the experimental result indicates that the mere action of injecting *A. tristis* with 0.5 µl bacteria culture (10E7 cells/ml) should not contribute to a difference in pathogen proliferation within *A. tristis*. This can serve as a control and be compared with results from other experiments that involves introducing a pathogenic bacteria into *A. tristis*.

A significant difference in *S. marcescens* proliferation between symbiont-containing and symbiont-lacking *A. tristis* was observed at 6 hour time point (Figure 2.3B). Symbiont-containing *A. tristis* had a lower load of *S. marcescens* than symbiont-lacking *A. tristis*. With the same fluorescent *S. marcescens* Z01 strain, a similar trend also appears at 18 hour time point —— symbiont-containing *A. tristis* exhibit lower *S. marcescens* Z01 establishment than symbiont-lacking *A. tristis* (Figure 2.3D). The non-

significant p value might be due to an extremely small sample size. It should be noted that these results were obtained from experiments in which an unstable fluorescent *S. marcescens* Z01 strain was used. Losing the GFP-gene may have affected the experimental results. However, at the same time, the rate of losing GFP-label should be consistent across different *A. tristis* treatment groups, and thus may not affect the difference of pathogen proliferation across groups.

Nevertheless, difference in *S. marcescens* Z01 proliferation was not observed in a later experiment (Figure 2.4), in which a stable GFP-labeled *S. marcescens* strain was used. Thus, it is possible that losing-GFP label did confound the results from previous experiments, and that there is actually no significant difference in *S. marcescens* Z01 proliferation 6 hours after injection. However, it should be noted that the concentration of *S. marcescens* Z01 bacteria introduced into *A. tristis* was 5E6 cells /ml, half of the concentration used in previous experiments. I introduced a lower concentration of bacteria culture in the hope of observing a greater difference of pathogen proliferation between symbiont-containing and symbiont-lacking *A. tristis*. This bacterial concentration, however, might have been so low that symbiont-lacking *A. tristis* is as capable of clearing this amount of bacteria as symbiont-containing *A. tristis*. With a higher *S. marcescens* Z01 concentration, there might be a significant difference between *S. marcescens* proliferation within symbiont-containing and symbiont-lacking *A. tristis*. In addition, even if there is actually no significant difference in *S. marcescens* proliferation across groups at the 6 hour time point, it is still not enough to conclude that whether the presence of symbiont *Burkholderia* influences *A. tristis*'s anti-growth resistance. According to the survivorship assay, *A. tristis* do not start to die until 18 hours after injection. A 6-hour time point might just be too early to exhibit a significant difference in pathogen proliferation. Thus, further experiments should be carried out at the 18-hour time point.

In all, the presence of symbiont *Burkholderia* SQ4A increases *A. tristis*'s survivorship after being infected with *S. marcescens* Z01 through injection. It is possible that nutrient depletion in symbiont-lacking *A. tristis* caused lower survivorship post-infection. Alternatively, it is also possible that the presence of symbiont *Burkholderia* SQ4A enhances *A. tristis*'s general immunity against invading *S. marcescens* Z01. This up-regulation of general immunity might also contribute to the lower *S. marcescens* establishment in symbiont-containing *A. tristis* (Figure 1.1 A-C). The current data is not enough to conclude whether there is an up-regulation of anti-growth resistance in symbiont-containing *A. tristis*. Further experiments should be carried out in order to see whether the presence of symbiotic *Burkholderia* enhances *A. tristis*'s anti-growth resistance.

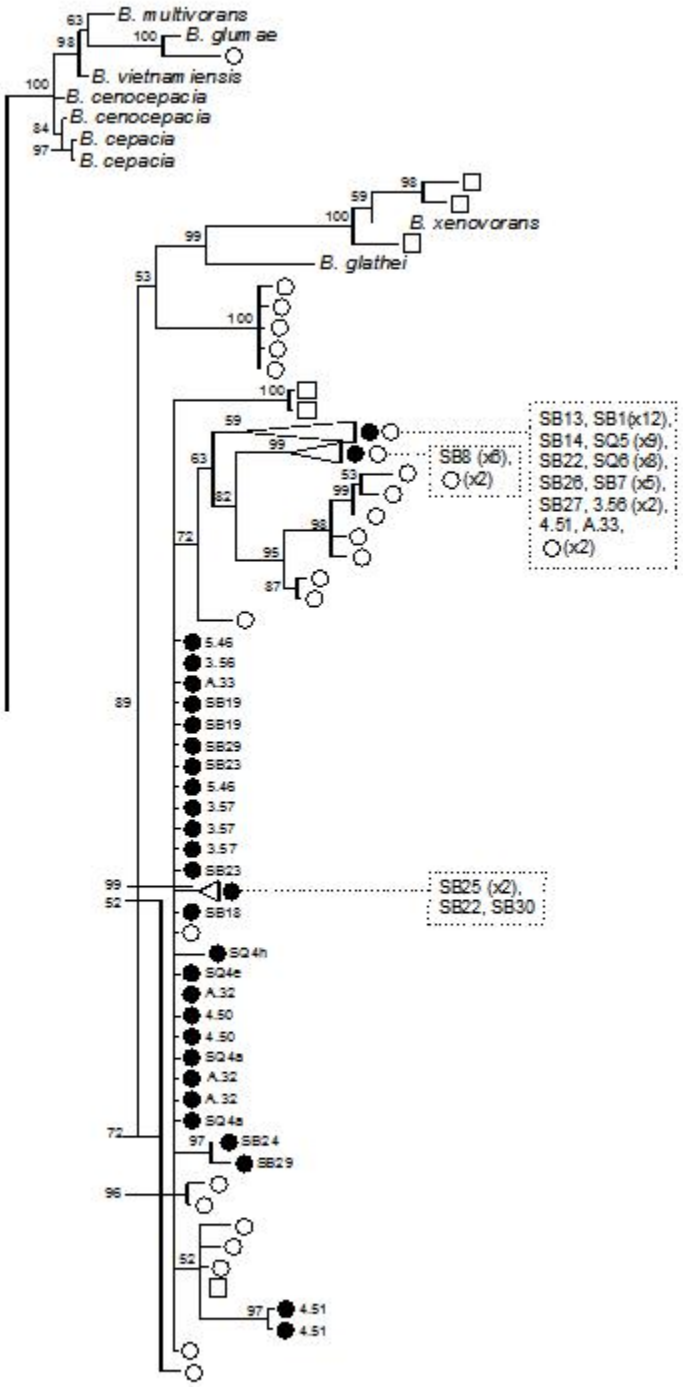
Conclusion and Future Directions

The presence of symbiotic *Burkholderia* is associated with lower *S. marcescens* establishment in *A. tristis*. This indicates that the presence of symbiotic *Burkholderia* might reduce *A. tristis*'s vectoring capacity. However, more experiments, such as assays that test *S. marcescens* transmission rates to plant when fed upon by symbiont-containing and symbiont-lacking *A. tristis*, need to be carried out in order to determine whether the presence of symbiotic *Burkholderia* can influence *A. tristis*'s ability to vector *S. marcescens*. Zone of inhibition assays showed no sign of direct inhibition between *Burkholderia* and *S. marcescens*. However, *Burkholderia* may inhibit *S. marcescens* through competing over a common resource or up-regulating *A. tristis*'s immune system. The latter is consistent with results in chapter 2, where I found *A. tristis* had higher survivorship when injected with *S. marcescens*. However, it is still unclear whether *Burkholderia* can enhance *A. tristis*'s anti-growth resistance towards *S. marcescens* infection. A higher concentration of the fluorescent *S. marcescens* bacteria for which the GFP is genomically integrated (and thus seemingly more stable) and a later time point in the pathogen proliferation assay may give rise to a significant difference between *S. marcescens* proliferation within symbiont-containing and symbiont-lacking *A. tristis* when injected. In addition, more strains of *Burkholderia* and *S. marcescens* should be examined in order to see whether the presence of symbiotic *Burkholderia* can influence *A. tristis*'s response towards the subsequent introduction of *S. marcescens*. Further experiments need to be carried out to elucidate the mechanisms underlying the differences we see in both experiments.

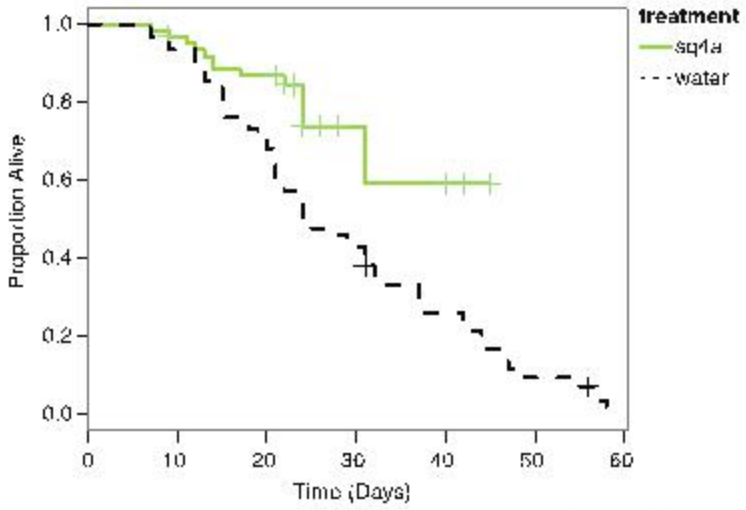
Since the presence of symbiotic *Burkholderia* decreases the establishment of *S. marcescens* in *A. tristis*, allowing *A. tristis* to acquire *Burkholderia* seems desirable for creating symbiont-mediated biocontrol strategies. However, previous data in the Gerardo lab indicate that the presence of symbiotic *Burkholderia* also increases *A. tristis*'s survival into adulthood during development and reduces *A. tristis*'s developmental time. This would give *A. tristis* more opportunity to damage curcurbit corps and transmit

CYVD. Therefore, it cannot be easily determined whether the presence of symbiotic *Burkholderia* is favorable for controlling CYVD transmission. Genetically modified *Burkholderia* that both suppresses *S. marcescens* establishment and shorten *A. tristis*'s life span would be an ideal biocontrol strategy, and further understanding the mechanisms underlying both are needed to facilitate such and approach.

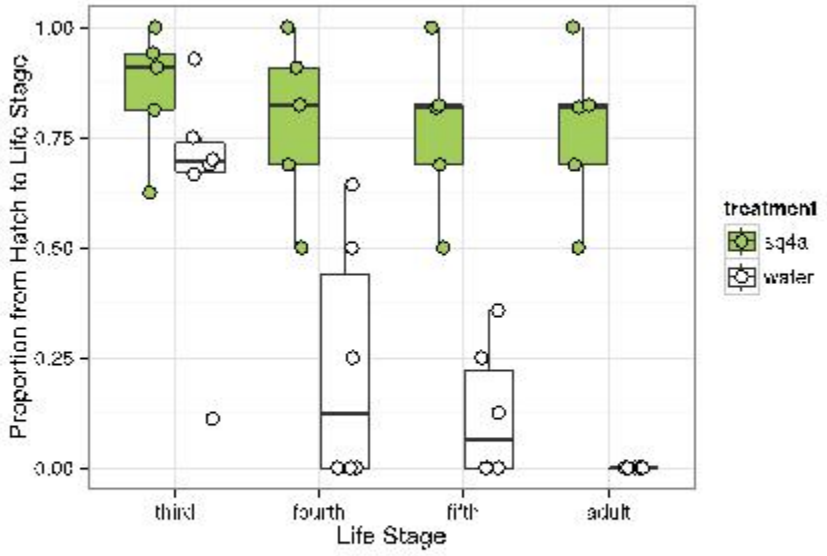
Supplemental Figures



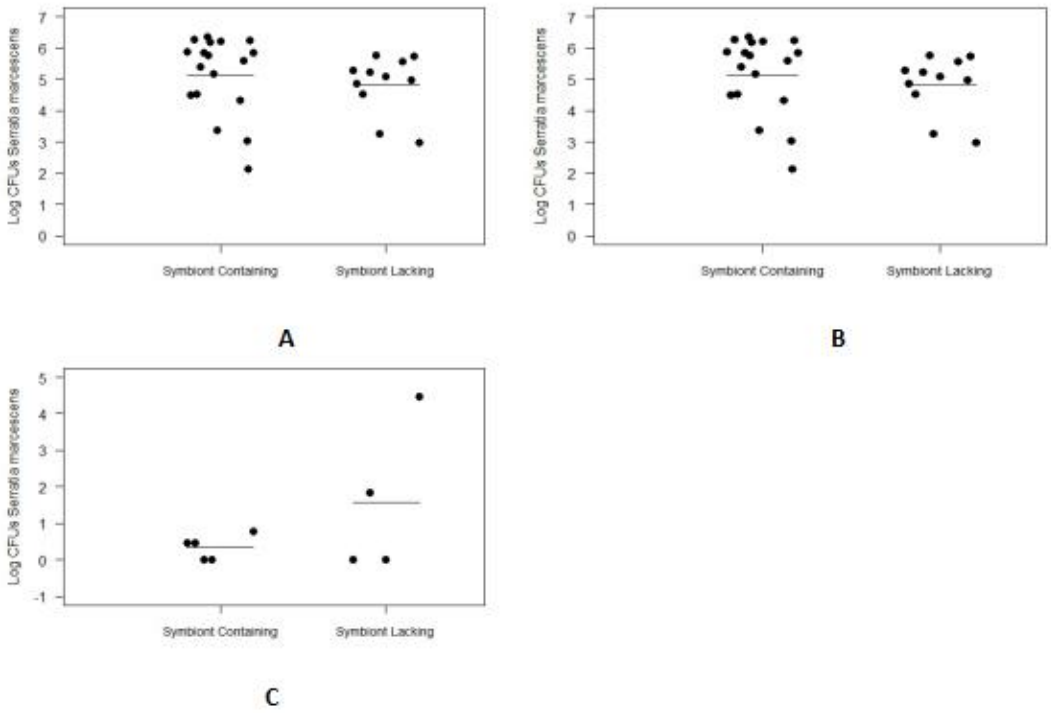
Supplemental Figure 1. *Burkholderia* phylogeny constructed based on sequencing a portion of 16s rRNA gene, The sequencing was done using basyesiam methods. Support values indicates posterior probabilities.



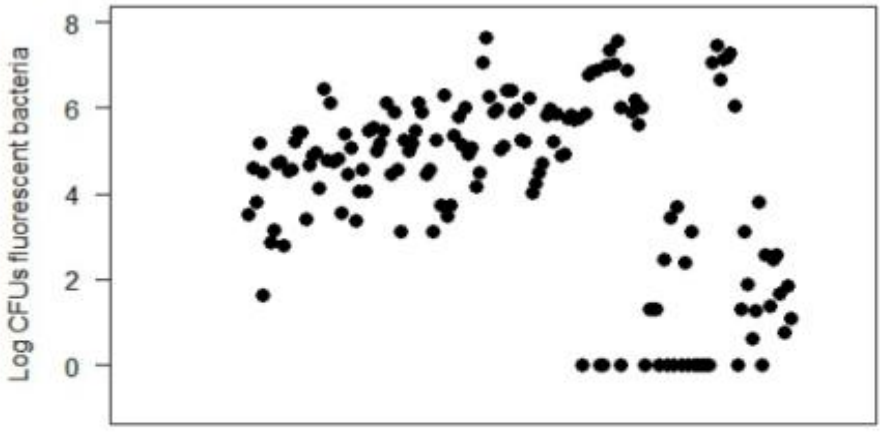
Supplemental Figure 2. Symbiotic *Burkholderia* SQ4A significantly increases *A. tristis*'s survival. Data provided by T. Acevedo.



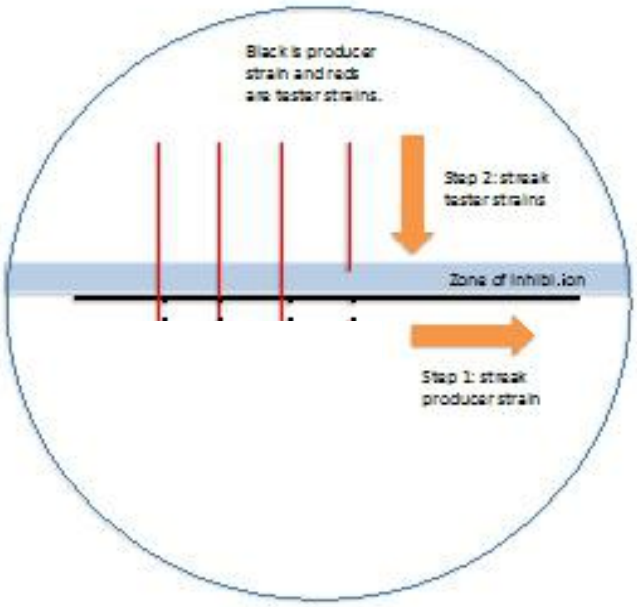
Supplemental Figure 3. Symbiotic *Burkholderia* SQ4A significantly increase the number of *A. tristis* that achieve later developmental stage. Data provided by T. Acevedo.



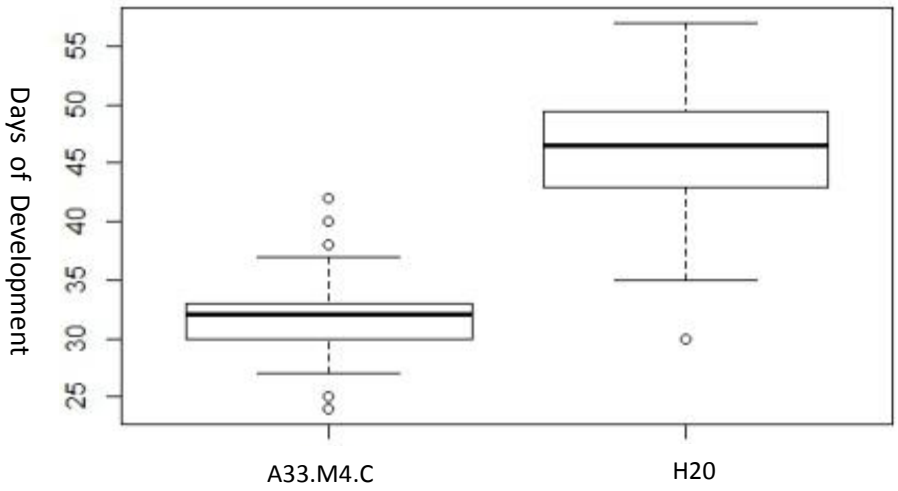
Supplemental Figure 4. The estimated amount of *S. marcescens* remaining in *Burkholderia* symbiont-containing and symbiont-lacking *A. tristis* at 0 hour time point after orally introducing *S. marcescens* into *A. tristis*. A) *Burkholderia* SQ4A and *S. marcescens* Z01. Data provided by T. Acevedo. B) *Burkholderia* A33 and *S. marcescens* Z01. Data provided by T. Acevedo. C) *Burkholderia* A33.M4.C and *S. marcescens* P01.



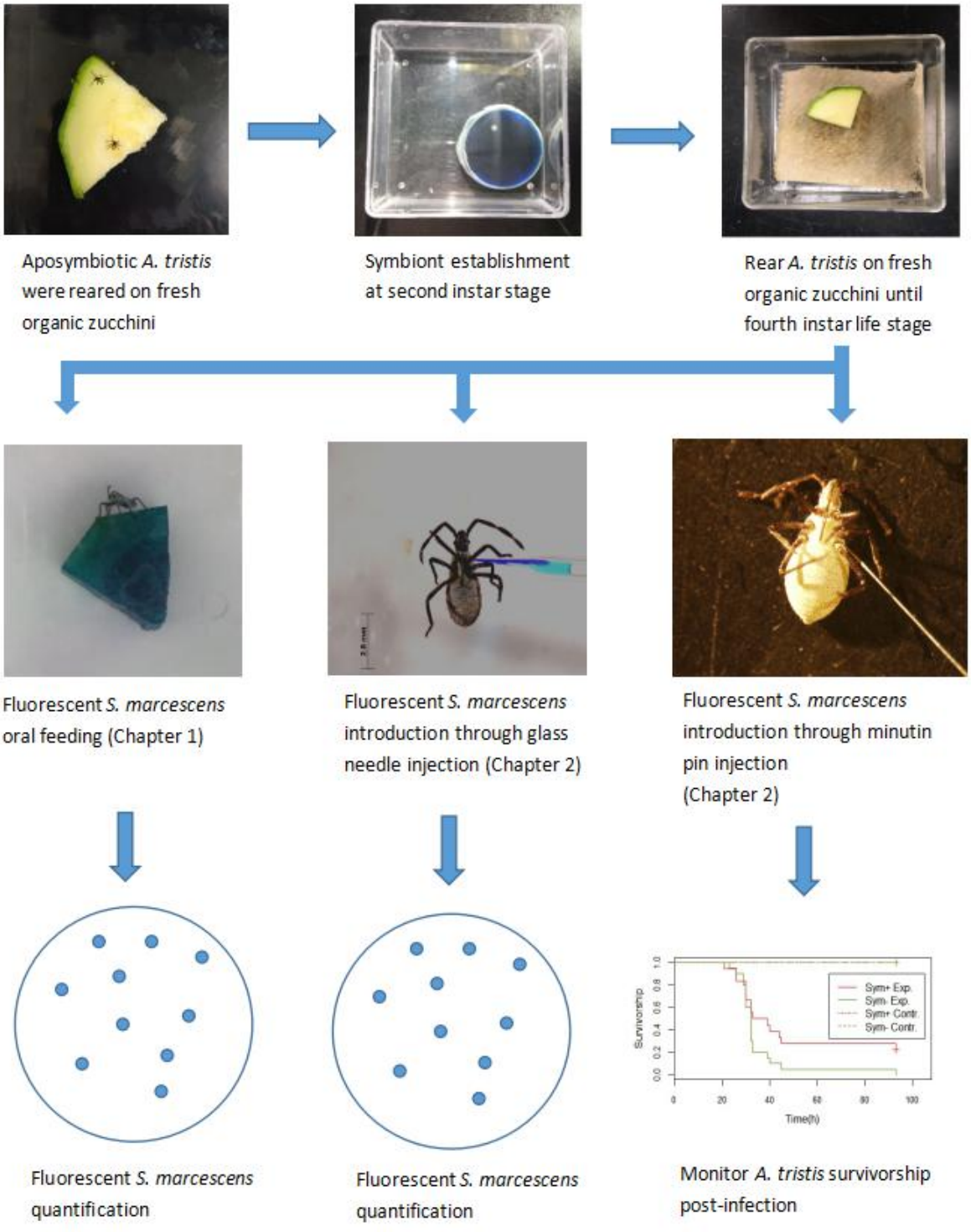
Supplemental Figure 5. The estimated amount of symbiotic bacteria in adult *A. tristis*. Symbiotic bacteria strains include *Burkholderia* SQ4A, *Burkholderia* A33.M4.C, and *S. marcescens* SMT4A. Data provided by T. Acevedo.



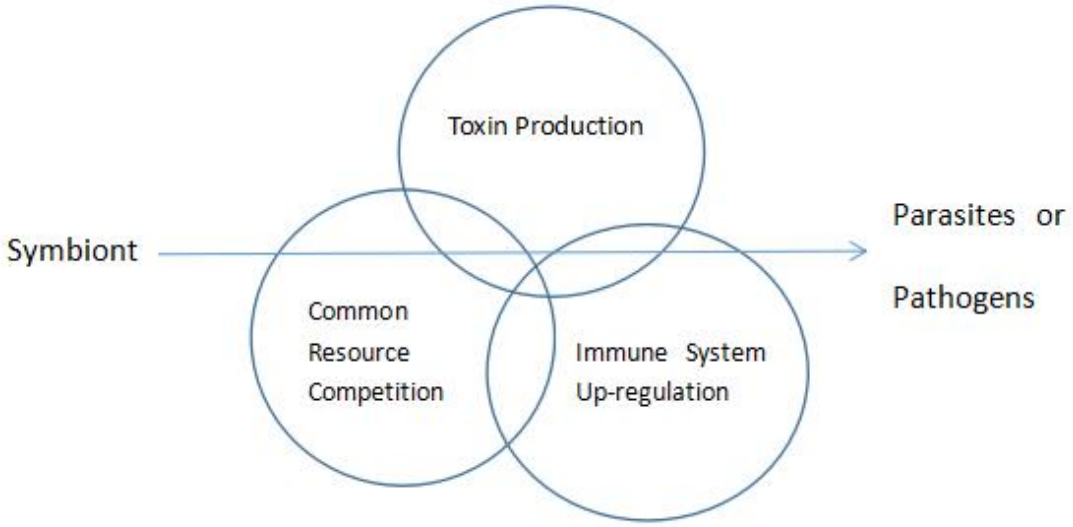
Supplemental Figure 6. Bacteria zone of inhibition design. Tester bacteria strains were streaked perpendicular to the producer strain. If the producer inhibits a tester, a zone of inhibition will appear, as indicate in the figure.



Supplemental Figure. 7. The number of days for *Burkholderia* A33.M4.C. symbiont-containg and symbiont-lacking *A. tristis* to develop into adulthood.



Supplemental figure 8. *A. tristis* rearing, symbiont establishment, and experiments.



Supplemental figure 9. Three mechanisms of symbiont-mediated host defense.

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