EFFICACY OF DIFFERENT LABORATOY METHODS TO INFECT *LEPTOGLOSSUS PHYLLOPUS* WITH SYMBIOTIC *CABALLERONIA* BACTERIA

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ABSTRACT

EFFICACY OF DIFFERENT LABORATOY METHODS TO INFECT *LEPTOGLOSSUS PHYLLOPUS* WITH SYMBIOTIC *CABALLERONIA* BACTERIA

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The insect *Leptoglossus phyllopus* and some other members of the true bug family must acquire their bacterial symbiont every generation from the environment during their second instar. This poses a problem for researchers wanting to study the bugs *in vitro.* We set out to assess five different methods of introducing bacteria into the insect's environment. We tested the introduction via inoculated water, broth, plant sprig, potting soil, and uninoculated agricultural soil. No significant difference was seen in average adult weight or the development time between treatments. The method of infection did affect the number of insects that reached adulthood which also correlates to how many insects were infected. Insects infected via inoculated water and agricultural soil were significantly less likely to reach adulthood than insects infected via inoculated broth, plant sprigs, or potting soil.

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CHAPTER 1

INTRODUCTION

There are at least six insect families known to form a close symbiotic relationship with the bacteria *Caballeronia* (previously classified as *Burkholderia*) (Kikuchi et al., 2011a). This includes *Coreidae*, the family containing *Leptoglossus* (Kikuchi et al., 2011a). *Leptoglossus*, a true bug commonly called the Leaf-footed bug, is a common agricultural pest (Xiao and Fadamiro, 2009). For this reason, it is often studied in the laboratory setting and there is a need to introduce the symbiont into the insect's lab made environment because they acquire the symbiont from their environment. However, no comparative study of the efficacy of different introduction methods has been performed.

Acquisition of the symbiont is important for proper insect development. Insects that do not acquire a bacterial symbiont have slower growth, increased death rates, and may not be able to reproduce (Kikuchi et al., 2011b). This is likely because inside the host, *Caballeronia* breaks down nitrogenous waste, returning it to a usable form of nitrogen and makes many essential nutrients for the insect (Ohbayashi et al., 2019). Caballeronia exists as both a free-living organism and a symbiote (Ohbayashi et al., 2019). As a symbiote it is provided with a variety of nutrient sources for carbon, nitrogen, and sulfur (Ohbayashi et al., 2019).

Despite its importance, *Caballeronia* is not passed from parent to offspring. Analysis of eggs laid by bugs known to form a symbiotic relationship with *Caballeronia*

shows little to no evidence of *Caballeronia* presence within or on the egg (Kikuchi et al., 2011a). However, within populations of insects that experience symbiosis with *Caballeronia*, infection rate is high, ranging from approximately 88-100%, likely due to the importance of the symbiont to the insect's development (Kikuchi et al., 2011a). In nature, insect hosts acquire *Caballeronia* from their environment, but in a laboratory setting it must be introduced into their environment. After hatching, true bugs pass through five instars before molting into adults (Kikuchi et al., 2011b). *Caballeronia* acquisition occurs most frequently during the second instar which generally lasts for three days (Kikuchi et al., 2011b).

Studies examining the relationship of *Caballeronia* with various insect hosts have utilized different methods to introduce *Caballeronia* to the host. One method reared *Leptoglossus zonatus* (*Coreidae*) insects on fruits that they are known to feed on in nature (Xiao and Fadamiro, 2009). *L. zonatus* nymphs were hatched and reared to second instar in a clean container (Xiao and Fadamiro, 2009). Once the insects had molted to the second instar, they were moved to a second container with a satsuma fruit until they had reached adulthood (Xiao and Fadamiro, 2009). A second introduction method used was introduction through potted soybean plants (Kikuchi et al., 2007). *Riptortus pedestris* (*Alydidae*) nymphs were reared to adulthood on a potted soybean plant and then tested for the presence of *Caballeronia* (Kikuchi et al., 2007). A third introduction method was inoculation of the *R. pedestris* (*Alydidae*) nymph's drinking water with *Caballeronia* (Kikuchi et al., 2011b). These insects were provided with drinking water throughout their rearing and during the desired instar, the drinking water was inoculated with 10^7 colony forming units/mL of *Caballeronia* for two days (Kikuchi et al., 2011b). The use of

inoculated drinking water seems to be the most common method used in research (Hunter et al., 2022; Itoh et al., 2019, Kikuchi et al., 2011b; Kikuchi and Yumoto, 2013; Ohbayashi et al., 2015; Ohbayashi et al., 2019).

In the following experiment, we set out to discover if a difference in efficacy of introduction methods exists. Second instar nymphs were exposed to *Caballeronia* using inoculated drinking water, potting soil, plant sprigs, broth, and fresh agricultural soil (uninoculated) from a farm with known previous *Leptoglossus* presence. The infection methods chosen for our research were based on literature, our own observations of *Leptoglossus* and their habits, and consideration of what environment would benefit the bacterial cells. Water was selected due to its common use and its seeming efficacy as seen in previous literature (Hunter et al., 2022; Itoh et al., 2019, Kikuchi et al., 2011b; Kikuchi and Yumoto, 2013; Ohbayashi et al., 2015; Ohbayashi et al., 2019). The broth method offers a benefit to the *Caballeronia* symbiont over the water infection method. In the broth infection method, the symbiont remains in a nutrient rich environment and should not be subjected to the osmotic shock that may be experienced by the cells moved into water. Dipped plants were used because it is common to see insects gathered on the leaves of the plant in their enclosure, particularly in the early instars. The plant provides a source of both water and nutrients for the developing insects, so it is likely that they could acquire *Caballeronia* from plant leaves if there are cells present. Potting soil was chosen as an infection method for two reasons. One, it has been successfully used as an infection method to maintain lab colonies (Ravenscraft et al., 2020). Two, soil is the accepted natural reservoir of *Caballeronia* and in the wild it is assumed this is where wild *Leptoglossus* obtain their symbiont. Theoretically, potting soil may allow the *Caballeronia* population

to thrive, and the second instar nymphs might be attracted to the soil as it is the natural reservoir for their symbiont. Agricultural soil was selected because of the assumed presence of *Caballeronia* and the nymph's natural ability to acquire its symbiont this way. Soil was specifically selected from a farm where there had been a *Leptoglossus* population in the past because presumably *Caballeronia* would be present in that soil.

After rearing, we performed a comparative analysis of survival to adulthood, development time, adult weight, and infection rate. We hypothesized that there would be a difference in infection rate and thus the rate of survival to adulthood. For insects that were successfully infected, we did not expect a difference in adult weight or development time across treatments. We found that survival to adulthood was affected by treatment while adult weight and development time were not. Infection via broth, plant, and potting soil resulted in a higher survival rate than infection via water or agricultural soil.

CHAPTER 2

METHODS

2.1 General Insect Rearing

Leptoglossus insects were reared from first instar to adulthood or death in clean plastic boxes with four to six individuals per box. The initial round of the experiment was conducted at room temperature, then a second round was conducted in a temperature- and humidity-controlled enclosure. For both rounds, the light:dark cycle was 16:8. For the first round, the temperature was approximately 20°C, but fluctuated with the building's HVAC system and humidity was not controlled. In the enclosure, temperature was maintained at approximately 26°C and the humidity was 40-60% during the day and 80% at night. During the first instar, the box contained only a single cow pea plant seedling (*Vigna unguiculata*) with one to three true leaves in a capped growing tube filled with 0.25% water agar. When most insects in a box molted to second instar, peanuts were added to the boxes as a food source for the nymphs and infection began.

2.2 General Infection Protocol

For infection purposes, *Caballeronia* strain LP006 was selected. LP006 was isolated from *Leptoglossus* collected in Texas and is maintained as a lab species in Ravenscraft Lab. A standard optical density (OD) of 0.3 was used whenever adding *Caballeronia* to any infection box; this is equivalent to approximately 6×10^7 colony forming units/mL. The presence of 3.5×10^3 colony forming units suspended in the drinking water of a single nymph consistently results in successful infection (Kikuchi and Yumoto,

2013). The infection period lasted a total of five days. *Caballeronia* was introduced the day the nymphs molted to second instar. This was considered day one. The insects were then exposed to the bacteria for three full days, days two through four. On the fifth day, the *Caballeronia* was removed.

2.3 Inoculated Water Infection Method

For boxes with inoculated drinking water, 1mL of standardized broth culture was added to two 1.5mL microcentrifuge tubes and centrifuged. Initially, the centrifugation was performed at 15,000 RPM for 1 minute. Later, the centrifugation speed was reduced to 5000 RPM, but the time of centrifugation remained the same. The supernatant was removed, and the bacterial cells were resuspended by vortexing in 1mL of 0.5% vitamin C water (It is standard practice to provide vitamin C in these insects' drinking water). The tubes were stopped with a small piece of cotton wool. They were replaced daily for the first three days of the infection period.

2.4 Inoculated Broth Infection Method

For boxes with broth instead of drinking water, two 1.5mL microcentrifuge tubes were filled with 1mL standardized broth culture. Insects were not provided with drinking water to incentivize them to drink from the broth tubes. The tubes were stopped with a bit of a cotton ball. They were replaced daily for the first three days of the infection period.

2.5 Inoculated Plant Infection Method

For boxes with plants inoculated with *Caballeronia*, a 50mL beaker of standardized broth was prepared. The seedling was dipped in the broth deep enough to cover all leaves (seedlings had 1-3 true leaves) completely to ensure full coverage by live *Caballeronia* cells. Dipped seedlings were then left out to dry before being added to the box. Plant sprigs

were re-dipped in a fresh culture of *Caballeronia* daily for the first three days of the infection period. Wild *Leptoglossus* obtain their water by drinking xylem sap, so drinking water was not provided in plant infection boxes to incentivize insects to drink from the plant. (Mitchell, 2006).

2.6 Inoculated Soil Infection Method

For boxes with inoculated soil, potting soil was autoclaved in a foil lined bin for two consecutive days to ensure the soil's sterility. The first day the soil was put through a 15-minute cycle. The bin was kept closed in a dark cabinet at room temperature overnight. The second day the soil was put through a 60-minute cycle. After autoclaving, the soil was moved into sterile jars for storage. To prepare an infection box, a thin layer of sterile potting soil, enough to cover the bottom of the box, was added. Then 2mL of standardized broth was mixed with the soil to ensure inoculation with live bacterial cells. The soil was checked daily for dryness and sprayed with reverse osmosis (RO) filtered water when necessary. No additional *Caballeronia* was added to the potting soil during the infection period. A vial of uninoculated 0.5% vitamin C water was provided as a source of water.

2.7 Agricultural Soil Infection Method

Agricultural soil samples were collected from a farm in Venus, Texas that grows tomatoes. The farm previously had infestations of *Leptoglossus* for many years although no visible insects were present at the time of collection. Two collections were performed during the insect rearing phase of this project. After collection, soil was stored in a mason jar with a modified lid that allowed air flow and was occasionally wetted to assist bacterial survival. The jars were kept in the same environment as the insects. Soil was stored in this manner for up to five weeks before being introduced into infection boxes. During the

infection period, a thin layer of soil was added so it covered the entire bottom of the box. Insects in the agricultural soil boxes were also provided with a vial of uninoculated 0.5% vitamin C water as a water source. Soil was wetted when added and sprayed with RO as needed to ensure it remained damp during the infection period.

2.8 Post-Infection Insect Rearing

When the *Caballeronia* was removed, uninoculated plant sprigs and 0.5% vitamin C water were added if not already present. After infection, insects and enclosures were checked regularly. The insect's plant sprig, water, and peanuts were kept fresh. Insect development and mortality were monitored. If an individual died before reaching adulthood, its instar was noted. Dead insects that had developed past the second instar were frozen and retained for diagnostic PCR. Occasionally, an individual would stall during development. It is unusual for a nymph to remain in any given instar for longer than one week; longer delays usually indicate that the nymph does not have a normal *Caballeronia* infection (Hunter et al., 2022). Therefore, if two or more weeks passed and a *Leptoglossus* nymph had not molted to the next instar, it was humanly euthanized. Insects that reached adulthood were weighed and a selection was frozen for PCR.

2.9 DNA Extraction

Two individuals per box were selected to test for the presence of *Caballeronia*. For boxes where all individuals reached adulthood, two adult samples were tested. When possible, one female and one male were selected. For boxes where some individuals reached adulthood and some did not, one nymph and one adult sample were tested. For boxes where no individuals reached adulthood, two nymph samples were tested. Samples were surface sterilized in bleach and ethanol. They were then homogenized in 180µL of 1x

Phosphate Buffered Saline. DNA extraction was then performed according to the QIAGEN DNeasy Blood and Tissue Kit. Two elutions with 25µL were performed in place of the final 100µL elution for a final product with a 50µL volume.

2.10 PCR

We performed diagnostic PCR on the extracted DNA to test for the presence of *Caballeronia*. We used primers developed by Spilker and colleagues (2009) that amplify the recombinase A gene of *Caballeronia senu lato*. We confirmed that these primers are specific to the *Caballeronia* group by searching for matches on NCBI. For a single reaction, the following recipe was used: 12.87µL PCR water, 4.00 5x New England BioLabs buffer, 0.4µL 10mM dNTPs, 0.8µL 5µM RecA forward primer, 0.8µL 5µM RecA reverse primer, 0.13µL New England BioLabs Taq polymerase, and 1µL of template DNA. Each reaction tube contained a total of 20.0µL of reactants. Samples were then run on the following PCR program: 1 cycle of 30 seconds at 94°C; 30 cycles of 30 seconds at 94°C, 1 minute at 58°C, and 1 minute at 68°C; 1 cycle of 68°C for 5 minutes. A positive and a negative control were run for each round of PCR.

2.11 Gel Electrophoresis

Samples were visualized using gel electrophoresis. Each well was loaded with 3µL of PCR product mixed with 1µL of GelRed with loading dye. Along with the PCR products, a 50bp ladder was run in its own well. Samples were run on a 1% agarose gel. The gels were run at 80V for 40 minutes. After running, gels were visualized under UV light using a GelDoc machine. Imaged gels were analyzed to determine if the tested individual was infected with *Caballeronia* or was aposymbiotic.

2.12 Sanger Sequencing

Samples from adult bugs reared on agricultural soil were put through additional testing. After confirming the presence of Caballeronia DNA using diagnostic PCR, two samples were also run through Sanger sequencing to more accurately identify what bacteria they had acquired. Sequences were then identified using NCBI Blast.

2.13 Data Analysis

We used linear models to assess test for correlation between the treatments and rearing environment and, respectively, insect mortality, mass at adulthood, and development time. In all cases, we included a random effect of cage. We used a binomial model to assess whether the infection treatments or the rearing environment was correlated with survival to adulthood. We also tested whether the infection treatments or rearing environment were correlated with the time it took a nymph to develop to adulthood. For body mass, we added a term for sex in addition to treatment and environment, since females are known to be larger than males. We found the best-fit fixed effects structure for all three models using backward model selection with likelihood ratio tests.

CHAPTER 3

RESULTS

First-instar nymphs were assigned to one of five infection treatments. For each treatment, 12-13 boxes of 4-6 nymphs were reared. Each treatment began with a sample size of at least 60 first-instar nymphs (Table 1). Four metrics were used to compare the different treatments efficacy: mortality, development time, adult weight, and infection success.

Table 3.1: Sample size of each treatment

	Water	Broth	Plant	Potting Soil	Agricultural Soil
Total Sample Size	67	68	70	63	63
Survival to Adulthood	57	65	66	58	49
Development Time	6	44	40	30	4
Adult Weight	6	44	40	30	4
Infection Status	12	14	14	14	14

3.1 Survival to Adulthood

Survival to adulthood showed significant differences among the five treatments (Fig 1; df=4, LRT=38.2, $p<<0.001$). Specifically, an average of 5% of nymphs infected via water or agricultural soil survived to adulthood, while 61% of nymphs infected via a broth

suspension, dipping the plant in broth, or inoculated potting soil reached adulthood. The conditions in which insects were reared (a standard indoor environment versus a temperature-controlled enclosure) did not affect their survival to adulthood (*df*=1, LRT=0.001, p=0.98). Individuals that were euthanized as nymphs because they stopped developing properly (10 water, 3 broths, 4 plant, 5 potting soil, 14 agricultural soil) were not included in these analyses but including them did qualitatively change the results.

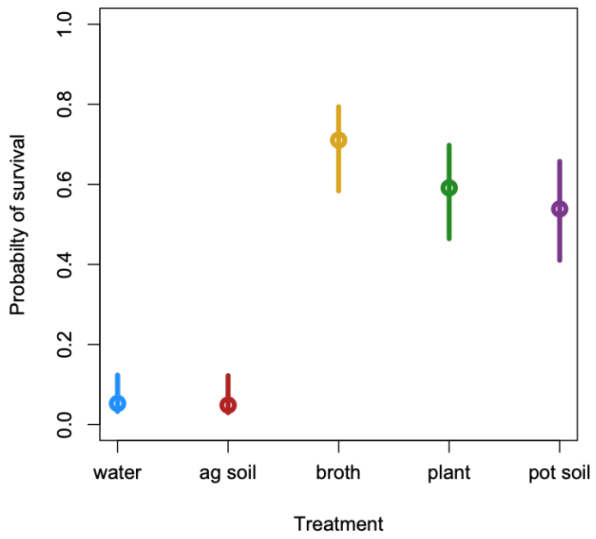

Figure 3.1**:** Survivorship of second instar *Leptoglossus phyllopus* nymphs to adulthood in five different infection treatments. Plotted are model probabilities that a second instar nymph survived to adulthood based on treatment. Estimates are based on a mixed logistic regression model that controls for cage as a random effect. Circles show estimated group means; error bars show estimated standard errors (the standard errors are asymmetric because the model was on a logit scale, but the y axis is not).

3.2 Development Time

The time it took for a nymph to develop from second instar to adulthood did not vary with infection method (Fig 2a; *df*=4, LRT=4.6, p=0.33). However, insects reared under ambient indoor conditions developed 1.4 times slower (23 vs 32 days) than insects reared at a warmer semi-controlled temperature (~26 C) and humidity (Fig 2b; *df*=1, LRT=17.0, p<<0.001).

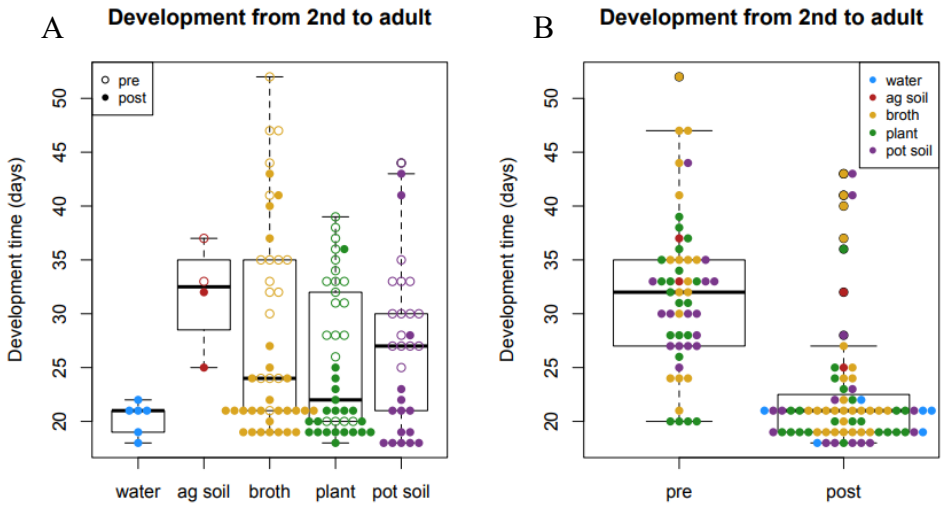

Figure 3.2: Development time of nymphs from the beginning of the second instar to adulthood by infection treatment (a) or rearing environment (b). Points show the raw data. Box plots depict medians and interquartile ranges of the data. Whiskers are placed at 1.5 times the interquartile range or, if all data fall within this range, they are placed at the most extreme value measured.

3.3 Adult Weight

The weight of nymphs that reached adulthood was not affected by the infection treatments (Fig 3; *df*=1, LRT=5.5, p=0.24) nor the rearing conditions (Fig 3; *df*=1, LRT=0.3 p=0.58). As expected, we observed that female *L. phyllopus* were about 14%

larger than males, on average (*df*=1, LRT=17.2, p<<0.001; average female was 137 mg and average male was 120 mg).

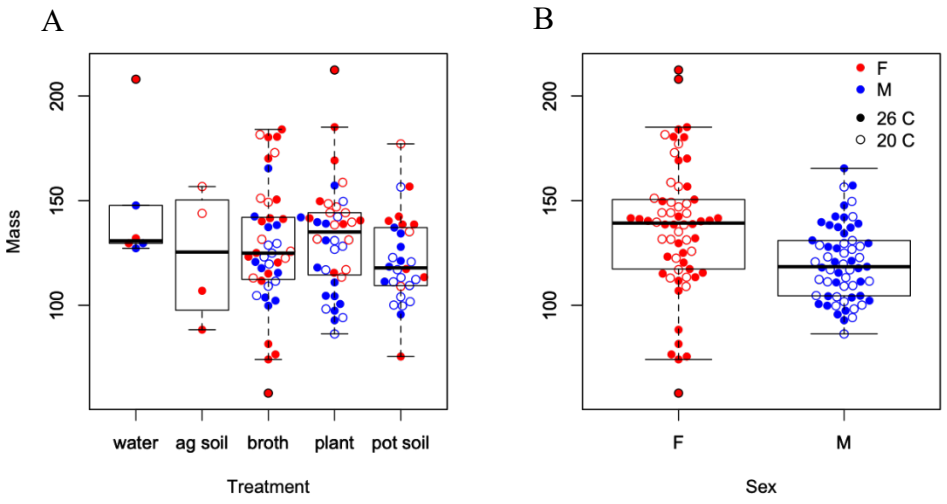

Figure 3.3**:** Fresh weight at adulthood by infection treatment (a) or sex (b). Points show the raw data. Box plot features are as in Figure 2.

3.4 Infection Status

Of the individuals reared in the environmental chamber, 29 adults and 39 nymphs ranging from third instar to fifth instar were tested for presence of *Caballeronia* DNA. Eight nymphs had an indeterminate result (a faint band) in diagnostic PCR; these were removed from the analyses, but their inclusion (as positives or negatives) did not qualitatively change the results. Nymphs that died were less likely to be infected than adults (Fig 4; *df*=1, LRT=32.3, p<<0.001); in fact, all the adults were infected with *Caballeronia*, while 67% of nymphs that died or stalled during development were uninfected. Infection status did not differ among the treatments (*df*=4, LRT=1.3, p=0.86). However, due to small sample sizes and the way in which samples were selected, we feel this does not accurately

reflect the difference in efficacy of the treatments. We will further analyze these results in the discussion section.

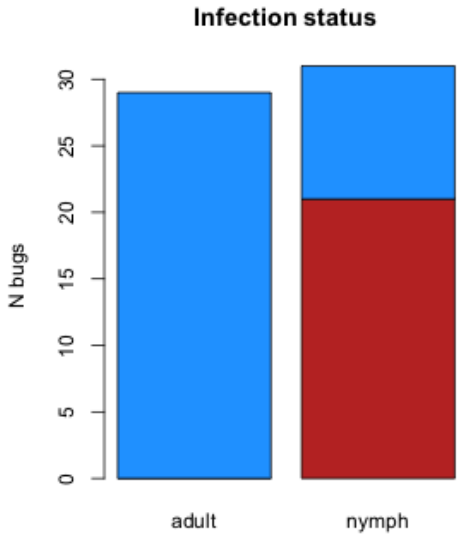

Figure 3.4: Infection status as determined by diagnostic PCR for individuals that successfully reached adulthood versus nymphs that died during development. Blue = *Caballeronia* detected; Red = no *Caballeronia* detected.

3.5 Sanger Sequencing

The two sequences from samples taken from adult insects reared on agricultural soil returned two different symbiont identities. One of the sequences was identified as a *Caballeronia* strain with 100% sequence match with LP006, our chosen lab inoculation strain, across 680 base pairs of the 16S rRNA gene. The other symbiont was identified as a *Pandorea* spp.

CHAPTER 4

DISCUSSION

To find the most effective method for infecting the bug *Leptoglossus phyllopus* with its beneficial bacterial symbiont, *Caballeronia*, in the lab, we tested five different exposure techniques: exposing the insects either to soil collected from an agricultural field, or to cultured *Caballeronia* cells that were suspended in water, suspended in broth, applied to a host plant by dipping, or poured into potting soil. We found that the method by which a nymph was exposed to *Caballeronia* had a significant effect on survival to adulthood, with nymphs exposed to agricultural soil or cultured cells in water dying at about 3.4 times the rate of nymphs exposed to cells in broth, potting soil, or on plant leaves. The exposure method did not have a significant effect on the development time or adult weight of the nymphs that reached adulthood, as expected. This indicates that once an individual is successfully infected, no matter the method of infection, it develops normally. We conclude that of the methods tested, use of cultured cells in broth, whether directly or via some surface vehicle, is the most effective for infecting *L. phyllopus* with its symbiont.

For researchers studying the *Leptoglossus*-*Caballeronia* symbiosis, we recommend the use of the broth infection method. It was among the most effective infection methods, and it was the simplest. Cells were taken directly from the overnight culture and introduced into the insect's environment. There was no need for any additional steps to prepare for the infection. Other researchers have also used similar methods and found comparable results. In a study of symbiosis between *Caballeronia* and *Riptortus clavatus*

researchers exposed nymphs to LB medium inoculated with *Caballeronia* (Kikuchi et al., 2007). In this study, the infection success rate ranged from 60-93% between enclosures (Kikuchi et al., 2007). There is a slight risk when introducing a rich growth media into an unsterile environment. There is a chance that other bacteria would be allowed to thrive if they were introduced to the broth. However, due to the small surface area of exposure and relatively short exposure time, this risk is minimal. We did not observe any bacterial contamination in the broth enclosures except some that originated from the original overnight culture.

While no other study has used live plant leaves dipped in culture symbiont, plant leaves have previously been used as a vehicle for *Caballeronia* infection. One study looking at the effect of acquiring *Caballeronia* on *Jalysus wickhami* introduced nymphs to the symbiont by first exposing their enclosure which included a single tomato leaf, to six infected adult males for five or more days (Ravenscraft et al., 2020). In theory, this exposed the leaf and all other surfaces to *Caballeronia* which remained there until picked up by the nymphs. In this experiment, the infection rate was 63% (Ravenscraft et al., 2020). Assuming all aposymbiotic individuals died before reaching adulthood, this is similar to our results of an approximate survival rate of 60% of nymphs reared on inoculated leaves. Insects are clearly capable of picking up *Caballeronia* from leaves, but this does not appear to be how they acquire it in the wild. Researchers studying symbiont acquisition in the wild reared insects on pomegranate trees covered in nets to isolate the insects from the soil (LT Sullivan, S Kelley & M Hunter unpub data). They found that about 33% of insects in 2019 and less than 10% in 2020 acquired *Caballeronia* when reared in this manner. This leads us to believe that the leaf's surface is simply acting as a vehicle for symbiont cells. This

infection method likely does not offer any special benefit over other successful infection methods.

It is interesting to note that in this experiment, infection via plant was the only method where the *Caballeronia* was dried out before introduction to the nymphs. After dipping, plants were allowed to stand, often near a burning Bunsen burner, until broth had mostly or completely dried. Since this method was relatively successful, it suggests that *Caballeronia* can dry out for a time while remaining viable symbionts. It also suggests that *Leptoglossus* are capable of acquiring *Caballeronia* in this dried-out state.

The use of potting soil to infect insects with *Caballeronia* has not been used directly in rearing experiments. However, it has been used to maintain and infect a colony population (Ravenscraft et al., 2020). It appears to be a relatively successful way to maintain a colony with high infection rates. Potting soil was thought to be a controlled way to imitate how *Leptoglossus* obtains *Caballeronia* in the wild. Although the exact mechanism of acquisition in the wild is not known, the leading theory is that the insects somehow acquire it from the soil in their second instar. Despite this, we did not see a statistically significant difference between it and the other broth-based infection methods. This seems to point to potting soil as a good vehicle for *Caballeronia,* like plant leaves, but it does not appear to have any specific advantages.

The low survival rate of insects infected via water was unexpected. Many other studies have used this and similar methods with greater success (Hunter et al., 2022; Itoh et al., 2019; Kikuchi et al., 2011b; Kikuchi and Yumoto, 2013; Ohbayashi et al., 2015; Ohbayashi et al., 2019). There are some differences in these papers that are worth noting. All of the cited papers were studying the colonization of *R. pedestris* while we were

studying the colonization of *L. phyllopus.* While the two systems share many similarities, there may be significant differences we are currently unaware of that make the colonization of *L. phyllopus* via bacterial cells suspended in water less optimal. Another difference noted in several studies is the purposeful introduction of cells in log phase (Itoh et al., 2019; Kikuchi et al., 2011b; Kikuchi and Yumoto, 2013; Ohbayashi et al., 2015; Ohbayashi et al., 2019). This is accomplished by transferring cells from an overnight culture into fresh broth and allowing them to grow for the required number of hours to reach log phase. These cells are then collected and suspended into water. Our cells were taken straight from an overnight broth, collected, and suspended in water. This may mean more of our cells were dead or near to death than anticipated. Older cells may also be more susceptible to the osmotic shock of being moved to water from broth. Some studies also dehydrated the second instar nymphs by depriving them of water overnight before introducing the inoculated water (Hunter et al., 2022; Itoh et al., 2019; Kikuchi and Yumoto, 2013; Ohbayashi et al., 2015; Ohbayashi et al., 2019). Dehydrated nymphs are more likely to drink from the infected water. We chose not to dehydrate our nymphs before introducing *Caballeronia* because it would not have been beneficial in all infection methods and was deemed unnecessary. While many papers do not specify in detail how the inoculated water is presented to the nymphs, Kikuchi and colleagues (2011b) placed nymphs in an enclosure with a small petri dish containing water and a cotton ball. This setup allows a larger open surface area for the nymph to interact with the inoculated water than our methods. In our set up, the water and cotton ball are in a microcentrifuge tube. This leaves a very small surface area exposed, and often the nymphs have to crawl into a small, enclosed space to

reach the water. This may discourage interaction with the inoculated water which would lead to a lowered infection rate.

In the second half of the experiment, the methods used for water infection were slightly modified. For the first round of experimental boxes, the centrifugation speed used to collect cells for suspension in water was 15,000 RPM. In the second round, the centrifugation speed was decreased to 5,000 RPM. The speed was decreased after concerns over high speeds causing cell damage arose. Based on preliminary mortality tests, the speed change did not affect the bacteria's ability to survive the higher speeds. Survival is not the only important factor. High centrifugation speeds can affect cell surface structures such as flagella (Peterson et al., 2012). *Caballeronia*'s flagella is key to its ability to colonize *Leptoglossus'* gut. At the entrance to the symbiotic organ there is a thick mucus plug (Ohbayashi et al., 2015). To pass through this plug, *Caballeronia* wraps its flagella around itself and moves in a corkscrew-like manner (Kinosita et al., 2018). So, the loss of their flagella would stop *Caballeronia'*s ability to colonize its host. This leads to the question, is there any centrifugation speed at which *Caballeronia* will keep its flagella? Because the water treatment was the only treatment that underwent centrifugation, it is worth considering that centrifugation itself is the key problem with the water infection method.

The low survival rate of insects infected via agricultural soil was also unexpected. Because soil is the assumed reservoir from which these bugs acquire *Caballeronia* in the wild and the soil was taken from an area where *L. phyllopus* have been observed, good infection rates were expected. It is possible that generally there was a low abundance of *Caballeronia* in the soil used in our study. Only one of the agricultural soil adults obtained a *Caballeronia* strain. The strain acquired matched LP006, our chosen lab inoculation

strain. It is not possible to determine if the presence of this strain was due to contamination from the lab or if it occurred naturally in the soil. LP006 is a known wild strain native to Texas, where the soil was collected. The other agricultural soil adult obtained a *Pandorea* species symbiont. While it is known that *Pandorea* species are capable of colonizing the *Leptoglossus* symbiotic organ, they generally colonize less completely (Itoh et al., 2019). Insects infected with *Pandorea* showed improved survival, development time, and adult mass over aposymbiotic insects (Itoh et al. 2019). Despite showing improvement over aposymbiotic insects, they did show slower development and lower mass than individuals infected with *Caballeronia* (Itoh et al., 2019). The only confirmed wild symbiont acquired by bugs infected by agricultural soil did not acquire a *Caballeronia* strain and the one insect that did acquire a *Caballeronia* strain may have been due to contamination. So, was *Caballeronia* even present in our soil? We assumed *Caballeronia* would be present because it is a common soil isolate and we obtained our soil from a location known to host *Leptoglossus*. However, it seems that *Caballeronia* may not be abundant across all soil as it was previously presumed.

It has been shown in other studies that *Riptortus pedestris,* another Heteroptera known to host *Caballeronia,* can be successfully infected when reared on agricultural soil (Kikuchi et al., 2012; Itoh et al., 2018). Neither of these studies report exact infection success rate or survival to adulthood, but they do appear to have been relatively successful. One study showed the infection of some individuals with *Caballeronia* and some individuals with *Pandorea*, similar to our results (Itoh et al., 2018). How the soil was maintained in these studies varied greatly from how we maintained our soil. In these studies, soybean seeds were planted in the collected soil and hatchling insects were added

(Kikuchi et al., 2012; Itoh et al., 2018). The presence of the plant and its maintenance would greatly affect the soil and its microbiota. In comparison, our soil was wetted and added to the bottom of the enclosure to about one-half inch deep. This difference may have affected the insect's ability to obtain a symbiont. Another difference is these studies both introduced pesticide to the soil during the rearing experiment. When analyzing the soil microbiota, *Caballeronia* and related species increased in relative abundance after the application of the insecticide fenitrothion while other species decreased in relative abundance (Itoh et al., 2018). This loss of bacterial competition may have also affected the probability of infection.

To test insect infection status, we performed diagnostic PCR using *Caballeronia*-specific primers on two individual insects per cage - two adults if all nymphs reached adulthood, two nymphs if none reached adulthood, and one adult and one nymph if some, but not all, individuals reached adulthood. Individuals that reached adulthood showed a 100% infection rate while individuals that died as nymphs showed only a 33% infection rate. This strongly suggests that most of the individuals that died as nymphs died because they failed to acquire their symbiont. This is consistent with prior work which has shown that the rate of survival to adulthood of aposymbiotic *L. phyllopus* is 16% (Hunter et al., 2022). In fact, more nymphs were infected than expected. Because diagnostic PCR was performed and not quantitative PCR, it is possible that some of the nymphs that appeared infected were not sufficiently colonized to develop properly. There are also other factors that could be causing the deaths such as disease.

We did not detect a difference in infection rates among the exposure methods, however this was likely due to the limited sample size of our PCR study. The number of

bugs tested for infection status was relatively low, only about one-fifth the total sample size, and we tested a widely different number of adults and nymphs in each treatment. For example, in the agricultural soil treatment two adults and twelve nymphs were tested, but in the broth treatment eleven adults and three nymphs were tested. Given that most nymphs that died were uninfected with *Caballeronia*, it seems likely that the difference in survival to adulthood between treatments was caused by differences in infection success, with the three broth-based treatments resulting in more effective infection, even though our limited sample size did not result in statistical significance.

We also tested two different rearing conditions - ambient laboratory temperature and humidity versus a 28°C chamber that fluctuated between 60% and 80% relative humidity. There was not a statistically significant difference in any of the metrics when comparing insects reared in each environment. Looking at Figure 2B we can see that all individuals reared in the environment-controlled enclosure, regardless of treatment, trended towards developing faster compared to those reared outside of the environmentally controlled enclosure. This is likely due to the increased temperature and humidity maintained in the enclosure. This trend also appears in Figure 2A. There appears to be a trend that individuals in the water treatment developed more quickly than those in other treatments, however, all individuals who reached adulthood in the water treatment were reared in the environment-controlled enclosure. Individuals reared in less optimal conditions still tended to develop normally. This indicates that an environmentally controlled enclosure is not necessary for the study of *Leptoglossus.* While insects did tend to develop slower in an uncontrolled environment, their growth was not so prohibitively slow as to hinder the progress of research.

There are still many avenues left to research regarding bug-*Caballeronia* symbiosis. While this study looked at different methods of infection, there are still many variables and factors left to test. Larger studies may reveal a difference between our three top infection methods. Testing different lengths of exposure may further improve infection rates. Field studies of symbiont acquisition may lead to improvements in lab methods. Considering the bacteria instead of the insect may also prove useful. Comparing how bacteria react to different environments used to infect and how this impacts their survival rate could lead to methods that are beneficial to both the insect and the bacteria. Further studies in this field can improve not only our understanding of bug-*Caballeronia,* but of symbiosis as a whole.

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BIOGRAPHICAL INFORMATION

Lillian Storm is the sixth child of Paul and Johanna Storm and grew up in Arlington, Texas. She and her husband met at a kid's summer day camp where they both worked. They now live happily in Arlington, Texas, with their two dogs, Naga and Suki. In their free time they love playing board games, rock climbing, and watching anime.

Lillian graduated from the University of Texas at Arlington with a Bachelor of Science in Microbiology and with a UTeach certification to teach secondary science in May 2023. During her undergraduate, she was a part of Ravenscraft Lab, a microbial ecology laboratory at UTA. She completed her research under the mentorship of Dr. Alison Ravenscraft and Alison Blanton, a PhD student. Their research of the *Leptoglossus-Caballeronia* symbiosis will hopefully help other researchers further our understanding of symbiosis.

Her goal is to become a junior high science teacher in the Fort Worth, Texas, area. She hopes through teaching she can help her students find a passion for science.