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Zayir Malik April 4, 2012

A Study of the *Alydus tomentosus-Burkholderia* Symbiont Interaction and *Burkholderia* Acquisition

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Abstract

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Alydus tomentosus, broad-headed bugs, house their symbiotic bacteria, *Burkholderia*, within their gut. While symbionts have been shown to confer positive benefits to the host in other broad-headed bug species, the effect of *Burkholderia* acquisition on *A. tomentosus* individuals remained unstudied. By monitoring the lifespans of multiple individuals, I show that *A. tomentosus* individuals that acquire *Burkholderia* survive at a higher rate than those that remain sterile. I also investigate the timing of bacterial acquisition. Through an experiment in which I expose *A. tomentosus* nymphs to *Burkholderia* during only one instar of their life, I demonstrate that *A. tomentosus* individuals are most likely to uptake their symbiont during their third instar. Finally, through a choice assay, I show that *A. tomentosus* do not actively seek out *Burkholderia*.

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Introduction

Nearly every organism comes into contact with bacteria on a regular basis. It is presumed that most of these bacteria are harmless, however some are inevitably harmful pathogens. A few may actually provide benefits for animals and other organisms that acquire them. In some cases, organisms will house certain bacterial species or groups in order to take advantage of whatever positive effect they may have; the bacteria, in turn, may benefit from this interaction because the host body provides a stable, persistent, nutrient-rich environment. This type of relationship is deemed "symbiosis," which translates from Greek as "living together." More specifically, the relationship between a host and an internal bacterial symbiont is termed endosymbiosis (Taylor, 2012). There are many documented cases of this phenomenon occurring for many hosts, including marine life, plants and even complex mammals (Margulis, 1991). However, the relationship between microorganism and host is most well studied in insects (Bourtzis, 2003).

For the relationship to be established, three criteria must be fulfilled. First, the host and the bacterial symbiont must come into physical contact. Second, the bacteria must be able to infect the host, overcoming any host defenses in the process. Finally, the relationship between the host and the microbe must be maintained over time (Combes, 2004). To allow the relationship to be maintained, the host organism must permit the microbe to survive and flourish within its body. To facilitate this, many organisms have specific organs or locations in which they house the bacteria. In certain species of squid, this organ is highly specialized to only house its specific symbiont (Schleicher, 2011). In

many insects, these organs are called crypts or ceca and they are generally located within the gut of the organism (Figure 1) (Dasch, 1984).

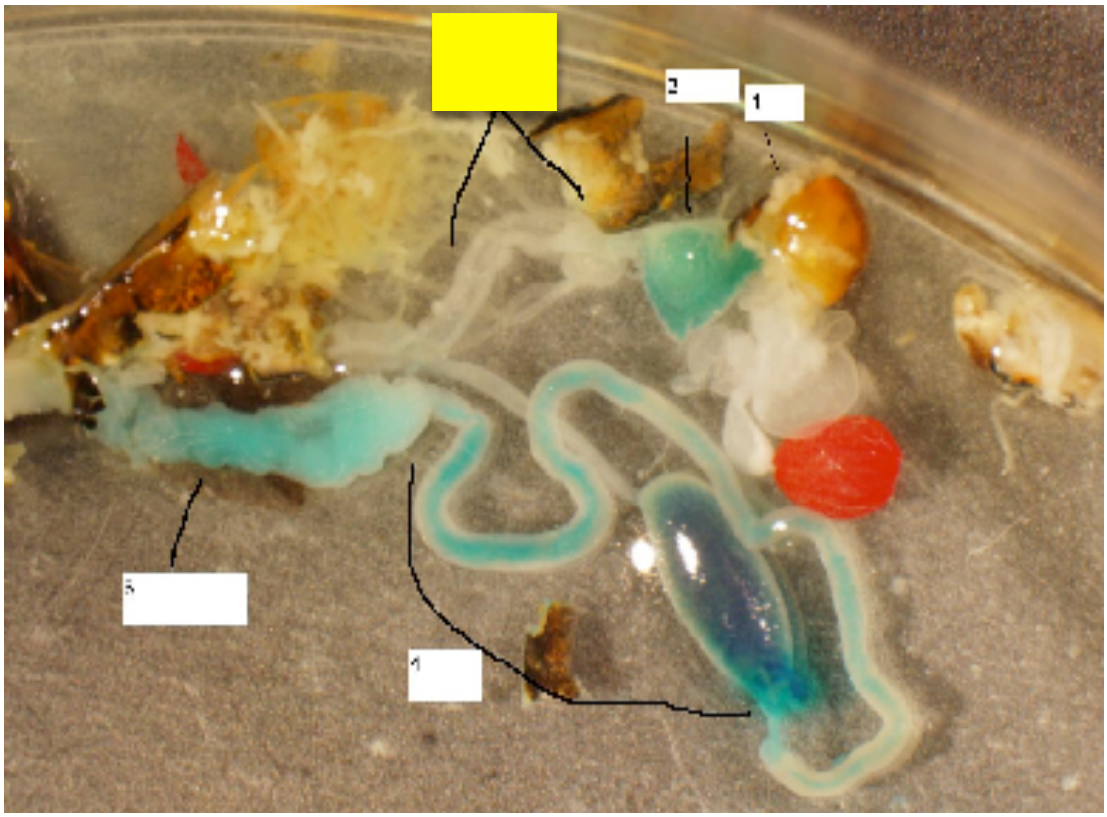

Fig. 1. Dissected gut of a broad-headed bug. The section marked by a yellow box is composed of the bugs' bacterial crypts.

The possible effects of a bacterial symbiont on its host vary greatly. On one hand, the symbiont can have negligent effects and act similarly to a parasite. On the other side of the spectrum, a microbe can drastically increase the reproductive fitness and/or survival of an organism (Hosokawa, 2006). In many relationships, these fitness benefits are mitigated through microbial symbionts that produce certain nutrients that the host then utilizes. A classic example of this is the legume-*Rhizobia* interaction, in which the bacteria produce fixed nitrogen to be used by the host plant; without these beneficial bacteria, plant growth is significantly retarded (Mortier, 2012). In addition to changes in

fitness, the presence or absence of a microbe can alter the behavior of an organism. For example, in *Megacopta punctatissima*, a stinkbug, the absence of the symbiotic bacteria causes nymphs to wander about in search for the appropriate symbiotic microbe. Upon finding it, they cease their wandering almost immediately (Hosokawa, 2008). In a different example, pea aphids can be infected with a symbiont that bestows increased resistance to parasitism. When infected with this symbiont, *Hamiltonella defensa,* aphids reduce their evasive maneuvers in the presence of a parasitic wasp (Dion, 2011).

In most mutualistic relationships, if the symbiotic organisms are removed from each other, they both suffer. In certain cases, this separation can be fatal; in others it may have a much lesser effect (Tada, 2011). In the former scenario, the symbiosis is said to be obligate, meaning that the symbiosis is required for most basic functioning. *Buchnera* in aphids and *Wigglesworthia* in tsetse flies both are great examples of obligate insect-microbe symbioses (Aksoy, 2003 and Baumann, 1995). In facultative symbioses the host and the microbe do not necessarily need one another, but at least one partner is better suited for survival together. Regardless of whether a symbiosis is obligate or facultative, there is a clear advantage for a host to pass on beneficial bacteria from generation to generation. There are a few ways that this can be accomplished. The mother host can expose her offspring to the bacteria while they are developing in her body. This manner of passing on the microbial symbiont is called transovarial transmission (Braendle, 2003). Another way to ensure the successful transmission of bacteria is by egg smearing, a tactic in which the parent somehow contaminates its eggs with bacteria after laying them. Alternatively, the parent can leave a deposit of microbe-infested feces for the nymphs to probe upon hatching; this habit is known as coprophagy (Kikuchi, 2010). Symbionts that

are absolutely essential for the survival of the host are often transmitted transovarially because of the high fidelity of this mode. However, in a few insect species, such as the broad-headed bug *Riptortus clavatus*, symbionts—though beneficial for growth and possibly reproduction—are not absolutely vital for survival; they are also not vertically transmitted from parent to offspring. Instead, in this system, each generation the nymphs hatch without any bacteria and later pick up the microbe from the environment. It appears that these bugs are more likely to pick up the bacteria at a specific stage in their lifecycle (Kikucki, 2007). It is unknown whether other broad-headed bug species also acquire their symbiont from the environment in a similar manner.

Alydus tomentosus is a broad-headed bug that is a member of the Heteroptera insect suborder. This specific suborder consists of over 38,000 species (Schuh, 1996). The insect is differentiated from its sympatric relatives (*A. conspersus* and *A. calcarotus*) by its color. *A. tomentosus* is generally the easiest to identify because of the jet-black color of its body (Figure 2). The bugs are known to feed on seeds of *Lespedeza* spp., common weedy legumes in much of the United States.

Fig 2. An *A. tomentosus* adult.

The life cycle of *A. tomentosus* is similar to that of many other insects. They are born quite small, measuring just a few millimeters. At this stage, they are known as first instars. Within a few days, they shed their exoskeleton in a process known as a molt. After they shed their exoskeleton, they are in the second instar. *A. tomentosus* individuals undergo four molts (and thus go through four instars) before they reach adulthood, at which time they are reproductively mature. The average time spent in each *A. tomentosus* molt has not been investigated previously, but for *R. clavatus*, a broad-headed bug that undergoes five molts before adulthood, bugs generally spend two to four days in each of the first four instars and five to six days in the fifth instar (Kikuchi, 2011).

The bacterial symbionts for *A. tomentosus* are bacteria in the genus *Burkholderia*. The symbiosis between these two is relatively unstudied, with no papers published that detail any aspect of this specific interaction. In two other broad-headed bug species, *R. clavatus* and *L. chinensis*, *Burkholderia* acts as the microbial symbiont that is housed within the crypts of the posterior gut of each species (Yoshitomo, 2005). Kikuchi *et. al.* (2007) found that *R. clavatus* individuals harboring the symbiont showed significant increases in body weight, body length, thorax width, and abdomen width. These findings suggest that there is a mutualism that exists between the two but that the relationship is not an obligate one since sterile bugs can still survive to adulthood. Surprisingly, rather than transmit the bacteria from mother to offspring, *R. clavatus* individuals generally acquire their bacteria from the environment within 2-8 days after hatching (Kikuchi 2011).

Here, expanding on previous work in other broad-headed bug species, I analyze the effects of *Burkholderia* on *A. tomentosus*. First, I investigate the relationship between

survival and the presence of *Burkholderia*. It has been seen in other insects that symbiont presence affects host growth and survival; I determine if this holds true for the *A. tomentosus-Burkholderia* system. Second, I confirm the mode of transmission in this system. Next, I explore the possibility that *A. tomentosus* nymphs are more able to acquire *Burkholderia* during certain instars. Finally, I look at the behavioral effects of symbiont acquisition on *A. tomentosus* individuals.

Methods

Collection. I collected *A. tomentosus* along with related broad-headed bug species from various sites in Georgia between the months of May and October 2011. The site most often visited was a patch of *Lespedeza* plants along the Stone Mountain Park Songbird Habitat Trail in Stone Mountain, GA. I also collected from Piedmont Park and Morningside Nature Preserve. To capture the insects, I trapped them individually in vials using either hands or a large net swung across the tops of the plants. The majority of the bugs caught were found dwelling on the upper regions of the *Lespedeza* plant, but some were found upon examination of the soil around the base of the plant. I rarely captured nymphs from the wild, but when I did, they were nearly all in the fourth instar. Younger bugs were never found; I suspect that they spend more time on the ground and are thus much harder to find. I also collected soil from each of these locations to be used in experiments. In addition, our lab cultured a strain of *Burkholderia* from this dirt and kept it alive by plating it on new LB plates each week. *Burkholderia* was grown at 25 degrees Celcius and then maintained at 4 degrees Celcius until needed.

Insect Rearing and Care. Mimicking the natural environment of *A. tomentosus*

was difficult in a laboratory setting. Following the model of Kikuchi *et. al.* (2007) for raising *R. clavatus*, I initially housed multiple insects in plastic boxes measuring approximately 7.5cm x 7.5cm x 2.8cm. They were raised along with a cotton ball soaked in a nutrient-rich solution containing L-cysteine, L-aspartic acid, and blue food dye (BHB solution), dirt from their environment (which served as their source for *Burkholderia*), and a combination of sterile peanuts and black-eyed peas to replace the *Lespedeza* seeds on which the bugs normally feed. Over time, I adjusted the protocol for care. To better facilitate the circulation of fresh air into the boxes, I punched holes in the top of the boxes using a soldering iron. I also eventually stopped using cotton balls and started using an organic sponge to reduce mold growth. Finally, I obtained *Lespedeza* seeds and fed the bugs a combination of seeds, peanuts, and peas. The bugs and boxes were stored in an incubator at 28 degrees Celcius and 50% humidity; they were exposed to light for 16 hours a day.

Experiment 1: Symbiont Acquisition Window. The premise of this experiment was to expose each individual to *Burkholderia* during a certain instar to test whether or not nymphs were more susceptible to symbiont acquisition during a certain period. From early June through July 2011, I monitored *A. tomentosus* eggs that were placed in a sterile plastic box. Before placement in this box, the eggs were sterilized. This was done via submersion of the eggs in a solution of 70% ethanol for 5 minutes followed by submersion in 10% bleach for 5 minutes, and finally submersion in filtered water for 5 minutes. Each day I removed the eggs that had hatched overnight and placed the new nymphs individually into new sterile boxes with a shelled peanut half and a cotton ball soaked with 1-2mL of BHB solution. I ultimately created 204 boxes with individual

nymphs. I exposed a cohort of individuals to *Burkholderia* (in the dirt from the wild) during only their first instar, another cohort was exposed only during the second, and another only during their third (Figure 3). Upon finding a shed exoskeleton in an individual's cage (indicating that a molt had occurred), I transferred that individual from a sterile cage to one containing a peanut half, supplemental BHB solution, and a small tray of dirt from the wild. After 48 hours in this environment, the individual was then transferred back into a sterile cage to live out its life. I also maintained positive and negative controls: the positive controls were exposed to dirt throughout life, and the negative controls remained in a sterile environment. For each individual, I also monitored lifespan and the time spent between each molt. I was unable to obtain sufficient nymphs to live to the fourth instar in a sterile environment, so I could not obtain data on whether or not the fourth instar insects were able to acquire bacteria. I also do not have survival data for individuals only exposed to bacteria in the fourth instar.

	1st Instar	2nd Instar	3rd Instar	4th Instar	Adult
1st Instar Cohort (n=27)	**Exposure** (n=10)	----------	----------	----------	----------
2nd Instar Cohort (n=30)	----------	**Exposure** (n=10)	----------	----------	----------
3rd Instar Cohort (n=18)	----------	----------	**Exposure** (n=10)	----------	----------
Negative Control (n=110)	----------	----------	----------	----------	----------
Positive Control (n=19)	**Exposure**	**Exposure**	**Exposure**	**Exposure**	**Exposure**

Fig. 3. Table outlining the symbiont acquisition window experiment. The first instar cohort was exposed to *Burkholderia*-infected dirt only during their first instar, the second instar cohort during the second instar only, and the third instar cohort during the third instar. Positive controls were always in the presence of dirt and negative controls remained in a sterile environment. Values under "Exposure" indicate number of PCR-verified nymphs that acquired *Burkholderia* during that time.

I used polymerase chain reaction (PCR) assays to confirm *Burkholderia* acquisition. I first sterilized each individual by soaking it in 70% alcohol for five minutes and then dissected each bug under a microscope. For larger specimens, I attempted to isolate the midgut bacterial crypts; for smaller bugs I simply removed appendages and isolated the entire gut. I then extracted the DNA from each specimen using a Qiagen DNA Extraction Kit. After isolating the DNA, I preformed a PCR using *Burkholderia* specific primers (Burk 16SR: GCTCTTGCGTAGCAACTAAG and Burk16SF: TTTTGGACAATGGGGGCAAC). I also used 0.2 μL of 5-prime Taq from a MasterTaq Kit. The PCR consisted of 35 cycles of a 30 second, 94 degree denaturation step followed a 1 minute, 55 degree annealing step and a final elongation step for 2 minutes at 72 degrees. Amplification was verified via gel electrophoresis (90V for approximately 40 minutes). Positive results displayed a bright band on the agarose gel at about 750 base pairs, confirming the presence of *Burkholderia*.

To analyze these data, I used the survival analysis package in R version 2.14.1. I also calculated the time spent in each instar and time to each molt to determine whether or not *Burkholderia* presence affected either; significance was verified by using t-tests to compare the mean times to and between each molt.

Experiment 2: Symbiont-Induced Behavior Modifications. If bugs must acquire bacteria from the environment, then they may actively seek out locations with those bacteria and preferentially feed on bacteria. To determine whether *A. tomentosus* showed a preference for *Burkholderia*-containing habitats, I took time-lapse photographs of nymphs moving within choice assay arenas. The choice assay consisted of two food sources: one sterile shelled peanut half and one shelled peanut half coated with

Burkholderia (Figure 4). I then set up a camera that captured images of the bugs' locations every 5 minutes. The location of the bug was scored as either on one of the nuts, near one of the nuts, or in a neutral location. I conducted 20 trials of which four were first instars, three were third instars, and 13 were second instars. Behavioral data were analyzed using a GLM with quasibinomial distributions in R.

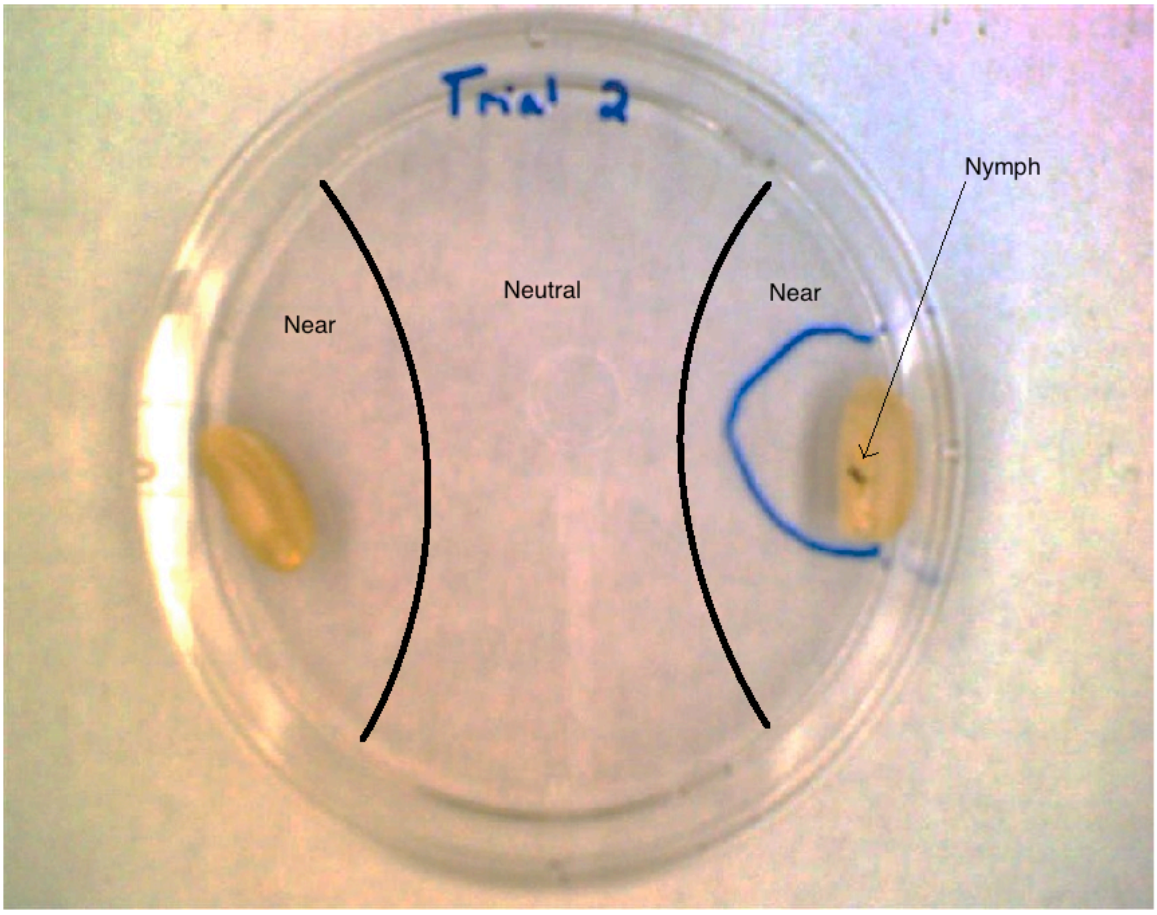

Fig 4. Choice-assay experiment. Peanut half with *Burkholderia* is marked with a blue outline. Sections for scoring are outlined.

Results

Symbiont Acquisition Window. Figure 5 shows the percent survivorship over time for each cohort of insects used in the symbiont acquisition experiment. The survival for

each of the groups after the experiment window (50 days) ended was: 14.8% for the first instar group (i.e., those bugs exposed to *Burkholderia* only in the first instar) (n = 27), 26.67% for the second instar group (n = 30), 16.67% for the third instar group (n = 18), 21.11% for the positive control insects exposed to *Burkholderia* their entire life (n = 19), and 8.33% for the negative control insects that were never exposed to *Burkholderia* (n = 110). Using a Coxph survival model (with censoring for bugs who lived beyond 50 days), I found that bugs exposed to bacteria in the second ($p < 0.001$) and third instar ($p < 0.01$) both showed a significant increase in survival compared to the negative controls. The first instar cohort, however, was not significantly different from the negative control ($p=0.26$). Comparing survival of all bugs that were determined (via PCR) to have obtained *Burkholderia* to survival of sterile bugs (Figure 6) revealed that there was a significant difference ($p < 0.05$) in survival; the sterile bugs survived at a rate of 9.49% whereas the bugs carrying *Burkholderia* survived at a rate of 17.78%.

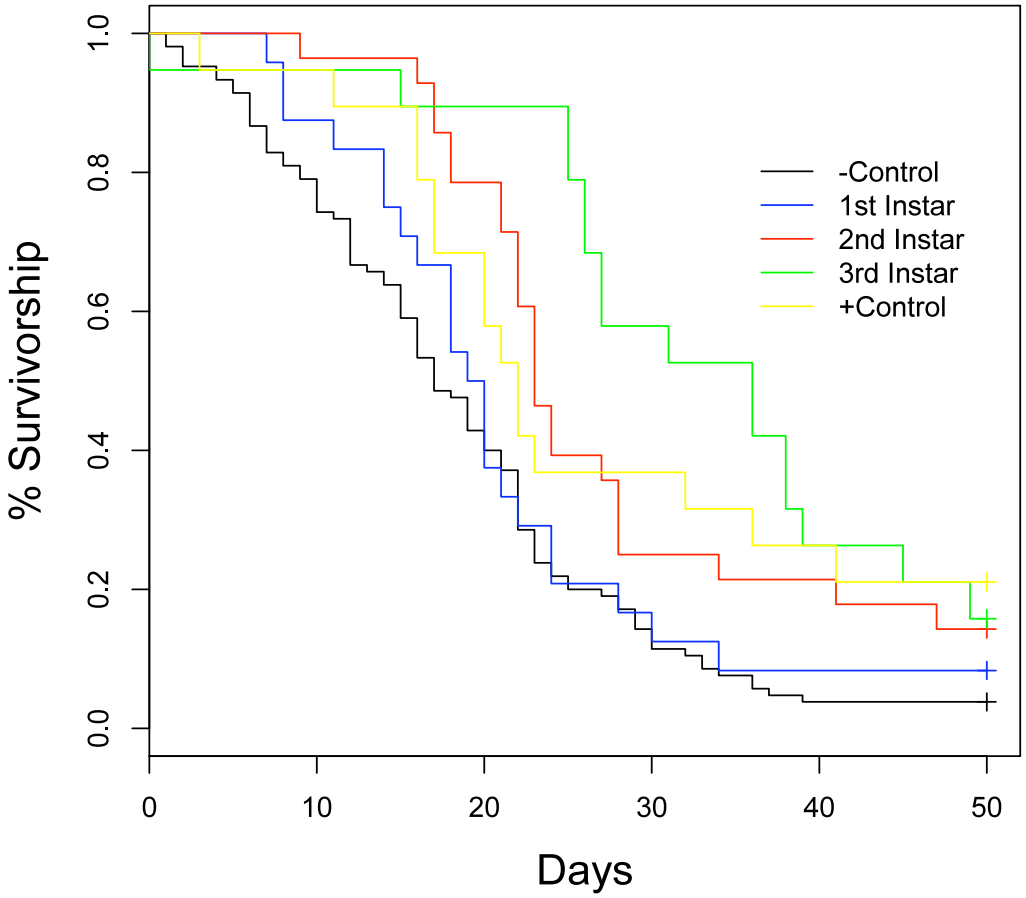

Fig 5. Percentage of bugs alive over time by exposure group. Second and third instar cohorts showed significant increases in survivorship as compared to the negative control.

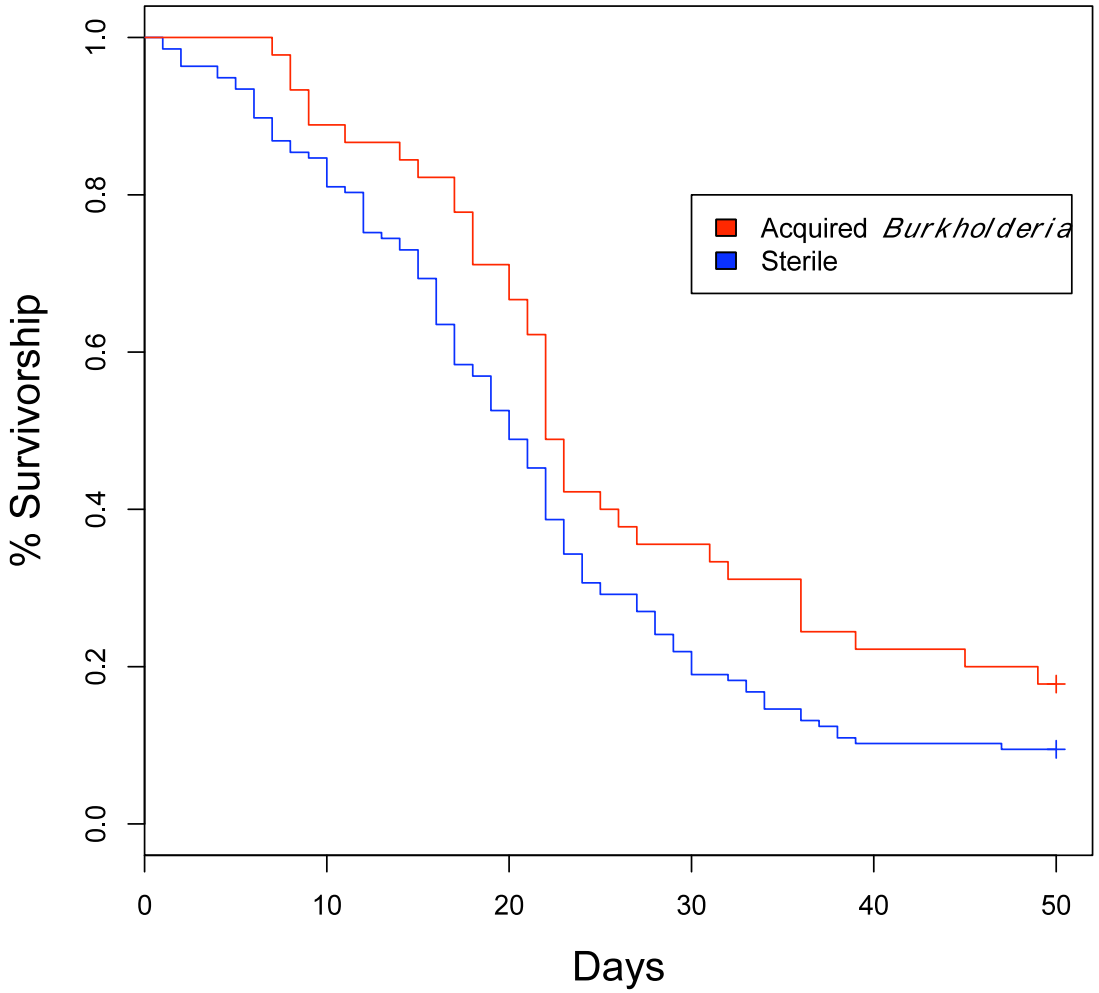

Fig 6. Percentage of bugs alive over time, with *Burkholderia* versus sterile. Bugs that had acquired the symbiont had significantly increased survivorship.

One of the main goals of the experiment was to determine if *Burkholderia* acquisition was limited to a certain instar. PCR analysis showed that, while the first and second instar cohorts acquired the bacteria, the third instar cohort did so at a significantly higher percentage. Forty two percent of first-instar-exposed bugs acquired the bacteria, and 44% of second-instar-exposed bugs acquired the bacteria; using a Welch two-sample t-test, I found that this difference was not significant ($p=0.9$). Within the third instar

cohort, 77% of bugs acquired the bacteria; this was significantly higher than the acquisition percentages for the first and second instars ($p<0.05$). Among the positive controls, 92% acquired *Burkholderia*, which was not significantly higher than the third instar cohort acquisition percentage ($p=0.3$). Less than 4% of negative controls were infected.

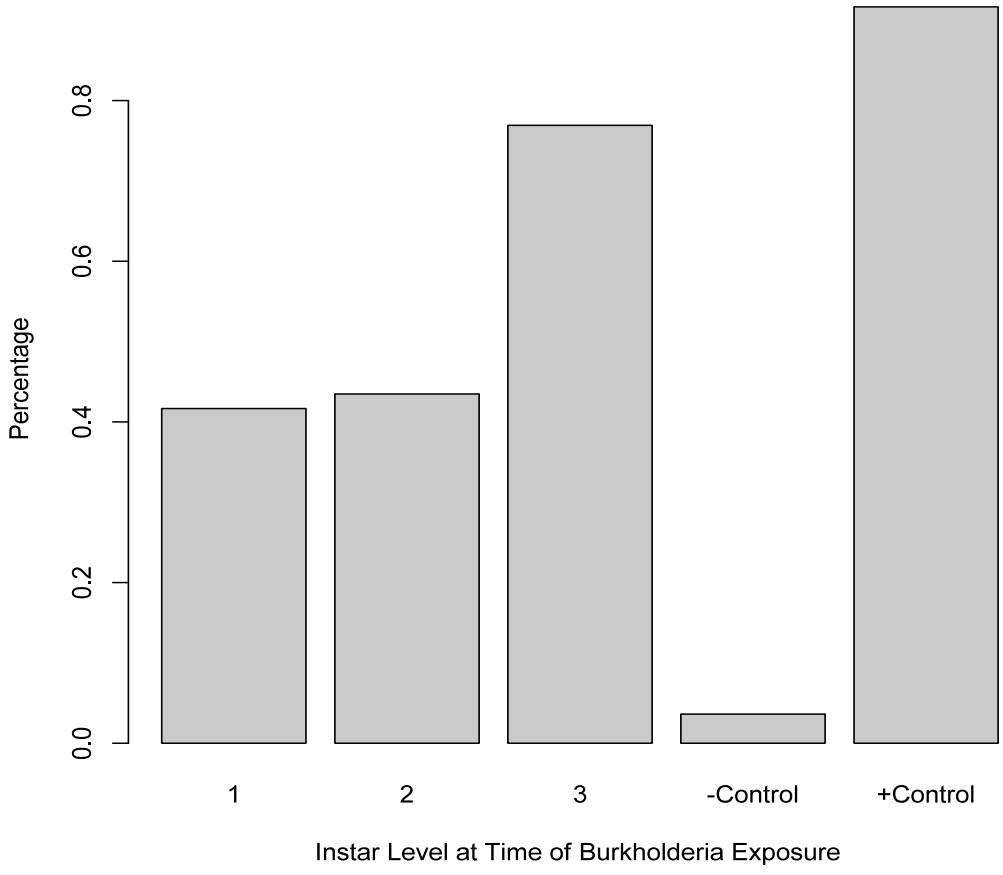

Fig 7. Proportion of each cohort infected with *Burkholderia* in symbiont acquisition experiment. Negative control bugs were never exposed to *Burkholderia* containing soil. Positive control bugs were always exposed to *Burkholderia* containing soil.

For each cohort, I also analyzed the time between each molt. To analyze these data, I found the mean aggregate days alive before a certain molt (*i.e.* time between birth

and molt 1, 2, 3, 4) for infected (n = 41) and sterile (n = 159) bugs (as confirmed via PCR). Using Welch two-sample t-tests, I found that the difference in time to each molt between the infected and sterile bugs was not significantly different for any molt number (first instar p=0.16, degrees of freedom=11; second instar p=0.37, degrees of freedom=12.5; third instar p=0.69, degrees of freedom=16; fourth instar p=0.96, degrees of freedom=3.1). Bugs that were infected during the first instar and those that were not took equally as long to reach the first molt. This holds true for the time elapsed before each different molt, as seen in Figure 8.

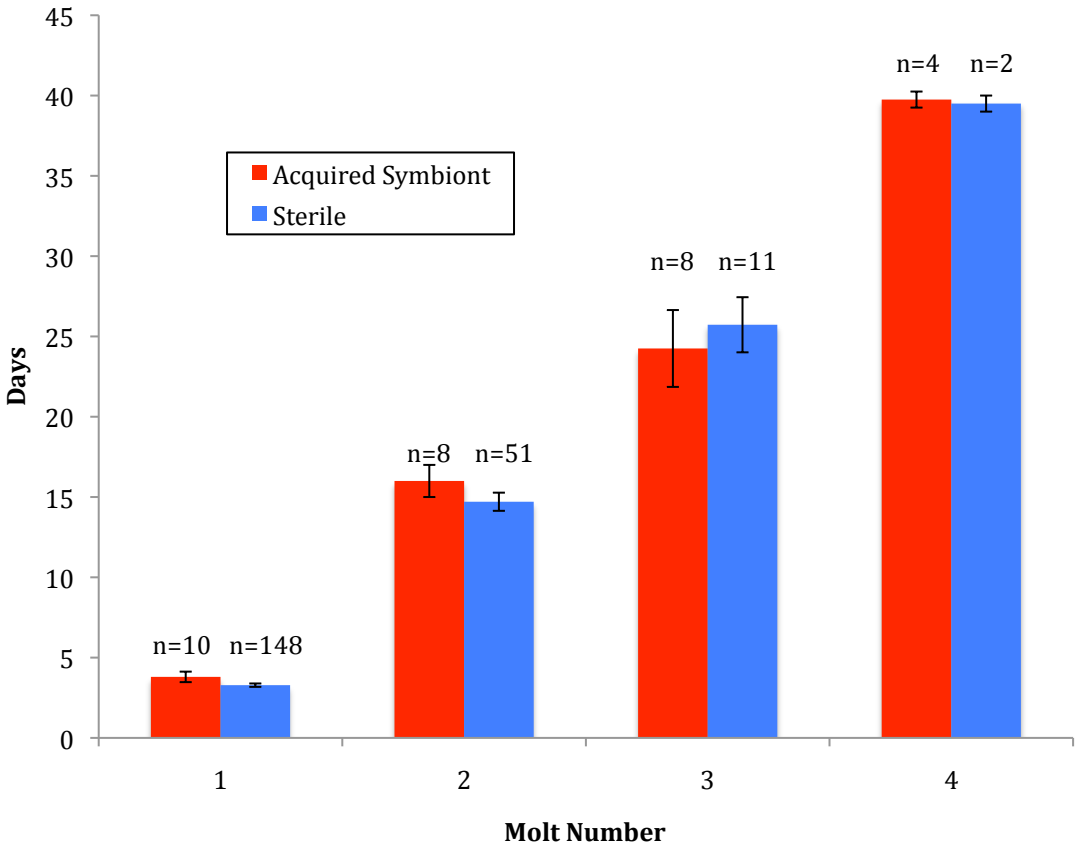

Fig 8. Comparison of mean aggregate time elapsed before each molt. There were no significant differences between infected and sterile insects. Error bars represent standard errors of the mean.

I also compared the time spent in each instar (*i.e.*, time between molts 1 and 2, 2 and 3, etc.) for infected versus sterile bugs. Again, the analysis with Welch two-sample t-tests showed that there were no significant differences between time spent in any instar for infected and sterile bugs (first instar p=0.16, degrees of freedom=11; second instar p=0.51, degrees of freedom=13.8; third instar p=0.74, degrees of freedom=11; fourth instar p=0.76, degrees of freedom=1.8).

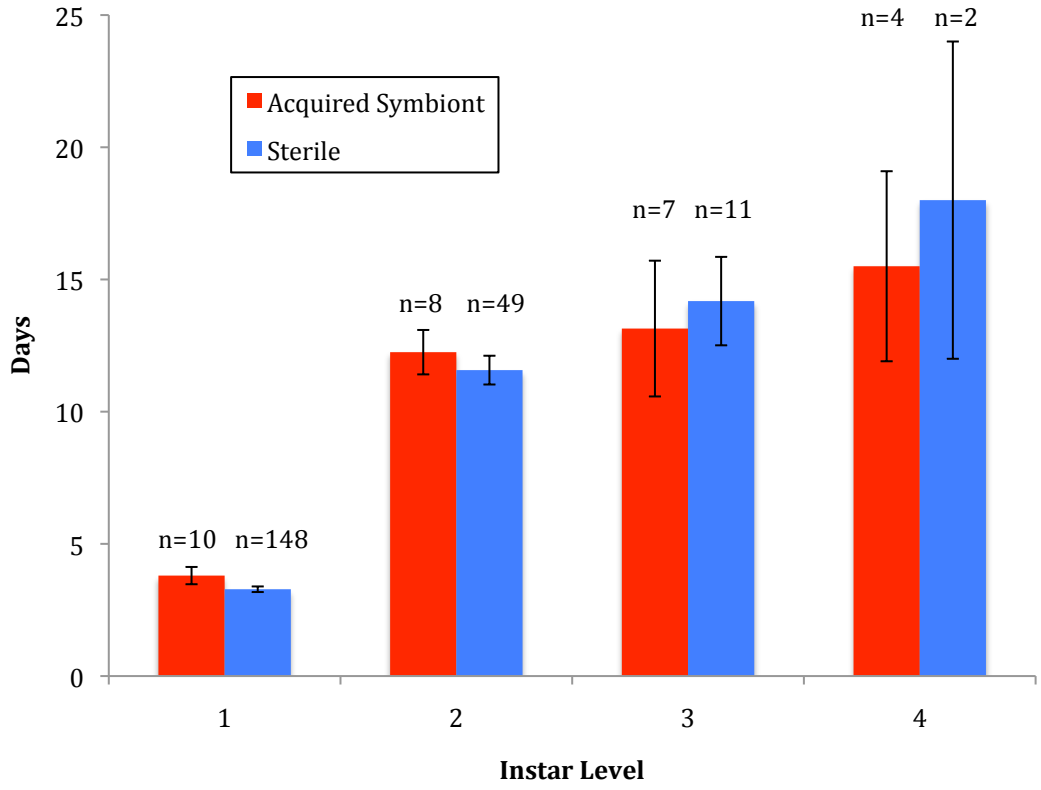

Fig. 9. Comparison of time spent in each instar for infected versus sterile bugs. There were no significant differences. Error bars represent standard errors of the mean.

Symbiont-Induced Behavior Modifications. In order to determine whether insect behavior was altered by the presence or absence of *Burkholderia*, I analyzed my data in three distinct ways. I first analyzed the proportion of total time spent on or near the *Burkholderia*-covered peanut relative to total time spent on or near either peanut ("total time"). To attempt to remove the affect of the bugs falling asleep for long periods of time, I also analyzed the proportion of time spent on or near the *Burkholderia*-covered peanut relative to the total time spent on or near either peanut, shortening long stretches in the same place to 10 time periods (50 minutes)("short time"). Finally, I analyzed the proportion of visits to regions on or near the *Burkholderia*-covered peanut relative to total visits ("visits"). All observations of the bugs on or near neither peanut (*i.e.,* in the neutral zone) were excluded. Figure 10 indicates the proportion of time spent on or near the *Burkholderia*-peanut relative to the bacteria-free peanut. There was no significant deviation of these proportions from a neutral expectation of 0.5, suggesting that the bugs exhibited no preference for being on or near either peanut in the choice assay ("total time": $p = 0.35$; "short time": $p = 0.92$; "visits": $p = 0.81$). Analyses of proportions based on only observations on but not near the peanuts did not alter significance.

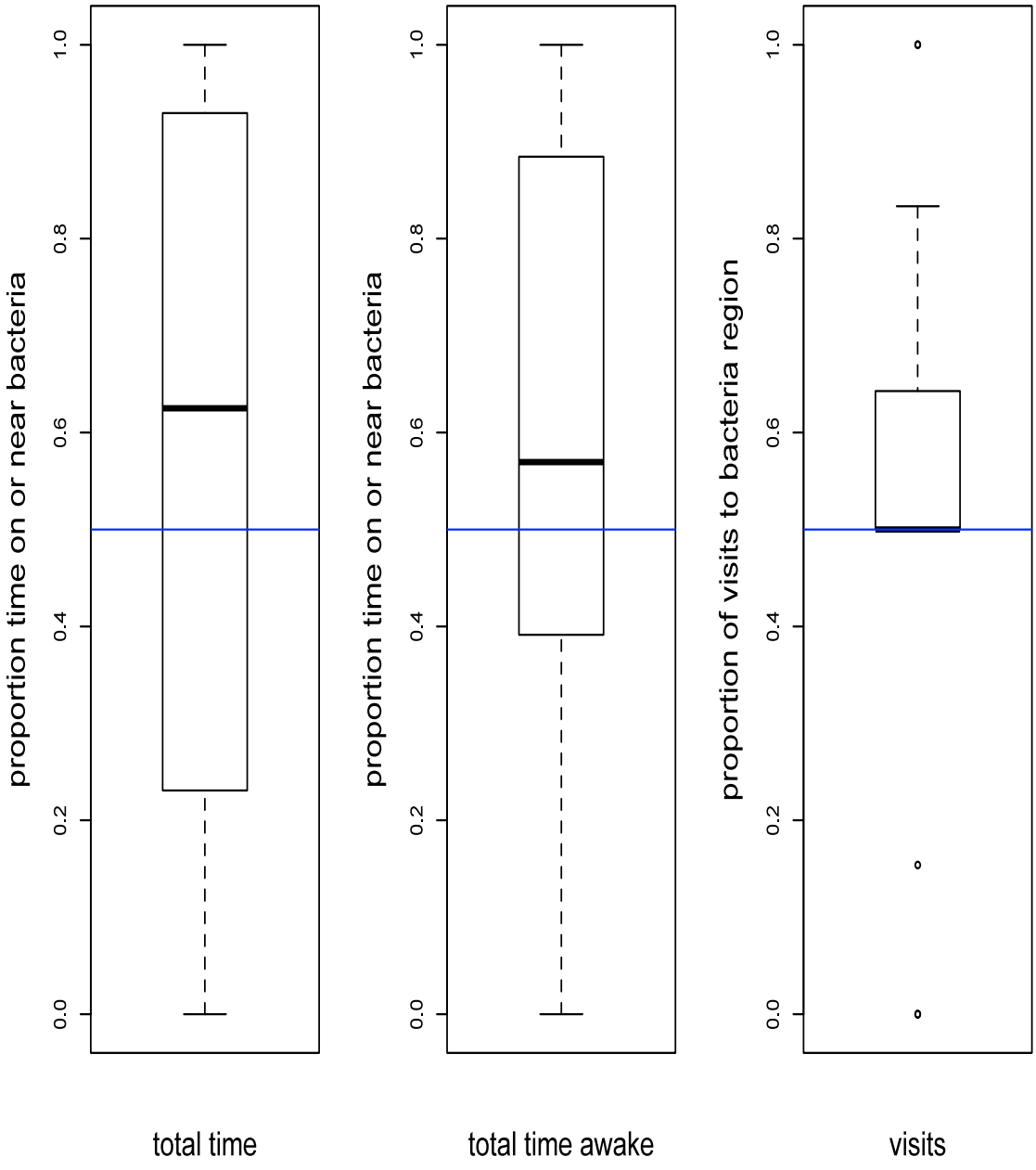

Fig. 10. Proportion of time spent near *Burkholderia* in choice assay. The left graph shows the proportion of total time spent on or near the *Burkholderia*-covered peanut relative to the total time spent near either peanut. The middle graph depicts the same data, excluding long stretches of stillness. The right graph depicts the proportion of visits made to or near the *Burkholderia* peanut relative to the total number of visit made to regions near either peanut. The blue line in all three represents the neutral expectation of a 0.5 proportion.

Discussion

Symbiont Infection Susceptibility Increases. *A. tomentosus* nymphs have a specific window of *Burkholderia* acquisition. This finding corresponds to what Kikuchi *et. al*. (2011) observed in *R. clavatus*. *R. clavatus* show increased acquisition rates during the second and third instar and decreased rates in the first, fourth, and fifth instar. Our results show that *A. tomentosus* individuals are more susceptible to symbiont infection during their third instar. This finding is quite rare among insect-microbe symbioses because most relationships of this sort are established very early in life. One of the few exceptions is the relationship between termites and their symbiotic bacteria that help digest cellulose. In this instance, the bacteria are lost each molt only to be reestablished quickly (Honigberg, 1970). Our experiments ruled out this possibility because the majority of insects that acquired the bacteria in one instar lived through at least one molt and maintained the *Burkholderia* within their bodies. However, one shortcoming of our data is the lack of trials for a fourth instar exposure cohort. Because the mortality rate for sterile bugs was so high, it was difficult to raise sufficient bugs for a group to be exposed to dirt in their fourth instar. Thus I was unable to confirm if the susceptibility to infection in *A. tomentosus* decreases after the third instar as it did in *R. clavatus*.

Several theories as to why these insects acquire their symbiont late in life were proposed by Kikuchi *et al*. (2011). In addition to determining which instar was most capable of *Burkholderia* acquistion, Kikuchi *et al*. (2011) also observed morphological changes in their focal species, *R. clavatus*, over time. They observed that the midgut crypts, which house the bacterial symbionts, were underdeveloped in the first instar. This underdevelopment could possibly be a result of simple developmental constraints, or it

could have arisen as a regulation mechanism that prevents early symbiont infection. While I did not observe specific developmental changes in the *A. tomentosus* midgut as part of this study, a similar pattern of development may occur, which would explain why I observed lower infection rates during the first two instars. Because I saw a spike in infection rates during the third instar, I hypothesize that in *A. tomentosus*, if midgut crypt development is critical to symbiont acquisition, then the crypts would become well-developed around the third instar.

Regardless of the mechanism regulating symbiont acquisition, the selective pressures that have shaped acquisition of symbionts late in life are perplexing. As stated previously, most other insect-symbiont relationships are established transovarially or immediately after birth. Thus, the fact that *A. tomentosus* do not follow this structure is surprising. It has been reasoned that termites, which similarly acquire their symbiont in later life stages, are able to do so because the environment that they are in is flush with their microbial symbiont. In an environment where the symbiont is always readily available, the need for a complex system by which bacteria are housed throughout life is unnecessary and possibly energetically unfavorable. Under such circumstances, it has been proposed for termites that developmental constraints on the timing of infection were relaxed and that this led to the evolution of continual microbial re-acquisition (Inoue, 2000). In *A. tomentosus*, we see a similar phenomenon. *Burkholderia* exists in rather high concentrations in the wild; previous studies have estimated that the concentration is around 10^5 colony-forming units per gram of soil (Ramette, 2005). In my experiments, even when I exposed individuals to a small amount of dirt I still saw infection. Because *A. tomentosus*, like termites, live in an environment where their symbiont exists in high

concentrations, they may have similarly lost the need to quickly and efficiently develop crypts.

Another possible explanation for why *A. tomentosus* individuals do not acquire their symbiont until later in life is that bacterial infection could possibly have negative effects in early life (Yamamura, 1993). Non-symbiotic bacterial pathogens could possibly infect younger nymphs if they were susceptible to bacterial uptake, or the presence of the symbiotic bacteria itself could have negative effects. If the latter were true, I should have observed reduced survival of nymphs infected with *Burkholderia* in the first instar. The survival rate of those infected during their first instar was not significantly different from the survival of uninfected insects ($p = 0.97$) so I theorize that early *Burkholderia* acquisition does not have negative impacts. However, the sample size for these data was relatively small ($n = 10$).

Increased Survival with Infection. One of the main purposes of this thesis was to uncover any effects that *Burkholderia* presence might have on *Alydus tomentosus* individuals. From the results of the survival analysis, I can conclude that *Burkholderia* infection does increase survival of *A. tomentosus* individuals. Irrespective of when the symbiont was acquired, its presence increased the survival of the host. Although the overall survival rate in this experiment may seem low, it actually aligns with the survival rates of similar species (Evangelista Jr., 2011). In the survival analysis of each experimental cohort of insects, I observed that those bugs that were exposed to dirt during the second instar saw significantly increased survival when compared to the first instar cohort and negative controls. This would suggest that first instars rarely acquired the symbiont and that second instars did so at a higher rate. However, the results of PCR

analysis showed that the first and second instar cohorts were infected with *Burkholderia* at similar rates, both of which were significantly higher than the negative controls and significantly lower than the third instar cohort. This survival data is somewhat confounding, thus a repeat of this experiment is merited.

While it has been observed that symbiotic bacteria can increase the size of an individual (Kikuchi, 2007), well-documented cases of survivability increases due to symbiont infection are less common. Increased host survival due to a symbiont generally is caused by one of two bacterial traits: the symbiont can confer resistance to certain pathogens or the symbiont can allow for some sort of nutritional advantage. In this case, since the bugs were raised in sterile environments (except when they were exposed to the dirt), I assume that pathogens were not present, thus any sort of pathogenic resistance conferred by *Burkholderia* would not result in increased survival. Thus, it seems likely that the bacteria increase survival of the hosts by increasing or supplementing some nutrient. *Burkholderia* are utilized by plants as well. The plant-associated *Burkholderia* have genes responsible for nitrogen-fixation, a tool common in many plant symbiotes. Nitrogen fixation is a process by which bacteria convert the chemically stable atmospheric nitrogen (N_2) into biochemically useful ammonia (Suarez-Moreno, 2012). Therefore, one possibility is that *Burkholderia* acquisition leads to increased survival because of the bacterias' ability to fix nitrogen or because of some other ability that increases the nutrient intake of the hosts.

Lack of Symbiont-Induced Behavior Modifications. I had hypothesized that individuals would show behavioral preference to be near their symbiont but the results of my experiments showed that *A. tomentosus* individuals exhibit no preference for being

around *Burkholderia*. This finding can be explained in three possible ways. The bugs could be completely nonselective and simply uptake and maintain any and all bacteria that they encounter. If this were true, the individuals would not actively seek out one type of bacteria, but would rather wander about while acquiring whatever bacteria they encounter. This hypothesis is logically sound, but our lab has sequenced a small amount of bacterial samples harvested from *A. tomentosus* crypts and these bacteria have yet to be anything but *Burkholderia* (unpublished data), suggesting that there is selectivity. Selectivity could arise in a number of ways. It is possible that nymphs do acquire all bacteria they encounter but that *Burkholderia* kill or outgrow any other bacteria present within the midgut crypts, or that the bugs' gut is only hospitable to *Burkholderia*. This would parallel the acquisition of *Vibrio* bacteria by squid, which uptake diverse bacteria from seawater and then kill all other bacteria besides their symbiont (Schleicher, 2011). Another possibility is that the nymphs are selectively picking up only *Burkholderia* from the soil but that they are infected with *Burkholderia* so quickly that they do not need to dwell on or near the bacteria. Since our camera was set to record the position of the insect every five minutes, an infection event would go almost unnoticed if it took very little time to take up the symbiont.

Future Directions. The *A. tomentosus – Burkholderia* system is an excellent system to investigate how organisms establish highly selective symbioses upon environmental acquisition. My results indicate that the symbionts increase survival of their hosts, which has not been seen in other broad-headed bug species studied to date. This indicates a benefit for the hosts that would select for reliable acquisition. However, the timing of acquisition suggests that there has not been strong selection to acquire the

bacteria early in life or suggests fundamental developmental constraints that have prevented evolution of earlier acquisition. To help answer the mystery of why these insects acquire their symbiont so late in life, a morphological examination of midgut crypts would be useful. Monitoring when the crypts become susceptible to infection would help clarify how and why *Burkholderia* acquisition is established.

I used a mixture of instars while conducting the behavioral choice assay trials; thus a more age-specific version of this experiment should be conducted. I determined that individuals do not show a preference for being around their *Burkholderia* symbiont, suggesting that specificity of the symbiosis is not dictated by host preference to inhabit areas with *Burkholderia*. The mechanisms behind the specific acquisition therefore remain unclear. More sequencing of bacterial samples obtained from crypts should be done to confirm that only *Burkholderia* is found in them. An interesting experiment that could be conducted would investigate whether or not it is even possible for another type of bacteria to flourish in *A. tomentosus* crypts. Yet another experiment that would shed light on this mechanism would expose nymphs to the bacteria for different amounts of time to determine whether or not *Burkholderia* infection is the result of a short exposure or a prolonged one.

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