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Gregory Paul Fricker April 14, 2015

Specificity, Choice, and Variation in an Environmentally Acquired Symbiosis: The Squash Bug, *Anasa tristis,* and the Bacterium, *Burkholderia*

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Abstract

Specificity, Choice, and Variation in an Environmentally Acquired Symbiosis: The Squash Bug, *Anasa tristis,* and the Bacterium, *Burkholderia*

By Gregory Paul Fricker

Symbioses and in particular, endosymbioses, are prevalent interactions throughout many plant, animal, bacterial, and fungal systems (McFall-Ngai 2000; Bever 2015). These interactions can affect host fitness both positively and negatively via a variety of mechanisms (Kikuchi et al. 2012; Nyholm and McFall-Ngai 2004; McCutcheon and von Dohlen 2011). The squash bug, *Anasa tristis*, is a common agricultural pest in the order Hemiptera, a classification of true bugs within which some species have been known to commonly harbor a bacterial endosymbiont (Cook and Neal 1999; Kikuchi et al. 2011). Little is known about whether squash bugs harbor a bacterial endosymbiont, how diverse this symbiont is, what selective mechanisms would stabilize these interactions, and what impact different strains of symbiont would have on host fitness. Here, we answer these questions and show that squash bugs harbor multiple genetically distinct strains of bacterial endosymbiont from the genus *Burkholderia*. We also demonstrated some, limited support of host preference in feeding on different strains of symbiont. Additionally, we illustrate the differential fitness effects of multiple strains of symbiotic bacteria on squash bugs.

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Chapter 1

Introduction

Symbionts and Hosts

Symbiosis refers to intimate interactions between any two organisms. While these interactions exist on a continuum ranging from parasitism to mutualism, interactions referred to as symbiotic are often mutualistic, benefiting both partners. Symbioses have been observed and studied in numerous animals, plants, fungi, and bacteria (McFall-Ngai 2000; Bever 2015). Of particular interest for this study are endosymbiotic interactions, those in which one organism, the symbiont, resides within another, the host. Endosymbionts, typically microorganisms, can affect their hosts in many ways, increasing host fitness by providing nutrients, protecting from pathogens and predators, or conferring resistance to toxins such as insecticides (Kikuchi et al. 2012; Garcia et al. 2014; Nyholm and McFall-Ngai 2004; McCutcheon and von Dohlen 2011). Endosymbionts can be acquired horizontally from the environment *de novo* each generation or vertically from parent to offspring.

Horizontally acquired symbioses involve populations of free-living bacteria that exist and prosper in the environment (Bright and Bulgheresi 2010). For example, Gram-negative proteobacteria in the genus *Burkholderia* occupy diverse environmental niches, including soil, the plant rhizosphere, and the human respiratory tract (Johnson et al. 2015; Angus et al. 2014). These bacteria, when host associated, can be pathogenic, mutualistic, or lie on the continuum in between (Coenye and Vandamme 2003). Strains of *Burkholderia* are known bacterial

endosymbionts of many true bugs (Kikuchi et al. 2011a; Kikuchi et al. 2011b). In some insect hosts, these bacteria can act as facultative symbionts that increase host fitness, thus selecting for greater association (Garcia et al. 2014). Such an interaction is seen with *Riptortus clavatus*, a true bug whose horizontally-acquired endosymbiont, *Burkholderia,* results in larger individuals with more mass (Kikuchi et al. 2007). Another true bug, the southern chinch bug, *Blissus insularis*, exhibits increased development rate and decreased mortality when harboring its *Burkholderia* endosymbiont (Boucias et al. 2012). Similarly, the absence of the gut endosymbiont in the green stinkbug, *Acrosternum hilare* negatively impacts nymphal development, survivorship, and reproduction (Prado 2009).

True bug associations with *Burkholderia* have a single evolutionary origin, and through evolutionary time, these associations have been lost in some families while being conserved in others (Kikuchi et al. 2011b). Members of the infraorder Pentatomomorpha that associate with *Burkholderia* possess tightly-folded portions of their digestive tract, herein referred to as midgut crypts, where food does not pass through and which are associated with the presence of microbial communities and in particular, *Burkholderia* symbionts (Goodchild 1963; Fukatsu and Hosokawa 2002). The conservation of this symbiosis is suggestive of distinct fitness benefits within this interaction for either host, endosymbiont, or both. Given the Red King evolutionary hypothesis, where more slowly evolving actors hold the evolutionary upper hand, insect hosts may select for bacterial endosymbionts that impart some fitness advantage upon the host (Bergstrom and Lachmann 2003).

Viewing the role of symbionts strictly through the binary of presence or

absence ignores the role that the diversity of bacterial endosymbionts may play in these symbioses. Genetic differences between symbionts may alter host fitness in different ways. In the bean bug, *Riptortus pedestris*, and its association with *Burkholderia*, for example, some strains confer pesticide resistance to their host (Kikuchi et al. 2012). In many systems, however, it is unknown, whether genetically distinct strains can alter their hosts' fitness.

The squash bug, *Anasa tristis*, a true bug, serves as an excellent study system in which to assay the benefits of symbiosis. Squash bugs can be found throughout North America and, as a vector of cucurbit yellow vine disease (CYVD), are considered a major pest of cucurbit crops, especially squash, pumpkin, melons, and cucumbers (Beard 1940; Cook and Neal 1999). Related hemipterans harbor *Burkholderia* endosymbionts (see above), suggesting that members of this true bug genus may also harbor these bacteria. *A. tristis* lay eggs that are aposymbiotic (without symbiont), meaning that any endosymbiont would have to be acquired from the environment each generation, providing the opportunity for individuals to pick up novel bacterial strains from the diverse microbial community within the soil near the bugs' hosts plants. Also, since *A. tristis* do not pass their symbionts to offspring vertically, it is possible to infect these bugs with a particular strain of symbiont. Furthermore, *A. tristis* are amendable to laboratory conditions and can thrive in healthy stock populations.

Overview of Thesis

In Chapter 2, based on collaborative work, we determine if squash bugs have symbioses with *Burkholderia* and estimate the phylogenetic diversity of these symbionts. In Chapter 3, I explore pre-establishment partner selection by the bugs through multiple no choice experiments. In Chapter 4, I compare the differential fitness effects of multiple strains from the genus *Burkholderia* on such host fitness measures as development time towards adult, weight, and size. Finally, in Chapter 5, I provide brief conclusions, placing these findings in light of other work in this field and suggest future directions.

Chapter 2

Phylogenetic Diversity of *A. tristis* Endosymbiont *Burkholderia* from Wild-caught Individuals

Modified from Acevedo, T., Garcia, J., Fricker, G. and N. Gerardo. Bacterial Symbionts of the Squash Bug, *Anasa tristis.* in prep.

Introduction

The squash bug, *Anasa tristis*, is a true bug of the order Hemiptera that is commonly considered a pest of cucurbit plants in the United States (Beard 1940; Cook and Neal 1999). Squash bugs can feed on a variety of cucurbit crops, but they preferentially feed on squash, pumpkin, melons, and cucumbers (Nechols 1987; Bonjour and Fargo 1989). Squash bugs hatch from eggs and are born aposymbiotic. While the symbiotic associations of other true bugs are known, comparatively little is known about *A. tristis* and the bacterial symbionts, if any, that they harbor. Like other members of the Hemiptera order (Kikuchi et al. 2005), *A. tristis* possesses a modified fourth section of the midgut with extensive invagination, called the midgut crypts, through which food does not pass. In many other species, the predominant bacterial species within the crypts are bacteria from the genus *Burkholderia,* though other genera of bacteria can occasionally inhabit the crypts as well (Fukatsu and Hosokawa 2002; Garcia et al. 2014; Kikuchi et al. 2005). It is currently unknown whether *A. tristis* harbors bacterial symbiont(s). Here, we investigate the bacterial

community within the midgut crypts of *A. tristis* by sequencing the 16S rRNA gene of crypt bacterial isolates and estimating a phylogeny using these samples and published reference samples. Here, we conclude that *A. tristis* forms a symbiosis with bacteria from the genus *Burkholderia* and that there is not only a diversity of possible symbiotic strains but also the possibility of co-infection of multiple strains within one individual.

Methods

Collection of Wild Populations

Table 1: *Anasa tristis* samples used in this study. * indicates 1 bacterial isolate with sequence below 600 base pairs. ** indicates 3 bacterial isolates with sequence below 600 base pairs.

A. tristis ID	Sample location	Total bacterial isolates	samples successfully sequenced
SB1	Crystal Farms	9	9**
SB12	Woodland Gardens	1	0
SB13	Woodland Gardens	1	1
SB14	Woodland Gardens	1	1
SB17	Oakhurst Garden	1	1
SB18	Oakhurst Garden	2	1
SB19	Oakhurst Garden	3	3
SB21	Oakhurst Garden	2	1
SB22	Crystal Farms	2	2
SB23	Crystal Farms	4	4
SB24	Crystal Farms	2	1
SB25	Crystal Farms	3	2
SB26	Crystal Farms	2	2
SB27	Crystal Farms	3	3
SB28	Missouri	4	2
SB29	Missouri	3	3*
SB30	Missouri	1	1
SB31	Missouri	2	2
SQ4	Oakhurst Garden	9	6*
SQ5	Oakhurst Garden	9	9
SQ6	Oakhurst Garden	9	9

A. tristis adults were collected from four yellow crookneck squash plots in Georgia, and from one garden in the state of Missouri (Figure 1, Table 1). Adult bugs were collected and transported alive to our laboratory with plant material from their location of origin. Bugs were either dissected immediately or were temporarily housed with only plant material and other squash bugs from the site from which they were collected until they could be dissected.

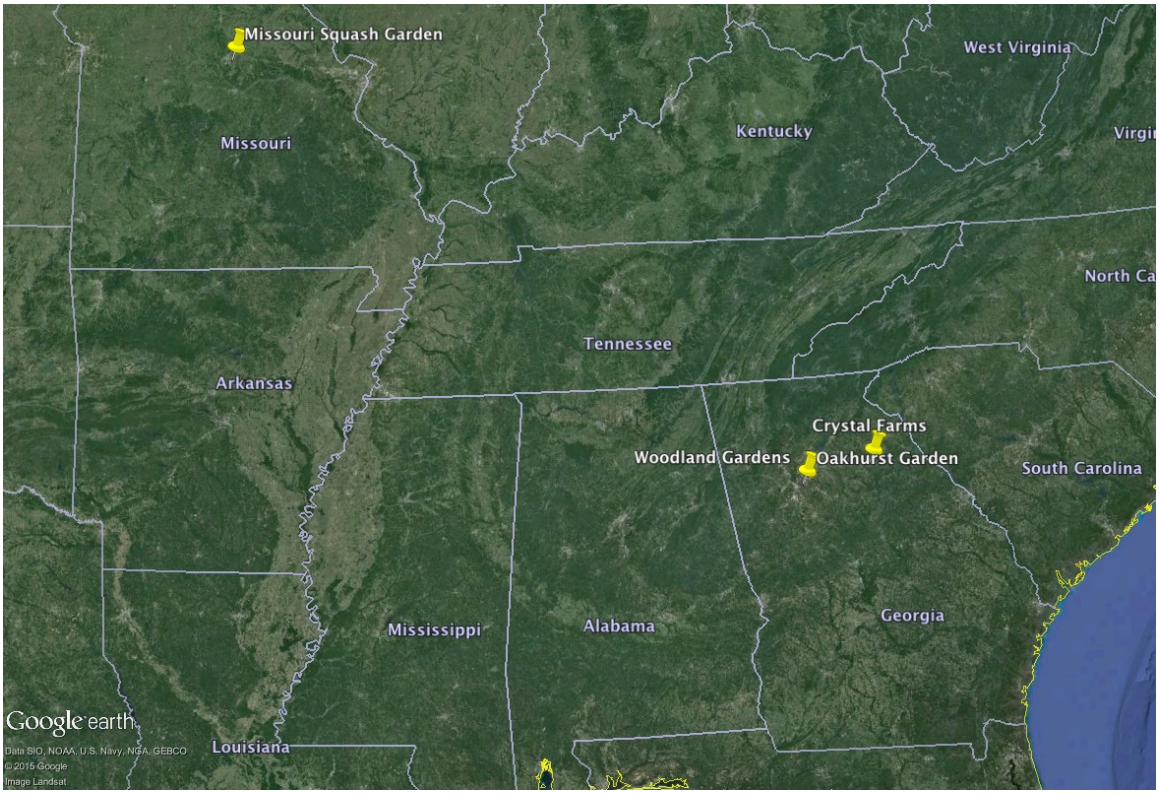

Figure 1: Squash bug collection sites.

Bacterial Isolation

The midgut crypts of adults were removed via dissection, crushed with a sterile pestle in Carlson's solution and cultured on Luria Broth agar plates within three days of collection. Luria Broth plates were used because other true bug symbionts in the genus *Burkholderia* grow readily on this media. These cultures

were incubated for 48 hours at 27° C. For the first four plated midgut crypts, nine bacterial colonies were isolated from the aforementioned plates. After this initial oversampling, only morphologically distinct colonies were isolated. All isolates were stored as glycerol stocks at -80° C for later use.

DNA Extraction

Single colonies of bacterial isolates from LB plates were used to inoculate LB broth, grown at 27° C and 200 rpm overnight, and then centrifuged at 3000 rpm for 5 minutes. The supernatant was removed, and bacteria were resuspended in 200 µl of CTAB lysis buffer and incubated at 60° C for 1 hour. Sodium dodecyl sulfate (SDS) was added to the lysed suspension at a final concentration of 10% and the mixture was incubated at 60° C for another hour. Nucleic acids were extracted using 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1). Residual phenol contamination was removed by re-extracting the nucleic acids with 200 µl of 100% chloroform. DNA was recovered by ethanol precipitation at -20° C overnight and was dissolved in 50 µl of molecular grade water.

Gene Amplification and Sequencing

Nearly complete 16S rRNA genes were amplified from each bacterial sample using the Mastertaq kit (5 Prime) and the universal bacteria primers 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3'). PCR amplifications were performed with an initial 4 minute denaturing at 94° C followed by 36 cycles of denaturing for 30 seconds at 94° C, annealing for 30 seconds at 55° C

and extending for 1 minute at 72° C, with a final 1 minute extension at 72° C. PCR amplicons were purified using the Qiagen PCR Purification kit and sequenced with the forward primer at Eurofins.

For nineteen samples isolated from three squash bugs that were highly similar based on 16s rRNA, we sequenced portions of 5 additional genes, described in Spilker et al. (2009), *atpD, gltB, gyrB, lepA,* and *recA*, following protocols in Garcia et al. 2014.

Phylogenetic Analysis

Sequences were visually inspected and trimmed using CodonCode Aligner 5.1.5 or SeqMan. The sequences were aligned using MUSCLE and manually corrected using Mesquite and CodonCode. JModeltest (version 2.1.6) was used to select a model of nucleotide substitution for phylogenetic analysis. A Bayesian phylogeny was estimated in MrBayes 3.2.0 using the HKY model with a proportion of invariable sites and a gamma shaped distribution of rates across sites. Two simultaneous runs were performed for 5,000,000 generations, with a burn-in fraction of 0.25. The phylogenetic trees were visualized using Figtree V.1.4.2.

Results

Diversity of Bacteria within *A. tristis* Populations

We examined symbiont diversity within squash bugs collected from four different locations (Table 1). Bacteria were cultivated from twenty-one dissected midgut crypt sections. Seventy-five bacterial isolates were sequenced; however,

only sixty three of those isolates returned with a sufficient base pair length, after trimming based on quality score, to be identified using NCBI's BLAST. Of these sixty-three 16s rRNA sequences, ninety-two percent of them were identified as *Burkholderia* samples, and *Burkholderia* was identified in twenty out of twenty-one dissected midgut crypts (Table 2).

Table 2: Sequencing results of bacterial isolates. Samples below 600 base pairs (BP) were used to identify bacterial species but not used for phylogenetic analysis. For all BLAST results, identity value was greater than 97%.

Isolate ID	Blast Results	Sequences < 600 BP	Sequences Failed
SB 1 c CV	*Burkholderia*		
SB1 b LB	*Burkholderia*		
SB1 d LB	*Burkholderia*		
SB1 e CV	*Burkholderia*		
SB1 e LB	*Burkholderia*		
SB1 f LB	*Burkholderia*	x	
SB1 f LB	*Burkholderia*	x	
SB1 g LB	*Burkholderia*	x	
SB1 h LB	*Burkholderia*		
SB12_M4_c			x
SB13_M4_a	*Burkholderia*		
SB14_M4_a	*Burkholderia*		
SB17_M4_b	*Burkholderia*		
SB18_M4_a			x
SB18_M4_b	*Burkholderia*		
SB19_M4_a	*Burkholderia*		
SB19_M4_b	*Burkholderia*		
Sb19_M4_c	*Paenibacillus*		
SB21_M4_a	*Bacillus pumilus*		
SB21_M4_b			x
SB22_M4_a	*Burkholderia*		
SB22_M4_b	*Burkholderia*		
SB23_M4_a	*Bacillus pumilus*		
SB23_M4_b	*Enterococcus fecales*		
SB23_M4_c	*Burkholderia*		
SB23_M4_d	*Burkholderia*		
SB24_M4_a	*Burkholderia*		
SB24_M4_b			x
SB25_M4_a			x

SB25_M4_b	*Burkholderia*		
SB25_M4_c	*Burkholderia*		
SB26_M4_a	*Burkholderia*		
SB26_M4_b	*Burkholderia*		
SB27_M4_a	*Burkholderia*		
SB27_M4_b	*Bacillus pumilus*		
SB27_M4_c	*Staphylococcus sciuri*		
SB28_M4_a	*Burkholderia*		
SB28_M4_b			x
SB28_M4_c	*Bacillus pumilus*		
SB28_M4_d			x
SB29_M4_a	*Burkholderia*	x	
SB29_M4_b	*Burkholderia*		
SB29_M4_c	*Burkholderia*		
SB30_M4_a	*Burkholderia*		
SB31_M4_a	*Klebsiealla oxycota*		
SB31_M4_b	*Burkholderia*		
SQ4a	*Burkholderia*	x	
SQ4b			x
SQ4c			
Sq4d	*Burkholderia*		
SQ4e	*Burkholderia*		
SQ4f	*Burkholderia*		
SQ4g	*Burkholderia*		
SQ4h	*Burkholderia*		
SQ4i			x
SQ5a	*Burkholderia*		
SQ5b	*Burkholderia*		
SQ5c	*Burkholderia*		
SQ5d	*Burkholderia*		
SQ5e	*Burkholderia*		
SQ5f	*Burkholderia*		
SQ5f	*Burkholderia*		
SQ5g	*Burkholderia*		
SQ5i	*Burkholderia*		
SQ6a	*Burkholderia*		
SQ6b	*Burkholderia*		
SQ6c	*Burkholderia*		
SQ6d	*Burkholderia*		
SQ6e	*Burkholderia*		
SQ6f	*Burkholderia*		
SQ6g	*Burkholderia*		
SQ6h	*Burkholderia*		
SQ6i	*Burkholderia*		

Diversity of *Burkholderia* Isolates

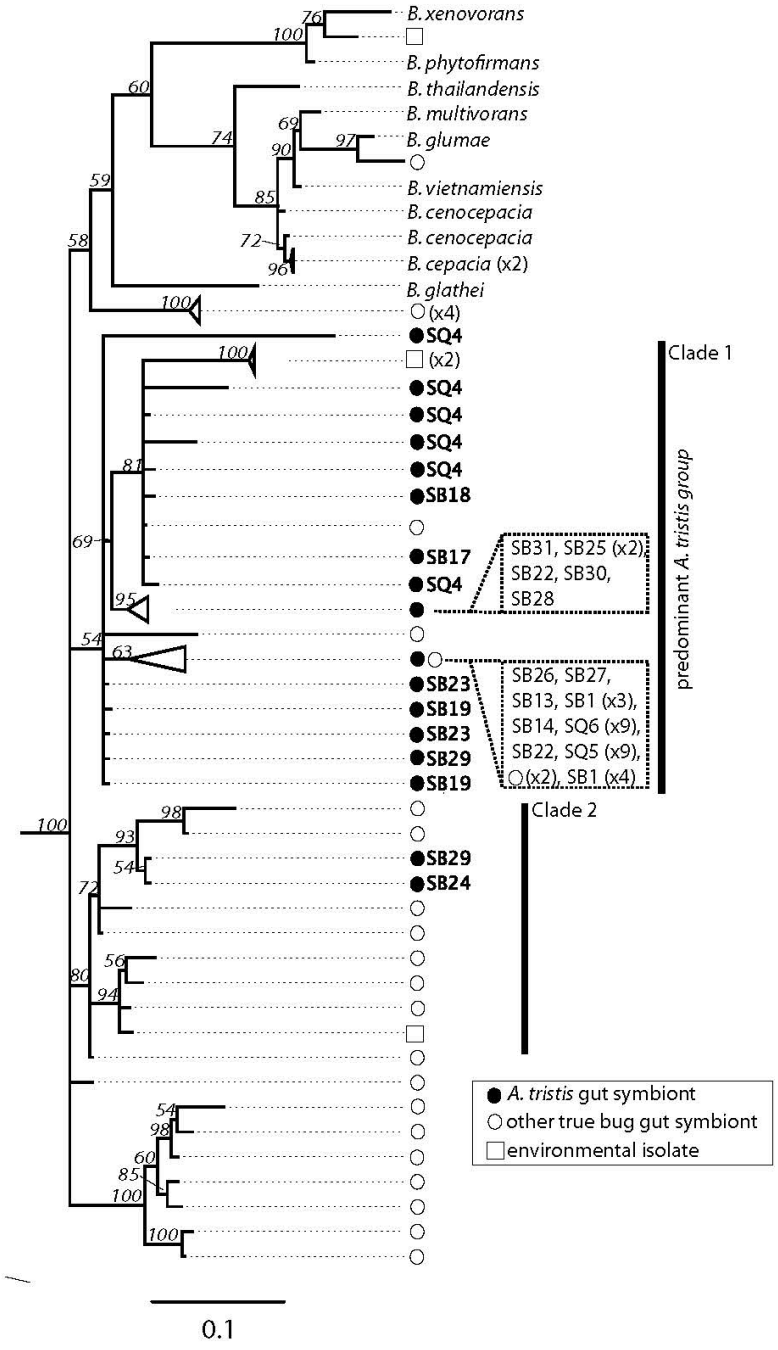

Figure 2: Bayesian analysis of *Burkholderia* isolates from *A. tristis* crypts on the basis of 16S rRNA gene sequences. *E. coli* was used as the outgroup taxon. Bayesian support values above 50 are above the node.

All *Burkholderia* samples isolated of sufficient length were included in a phylogenetic analysis with published *Burkholderia spp* found in other insects, plants, and the environment. The majority of the our isolates isolated from squash bugs grouped within a single monophyletic clade (Clade 1) that also contained *Burkholderia* isolated from the midgut crypts of other true bugs (Figure 2). Sequencing of additional loci revealed few differences between nineteen strains subjected to further analysis.

Discussion

The pervasiveness of *Burkholderia* isolated from wild-caught individuals suggests that squash bugs are engaged in a symbiosis with bacteria from the genus *Burkholderia* and that there exists some specificity in their association because the overwhelming majority of samples harbored *Burkholderia* and few crypt samples harbored other bacterial isolates. However, the presence of bacteria other than *Burkholderia* in dissected midgut crypts indicates that other microbial players may exert an influence, and non-culture dependent methods could reveal other bacterial strains in crypts.

Among our *Burkholderia* isolates, there was a high level of similarity, especially within Clade 1. Notably, however, genetically distinct strains of *Burkholderia* were occasionally isolated form wild-caught squash bugs. The implications of this diversity for host and symbiont fitness are yet unknown. These specialized associations suggests that either environmental or host mechanisms

select for a specialized association. The specialized nature of this association is consistent with a beneficial symbiosis.

Chapter 3

Pre-Establishment Partner Selection Mechanisms within the *Anasa tristis* Symbiosis

Introduction

The existence and proliferation of cooperation between two organisms has traditionally been difficult to understand given the potential for these systems to be infiltrated with cheaters that could take advantage of a benevolent partner while withholding some costly benefit for this partner (Sachs et al. 2010). Partner selection, the act of discerning between and actively choosing partners with which to interact, is one mechanism that can stabilize cooperative interactions by allowing an individual to associate with partners that provide benefits (Sachs et al. 2004). For example, fungus-growing ants have the ability to differentiate between symbiotic fungal cultivar strains, and these workers preferentially cultivate particular strains of fungus while avoiding others (Advani and Mueller 2006). We see similar interactions in *C. elegans*, where these nematodes are selectively attracted to certain species of beneficial bacteria and selectively avoid pathogenic strains (Kaplan et al. 2009; Chang et al. 2011).

Having answered the question, "Is there diversity in the *Anasa tristis* – *Burkholderia* symbiosis?" we now turn our attention to asking, "How does this diversity come about?" and "What selective pressures govern the diversity of these associations?" In systems with horizontal symbiont transmission, like the system

examined in this thesis, partner selection mechanisms would be expected to be important in selecting for beneficial symbionts. Hosts may impose selection actively, passively, or with some combination of active and passive mechanisms. Examples of active selective mechanisms include plant hosts preferentially seeking out particular strains of mycorrhizal fungi that provide nutritional benefits, and wasps selectively applying bacteria rich secretions to offspring when certain strains colonize (Kaltenpoth et al. 2014; Kiers et al. 2011). This mode of selection may also be refereed to as pre-establishment selection given that selective pressures are being exerted prior to bacterial uptake and establishment. Passive selective mechanisms are those in which host physiological processes limit growth of some potential partners but not others, or those in which competition between potential partners limits proliferation of some but not others, and include selection that is due to host gut lumen composition, gut pH, and establishment dynamics within the host gut.

Here, I study one potential mechanisms of active selection by *Anasa tristis*. I aim to determine if *Anasa tristis* preferentially feeds on certain strains of *Burkholderia* bacteria through no choice feeding experiments. I find little indication that the likelihood of feeding by an individual is impacted by the strain to which it is exposed, suggesting that other mechanisms must play a role in dictating specificity within this insect-*Burkholderia* association.

Methods

Egg Sterilization

Anasa tristis eggs laid in the laboratory were collected and pooled into a 1.5 ml tube. 500 µl of 70% ethanol was added to the tube and allowed to stand for two minutes with mild agitation. The ethanol was removed, and 500 µl of 10% bleach was added. The bleach was allowed to stand for two minutes with mild agitation. The bleach was removed, 500 µl of autoclaved water was added to the tube, and the water was allowed to stand for two minutes with mild agitation. The contents of the tube was then poured out onto a Kim wipe and placed into a small plastic bug cage cleaned with 70% ethanol.

Squash Bug Starving

Each day newly hatched first instars were placed with crookneck squash fruit, which had been sprayed with seventy percent ethanol and wiped with a Kim wipe, into a box containing bugs of the same age, measured in days post hatching. Twenty-four hours after these squash bugs molted into the second instar, these bugs were isolated without food for 24 hours.

Squash Bug Feeding

Starved second instars were placed individually in small Petri dishes with 0.5 µl drops of either a bacterial solution or water. The bacteria solutions included the squash bug derived strains SQ6c and SQ4a, the broad-headed bug derived strain BHJ32i (another species of insect that forms a symbiosis with *Burkholderia*), and a strain of *Burkholderia* isolated from soil, SMT4a. While both squash bug derived strains appear within Clade 1, they are genetically distinct and supported within

separate internal clades (Chapter 2, Figure 2). All bacterial solutions were grown in Luria Broth (LB) media and diluted with water to a working concentration of 2 X 10^7 CFU bacteria per ml of solution. Each bug was given four drops of the same solution, and in the event that the drops were not probed before they dried out, the bugs were allowed two more applications of the four drops. The number of individuals that fed was recorded.

Overall, three hundred and forty six squash bugs were divided up between multiple trials and allowed to either feed on or avoid either one of the four *Burkholderia* strains (SQ4a, SMT4a, SQ6c, BHJ32i) or sterile water. Sixty-eight individuals were given the opportunity to feed on strain SQ4a, eighteen individuals were given the opportunity to feed on strain SMT4a, one hundred sixty-four individuals were given the opportunity to feed on strain SQ6c, thirty-seven individuals were given the opportunity to feed on strain BHJ32i, and fifty-nine individuals were given the opportunity to feed on sterile water, the negative control.

Statistical Analyses

In the first analysis, all feeding proportions were analyzed using a Fisher's exact test, and then feeding on each bacterial strain was compared individually to feeding on water, again using the Fisher's exact test and a Bonferroni correction. All analyses were conducted in the statistical package R v2.13.

Results

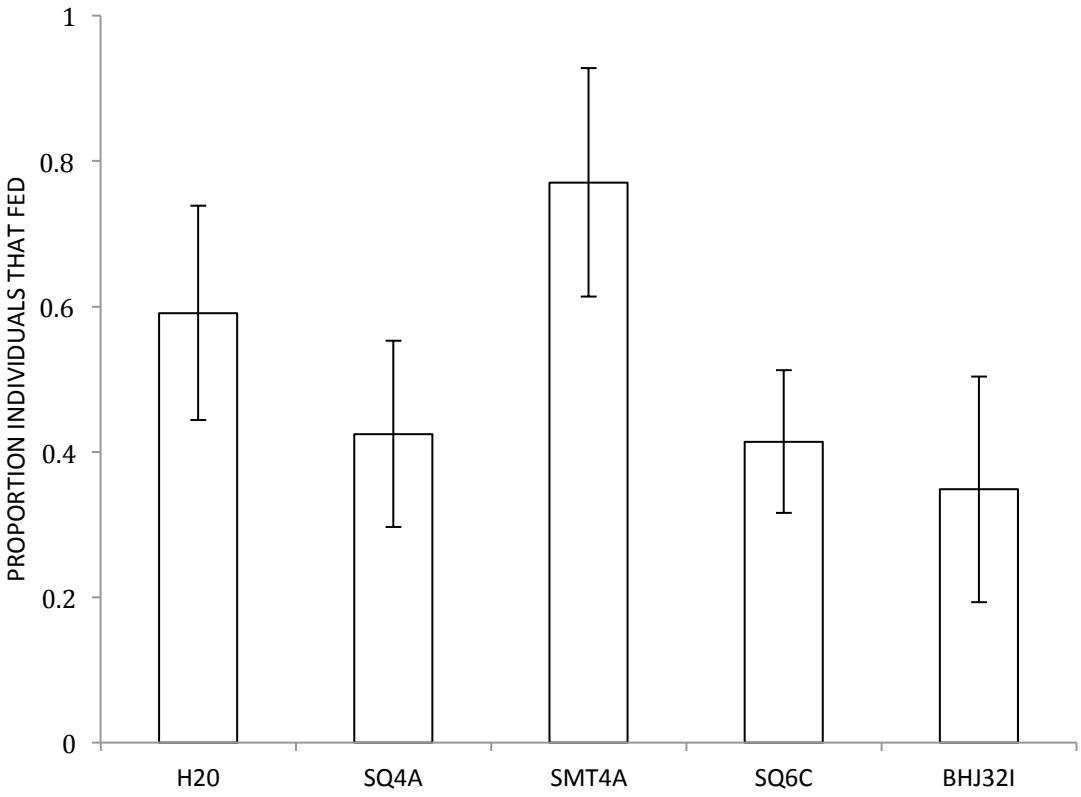

Figure 3. Proportion of individuals that fed within each bacterial treatment, averaged across feeding trials. Those trials with four or fewer bugs per trial were excluded. Error bars represent +/- s.e.m..

When we consider all trials compared to each other, we see that there is no significant difference in the proportion of individuals that fed in no choice trials based on the treatment they were allowed to feed upon (Figure 3). However, this difference is only marginally insignificant (Fischer's exact test, p=0.06355). When comparing each treatment individually to the water control, the proportion of individuals that fed on SQ4a was significantly lower than the proportion that fed on water (Fisher's exact test, p = 0.012). After Bonferroni correction, the likelihood of feeding on SQ6c was marginally non-significantly higher than the likelihood of feeding on water (Fisher's exact test, p = 0.02). The proportion that fed on BHJ32i

and SMT4a did not differ significantly from the proportion that fed on water (Fisher's exact test, $p = 0.29$ and $p = 0.78$ respectively).

Discussion

The significant differences in proportion that fed on SQ4a and SQ6c from the proportion that fed on the negative control, sterile water, provides some, limited evidence that individuals may prefer feeding on certain bacteria. This finding is consistent with a model of active partner choice, in which selection against or for different strains of symbiont occur prior to establishment. Implicit in this active partner choice is a mechanism for host discrimination of potential symbiont strain. Little is currently known about the signals that squash bugs respond to when discriminating various strains of symbiont. One limitation of this experiment is the lack of choices that hosts have when confronted with a strain of bacteria. In this experiment, hosts may choose to feed on a particular strain or to not feed at all. Building upon this work, future experiments should allow hosts the option of feeding upon multiple strains of symbiont.

Chapter 4

Differential Fitness Benefits of Strains of *Burkholderia* on *Anasa tristis*

Introduction

Endosymbiotic interactions, common throughout many systems, may be stabilized through multiple selective mechanisms. If one or both partners benefit from these interactions, selection would be expected to be a stabilizing force. Hosts and symbionts may act antagonistically, with either symbionts acting as parasite or with hosts trapping beneficial endosymbionts, or cooperatively, with both partners benefiting from the interaction. It is important to note, however, that the study of the effects of symbiotic interactions should not be seen through a binary of symbiont-positive and symbiont-negative. A more appropriate analysis recognizes the diversity of symbionts and explores the possible differential impacts of this diversity. These benefits can be seen either through the lens of the host or the symbiont (Garcia and Gerardo 2015), though measurements of size, weight, survival, fecundity, and other measures of fitness are often more easily made from the perspective of the host.

The squash bug system is an excellent system for studying symbiotic interactions. Aside from the amenability of these bugs to laboratory conditions, squash bugs, being born aposymbiotic, may be artificially fed particular strains of *Burkholderia*, their predominant symbiont type. Both survival and development can

be easily tracked upon controlled symbiotic infection, and thus we can examine the impact of genetically distinct strains of symbiotic bacteria on multiple host fitness traits.

We have shown that squash bugs have a symbiosis with *Burkholderia* (Chapter 2) and have indicated that squash bugs may preferentially feed on particular strains of *Burkholderia* (Chapter 3), but we do not know if *Burkholderia* affects the fitness of squash bugs and if this effect is positive or negative (Figure 1; Figure 2). Here, we explore the differential impact on host fitness of different strains of *Burkholderia,* and compare development and survival of Burkholderia infected bugs to those not provided a symbiotic partner. We conclude that one strain of *Burkholderia* significantly and positively impacts the survivorship of squash bugs, and that this strain also significantly and positively decreases the developmental time until fourth instar.

Methods

Rearing of the Squash Bugs

Squash bug eggs were pooled and placed into a 1.5 ml tube. In order to surface sterilize these eggs: 1) 500 µL of a 70% EtOH solution were added to this tube and the mixture was agitated for two minutes; 2) EtOH was removed, 500 µL of a 10% bleach solution were added to this tube, and this mixture was agitated for two minutes; and 3) bleach solution was removed, 500 µL of autoclaved H_20 were added to the tube, and this mixture was agitated for two minutes. The eggs in the tube were then poured out onto a Kim wipe and transferred into a clean plastic box.

Once these eggs hatched into first instar squash bug nymphs, they were transferred into another clean plastic box.

Burkholderia Infection

After first instar nymphs molted into second instars, squash bugs were starved for twenty-four hours and assigned to one of four infection treatments, with groups of eggs being partitioned across treatments to remove effects on host fitness of host genetic differences. These four infection treatments include SQ6c, a strain of *Burkholderia* isolated from squash bugs (Chapter 2, Figure 2), SQ4a, a genetically distinct strain of *Burkholderia* isolated from squash bugs (Chapter 2, Figure 2), SMT4a, a strain of *Burkholderia* isolated from the soil near lespedeza, a plant known to support other pentatomomorphans, and a sterile H_20 control. Each strain of *Burkholderia* being tested was labeled with green fluorescent protein (GFP) with a plasmid that also confers antibiotic resistance.

After the aforementioned starving, the second instar nymphs assigned to each treatment were placed into a plastic box with a small Petri dish filled with a solution of either bacteria and dye or water and dye. Each strain of bacteria was grown overnight in a Luria Broth solution and had its optical density at 600 nm measured. Using a conversion equation specific to each strain of bacteria we estimated the amount of sterile water and bacterial solution that would need to be added to each Petri dish in order to create a new solution of bacteria at a concentration of 10^7 cells per mL (Kikuchi et al., unpublished, updated by Gerardo Lab members). Sterile dye was added to this solution in order to provide a rough

indicator of squash bug feeding as this dye can be visually observed in the nymphs when they are placed under strong light. Each Petri dish was covered with stretched parafilm that allowed squash bugs to probe this solution and become inoculated with bacteria or sterile water. These bugs were allowed to probe this solution over the course of twenty-four hours. The presence of *Burkholderia* in the desired concentrations in feeding treatments was confirmed by plating this bacterial solution. These squash bugs were then placed into crookneck squash fruit surface sterilized with 70% EtOH before being moved into experimental cages. Each trial of each treatment was placed into its own experimental cage. All data was collected blind, such that those collecting data did not known what treatment was assigned to what cage.

Confirmation of successful infection was done by sacrificing, crushing, and subsequently plating contents of crypts from ten percent of squash bugs inoculated during one trial of each of the treatments onto antibiotic resistant plates. For each bacterial treatment, all plates showed growth on antibiotic resistant plates, while plates made from bugs fed from the negative control, water, showed no growth. Additionally, all squash bugs that become adults will be dissected and have their midgut crypts crushed and plated onto Luria Broth plates and antibiotic plates with the promoter IPTG to facilitate confirmation of GFP-labeling of isolated bacteria. Preliminary data on a subsample of adults dissected and plated illustrate that all bacteria-fed adults examined harbor antibiotic resistant bacteria that express GFP in their midgut crypts. These data provide evidence that squash bugs can be inoculated with particular strains of *Burkholderia* and that these strains can form

established communities within squash bug midgut crypts that are carried through different stages of instar development and into adulthood.

Experimental Cage Creation

The experimental cages provided squash bugs a sterile environment within which they were able to feed off of a crookneck squash plant, grown hydroponically. Each cage was comprised of an autoclaved mesh box with a small hole in its base that contains an autoclaved pot with autoclaved perlite (a soil stand in used in hydroponic systems) that extends into a separate, autoclaved nutrient water container filled with a dilution of 20 mL of Botanicare Pure Blend Pro Grow 3-2-4 in 4L of UV-C treated, filtered water. A crookneck squash plant was grown in each cage for two weeks before squash bugs were allowed to feed on the plant.

Crookneck squash seeds were surface sterilized by being immersed in 100% bleach and shaken for three to five minutes. These seeds were then rinsed with autoclaved H_20 and dried before being germinated in a Petri dish inside of an autoclaved coffee filter moistened with autoclaved water. Those seeds that germinated were then planted within the perlite of an experimental cage. Crookneck squash plants were grown in sixteen hours of full-spectrum light per day at 28° C and 75% humidity. Approximately every three weeks, the squash plants were pruned and large leaves were cut to prevent overcrowding. To provide evidence that these surface sterilized seeds do not harbor *Burkholderia* internally, and thus act as a reservoir, three crookneck squash seeds were surface sterilized, then crushed and plated onto Luria Broth media. The single colony that grew was

extracted, and its DNA was amplified via polymerase chain reaction using 16S primers and sequenced via Sanger sequencing. The resulting DNA sequence indicated this bacterium to be from the genus *Bacillus.*

Data Collection

Each data after feeding, the number of squash bugs at each instar stage within each experimental cage was recorded into a spreadsheet. Squash bug carcasses and molts were removed and noted as they were observed. As these squash bugs developed into adults, these adults were isolated and starved for twenty-four hours. Adult mass and length were then measured and any notes on its appearance were made. Adults were then immersed in 100% EtOH for four minutes and dissected within a pool of filter-sterilized Carlson's solution, a solution that allows bacteria to sustain themselves without extensively proliferating, using sterile instruments. The midgut crypts were isolated and stored in filter-sterilized Carlson's solution. These mixtures were homogenized using a sterile pestle and agitated using a vortex. This homogenized solution was then plated on a mixture of antibiotic and regular Luria Broth plates, both with IPTG. Currently, not all squash bugs have matured to adulthood, thus data presented focuses on development not to adult but to the fourth of the five nymphal instars.

Statistical Analysis

Survivorship was analyzed using a Cox PH model, and days from feeding until fourth instar were analyzed using the Kruskal-Wallis rank sum test, both within the statistical package R v2.13.

Results

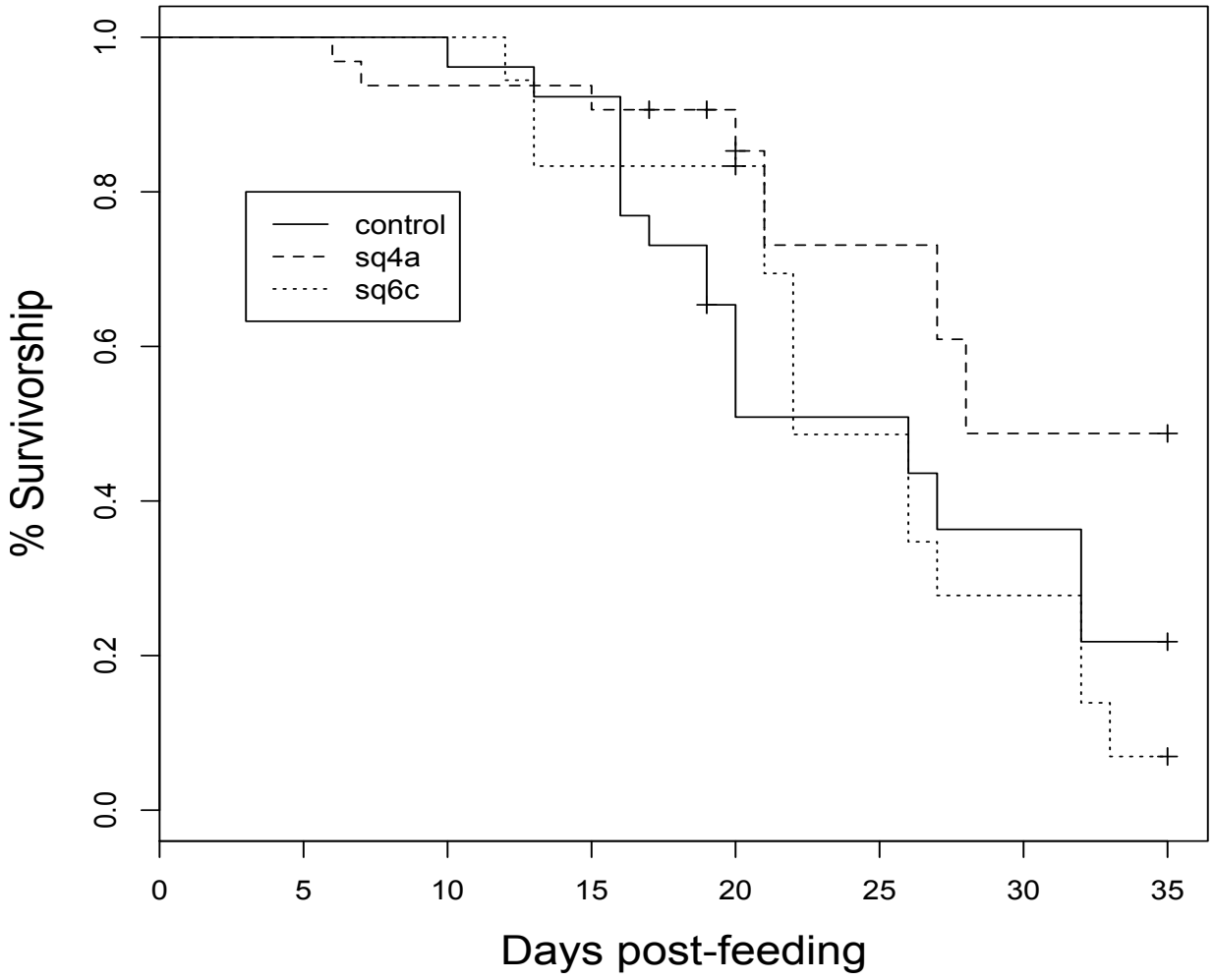

Figure 4. Percent survivorship over time as measured from date of feeding until the ending date of the experiment. Treatment SMT4a, containing only two individuals, was excluded from this analysis.

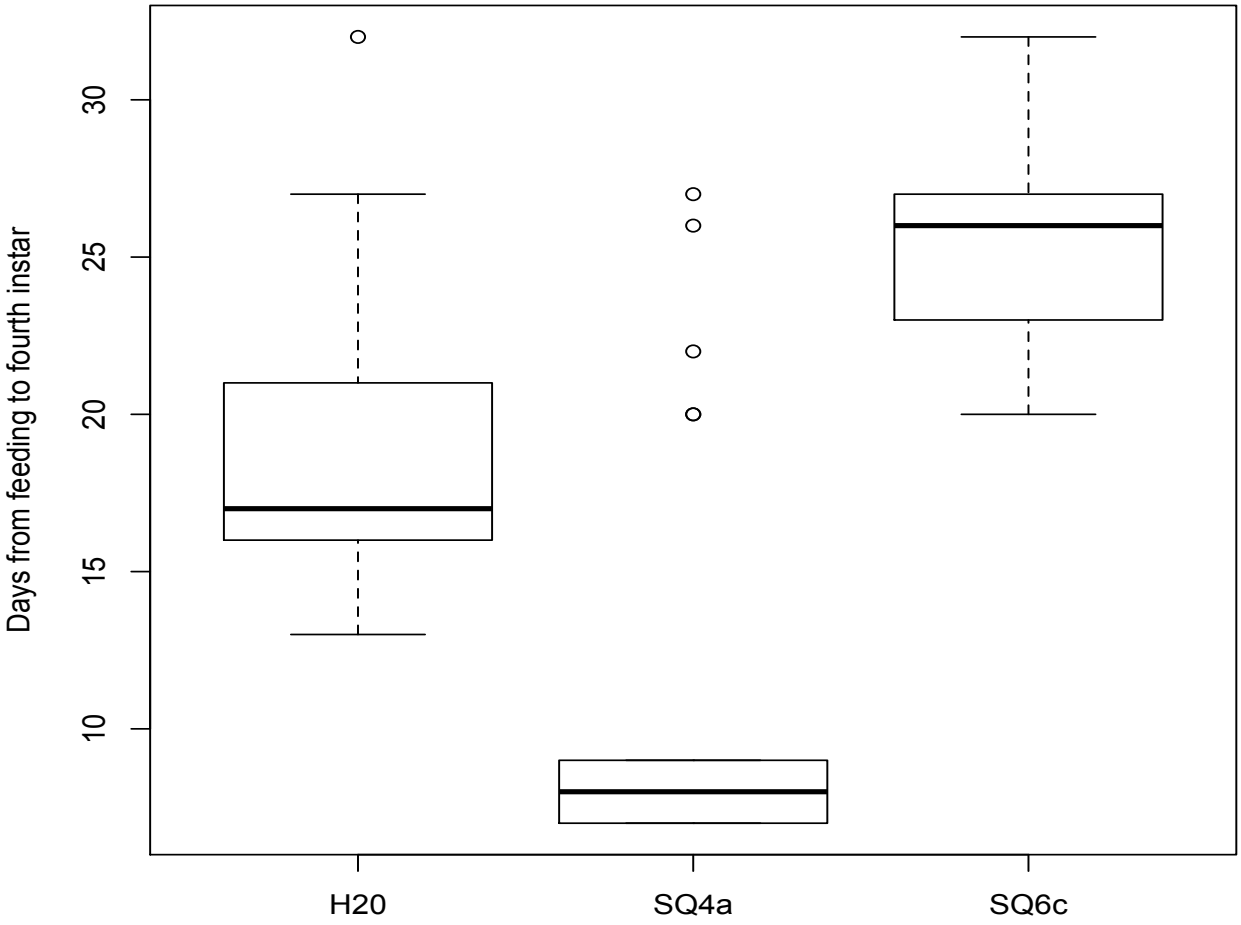

Figure 5. Time in days from date of feeding until squash bugs in different treatments reached fourth instar. Again, treatment SMT4a, containing two individuals, was excluded from this analysis.

Excluding those that died before being moved into the experimental boxes, eighteen squash bugs were inoculated with the SQ6c strain of *Burkholderia*, thirty-two squash bugs were inoculated with the SQ4a strain of *Burkholderia*, two squash bugs were inoculated with the SMT4a strain of *Burkholderia*, and twenty-five squash bugs were inoculated with the sterile H_20 control. Given the low number of bugs inoculated with the SMT4a treatment, this treatment was excluded from survivorship and developmental time analyses.

Overall, feeding treatment had no significant impact on survival (Cox PH analysis, p=0.07; Figure 4). However, squash bugs that fed on SQ4a had significantly higher survival than those that fed on water (p=0.05). This trend becomes visually apparent after day twenty in Figure 4 and becomes more pronounced as the experiment continues. While this experiment does not track individual bugs through their development, it is possible to tease apart the number of days until a given instar. The feeding treatment had a significant impact on the number of days it took individuals to reach the fourth instar post-feeding, with squash bugs fed SQ4a taking a significantly shorter period of time to develop to fourth instar (Kruskal-Wallis rank sum test, p=5.127e-5; Figure 5).

Discussion

The differences in survivorship and time to fourth instar support the differential fitness effects of different strains of symbiont on host fitness. Those squash bugs infected with SQ4a not only had higher survivorship over the course of this experiment, but also took less time to develop into fourth instars. Both of these findings indicate fitness benefits that are conferred onto the host, thus illustrating the beneficial impact of some *Burkholderia* strains on squash bug host fitness. Of note though, is that this fitness advantage is entirely strain dependent, with SQ6c not conferring benefits upon its squash bug hosts in terms of increased survival and decreased development. This finding sheds light on the complexity of the squash bug symbiosis with *Burkholderia*, in which the effects of genetically distinct,

naturally occurring symbionts are strain dependent and must not be seen through a more conventional binary lens of symbiont presence and symbiont absence.

Chapter 5

Conclusions

The squash bug system, *Anasa tristis*, is a great model with which to study environmentally acquired (horizontal) symbioses. Environmentally acquired symbioses are particularly of interest because out of the enormous diversity of the possible interactions and associations that may occur, certain associations persist. Here, we show that *Anasa tristis* does have a symbiosis with bacteria from the genus *Burkholderia* that includes multiple strains of *Burkholderia*. We also provide limited evidence that hosts actively discriminate in their decisions to feed on different stains of symbiotic bacteria in comparison to water. Finally, we show that different strains of *Burkholderia* illustrate alternative benefits to their hosts in total survivorship and in time through developmental instars. Paradoxically, the strain of *Burkholderia* that squash bugs prefer to feed on less than the negative control, water, is the same strain that increased survivorship and decreased development time. This finding was unexpected and underpins the importance of continued scholarship within the squash bug system. Future work in this system should further examine partner choice through experiments with that allow hosts to choose between strains of symbiont, examine fitness benefits or costs from the perspective of the symbiont, discern what governs establishment and persistence of a bacterial symbiont within a host exposed to multiple strains of symbiont, and elucidate how certain strains positively impact the fitness of their host more than other strains.

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