THE EFFECTS OF THE SYMBIONT *RICKETTSIA* ON THE INTERACTIONS BETWEEN A WHITEFLY PEST (*BEMISIA TABACI*) AND A GENERAL FUNGAL PATHOGEN (*BEAUVERIA BASSIANA*)

by

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STATEMENT BY AUTHOR

The thesis titled "The Effects of the Symbiont *Rickettsia* on the Interactions between a Whitefly Pest (*Bemisia tabaci*) and a General Fungal Pathogen (*Beauveria bassiana*)" by James Conway has been submitted in partial fulfillment of requirements for a master's degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

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Abstract:

Some intracellular symbionts of insects confer host protection from a variety of bacterial, fungal and viral pathogens as well as from predators and parasitoids. Within the cryptic species complex of whiteflies known collectively as *Bemisia tabaci* is a cosmopolitan invasive agricultural pest, which is commonly infected with the symbiont *Rickettsia* sp. nr. *bellii. Rickettsia* swept rapidly through southwestern USA whitefly populations of the MEAM1 species and has been associated in a genotype-dependent manner with increased whitefly fitness and female biased sex ratios. Here we sought to determine whether *Rickettsia* in MEAM1 might have a defensive role against the general entomopathogenic fungus, *Beauvaria bassiana*. Nymphs from two lines of whitefly, each with *Rickettsia* positive (R^+) and negative (R^-) sublines were exposed to different doses of *B. bassiana.* The results provided evidence of protection by *Rickettsia* in one genetic line (MAC1) but not in the other (MAC2). In a third experiment, females of the four sublines were each outcrossed for two generations with males from an outbred whitefly culture, derived from the field within the year, and F2 nymphs from these four new sublines were exposed to the fungus. In this experiment, *Rickettsia* was protective in both MAC1-O and MAC2-O lines. Taken together, our results suggest the symbiont *Rickettsia* can confer protection against a generalist entomopathogenic fungus, *B. bassiana*,

and that this protection is conditional on host genotype. To our knowledge, this is the first record of an insect symbiont conferring protection against a generalized and commercially available biological control agent. *Bemisia tabaci* MEAM1 is a global pest of warm temperate and tropical agriculture, and the prevalence of *Rickettsia* in many populations of this species could limit the predictability or efficacy of fungal pathogens as a potential management tool.

Introduction:

Insects have many intimate relationships with the microorganisms living alongside and within them. Although some of these microbes are pathogens, many are beneficial for their hosts (Engel & Moran 2013). Some live intracellularly, and are passed vertically from mother to offspring (Douglas et al. 2016). Some obligate intracellular symbionts supplement shortcomings of the host diet (Moran et al. 2003). Facultative symbionts may not be required, but can manipulate host reproduction in ways that enhance their transmission (O'Neill et al. 1997) or confer important benefits to their hosts.

The benefits include temperature tolerance (Russell et al. 2006) and defense (Moran et al. 2008, Dale et al. 2006). Symbionts with defensive roles can provide protection from many natural enemies, including parasitoids (Oliver et al. 2003), predators (Piel et al. 2004), viruses (Teixera et al. 2008, Hedges et al. 2008), and other microbial pathogens (Hughes et al. 2011, Moreira et al. 2009).

The generality of symbiont protection phenotypes may be either broad (e.g. *Wolbachia* in mosquitoes conferring protection to multiple viruses and *Plasmodium* (Moreira et al. 2009)) or narrow (e.g. *Hamiltonella* in pea aphids conferring protection to some parasitoid wasps and not to others (Oliver et al. 2013, McClean & Godfray 2015)). In this literature, however, some common insect pathogens appear to have been rarely used to challenge insects with protective symbionts, including the generalized and highly virulent entomopathogenic fungus *Beauvaria bassiana* (Ascomycota: Sordariomycetes: Cordycipitaceae) (Feng et al. 2004). *Beauveria bassiana* has been found in a wide range of habitats and it infects many insect taxa (Meyling & Eilenberg 2007). This fungus is commonly used in biological control efforts (Faria & Wraight 2001), so it's clearly relevant to know whether insect symbionts can confer protection against it. In one study, pea aphid symbionts that were successful in conferring protection against several other fungal pathogens did not provide protection from *B. bassiana* (Parker et al. 2013).

Rickettsia is a common genus of bacteria, well known as a vector-borne pathogen of vertebrates. Members of the genus are also regularly found in symbiosis with invertebrates (Perlman et al. 2006). *Rickettsia* sp. nr. *bellii* was found in the species complex of whiteflies collectively known as *Bemisia tabaci* in 2006 (Gottlieb et al. 2006). In the Southwestern U.S.A the *Rickettsia* infection spread rapidly in *B. tabaci* (MEAM1 species; Liu et al. 2012) from 2000-2006, and

was associated with benefits to whiteflies, including greater fecundity and shorter development time, as well as female bias (Himler et al. 2011). In recent work, benefits of *Rickettsia* symbionts have varied among whitefly genotypes (Cass et al. 2016, Hunter et al. 2016).

A *Rickettsia* in pea aphids, which is closely related to the whitefly *Rickettsia,* protects the aphid against the entomophthoralean fungus *Pandora neoaphidis* (Lukasik et al. 2013). In *B. tabaci*, *Rickettsia* provided protection against entomopathogenic strains of the ephiphytic bacterium, *Pseudomonas syringae* (Hendry et al. 2014). Here we address the question of whether *Rickettsia* in *B. tabaci* may confer protection against the general fungal pathogen *B. bassiana*, and whether symbiont-mediated defense is conditional on host genotype.

Materials and Methods:

Insect Cultures:

Whiteflies of the MEAM1 biotype were collected in 2006, 2009, and again in 2015, from a cotton field in Maricopa county, Arizona. In order to maintain a difference in infection status but homogenize the nuclear genetic background, paired sublines of *Rickettsia*-infected ("R^+") and uninfected ("R^-") were produced from each of the first two collections by outcrossing methods detailed in Himler et al. (2011). Briefly, for each line, R^+ and R^- whiteflies were identified and then

grouped in separate cultures. *Rickettsia*-infected females were then crossed with uninfected males. Backcrossing with uninfected males was repeated for six generations, at which point greater than >98% of nuclear alleles would be shared between the outcrossed R^+ subline and the original R^- subline. These lines will be referred to as MAC1 (from the 2006 collection, with sublines MAC1 R^- and MAC1R^+) and MAC2 (from the 2009 collection, also with R^- and R^+ sublines).

For assay three, we used a simpler, two generation outcrossing method to introduce outbred, wildtype genes from the 2015 whitefly collection into both laboratory lines. Females from all four MAC1 and MAC2 sublines were mated with males originally collected from the field in Semptember 2015, and kept in large ventilated cages in the laboratory on cowpeas without any selection until use in April and May of 2016. The progeny from this cross were then back-crossed to the wildtype males, and the progeny of this cross were used in the nymphal stage for exposure to the fungal pathogen. In these haplodiploid nymphs, an average of 75% of the nuclear genes of the females and 50% of the nuclear genes of the males are predicted to be derived from the wildtype whiteflies.

Experiments 1 and 2: Influence of *Rickettsia* infection on dose dependent mortality of two genetic lines of whiteflies exposed to *Beauveria bassiana*.

Whiteflies were reared on cowpea plants in ventilated Plexiglass cages in climate-controlled environmental chambers (27°C, 16L: 8D, ambient humidity).

To produce whitefly nymphs for fungal assays, cowpea seedlings were infested with adult whiteflies from one of the four whitefly sublines: MAC1 R^-, MAC1 R^+, MAC2 R^-, and MAC2 R^+. For logistical reasons, MAC1 and MAC2 sublines were examined in separate experiments. After inoculation, whiteflies were allowed to oviposit for 1-3 days until egg density was ca. 100-200 eggs per leaf, after which the adults were removed. The eggs were left to develop for approximately 2 weeks, until a majority of the whitefly nymphs reached the third instar nymphal stage.

Leaf discs bearing nymphs were cut to fit 35 mm Petri dishes. All unhatched eggs, dead nymphs, and nymphs close to the cut edge of the leaf were removed from the leaf discs. When all disks were cut and prepared they were immersed in either Mycotrol™ [http://www.bioworksinc.com] *B. bassiana* fungal solution (treatment discs), or in water (control). The fungal treatments were based on the recommended application rate for insect control (the "high" treatment,) and 10% ("low") and 50% ("medium") of that rate. The spore count for these doses was estimated by The original concentration of the product used, Mycotrol™, was calculated by the manufacturer to be 2×10^{13}. Using this count, unit conversions were made in order to estimate the number of viable spores applied to each dipped leaf disk. Dilutions were calculated for the three doses: high (50% the recommended rate, 2.5 ul/ml, 1.25×10^7 spores , medium (25% the recommended rate, 6.25×10^6 spores/ml) and low (10% the

recommended rate, 3 X 10^6 spores).Leaf discs were then dipped in the appropriate solution using a pair of forceps, until the leaf side bearing the nymphs was entirely covered with liquid and then removed and placed on filter paper in a 35 mm Petri dish. The Petri dishes were covered by a screen-covered lid, in which a second disk of filter paper was fitted, to allow for air circulation, but to raise the humidity for successful infection and contain any sporulation from the fungus. Dishes were then incubated in an environmental chamber (27°C, 16L: 8D, 65% humidity) for four days before being examined for nymphal mortality. Each of the two experiments (the MAC1 experiment and the MAC2 experiment) was performed in two temporal blocks. In each block a set of 6 replicates of leaf discs containing between 100-200 nymphs were used for each dose and control dishes.

Experiment 3: Influence of Rickettsia infection on susceptibility to fungus *Beauveria bassiana* in whitefly lines outcrossed with wild type whiteflies.

The results of the first two experiments suggested that *Rickettsia* was protective in one whitefly line but not the other. Therefore, we sought a method to better represent the nuclear genetic variation in whiteflies in the field, while maintaining distinct R^+ and R- infection status. To do this, we cultured whiteflies collected from the field and used them for two generations of outcrossing, as described above. For each of the original four sublines, two new outcrossed replicate sublines were created in separate cages to help distinguish

outcrossing effects from any shared environmental effects. Fungal susceptibility of these eight new sublines in two backgrounds, MAC1-O and MAC2-O, was compared after exposure to a single fungal dose corresponding to the "high" dose of the previous experiments. All other details of the bioassay remained the same as described in previous experiments, and the bioassay was performed in two temporal blocks. For each block a set of 6 replicates of leaf discs with between 100-200 nymphs were used for each sub-line and control dishes.

Statistical Analysis:

In each of the three experiments, we analyzed the percent of dead/total whitefly nymphs in a logistic regression, using the statistical software R (R Core Team 2015), and fungal treatment assays were analysed separately from water-dipped control treatments. In the first two experiments, explanatory variables included *Rickettsia* presence or absence, fungal dose, and block. In experiment three, a mixed general linear model with "cage" as a random effect was also run, using the R package lme4 (Bates et al. 2015). When cage was not significant in the mixed model, data from both cages were collapsed for each subline, and a linear model was performed, with *Rickettsia* presence or absence, line (MAC1-O or MAC2-O) and block as variables. In all analyses, overdispersion in the original models dictated the use of a quasibinomial model to correct for overdispersion, and an F test (Crawley 2007).

Results:

In the first experiment, focused on the MAC1 whitefly line, the percent of nymphs that were dead after treatment with *B. bassiana* was significantly less in the R^+ subline relative to the R^- subline (Fig. 1A; $F_{1,93} = 23.83$, P= 4.66e-06). Fungal dose was also significant, with lower doses resulting in lower mortality (Fig. 1A $F_{1,92} = 165.59$, P= 2.2e-16). Block was significant as well (Fig. 1A; $F_{1,92} = 1$, P= 0.006). In the water-dipped control treatments, few nymphs died, with a median of 1.9% dead in the R^- and 1.8% in the R^+, but mortality was significantly lower in the R^+ subline (Fig. 1B; $F_{1,21} = 4.795$, P= 0.04). Block was not statistically significant in the controls (Fig. 1B; $F_{1,22} = 2.095$, P= 0.163), nor was interaction between block and *Rickettsia* infection (Fig. 1B; $F_{1,20} = 1.559$, P= 0.266).

In the MAC2 line experiment, the percent of nymphs that were dead after treatment with *Beauveria bassiana* was similar in both the R^+ and R^- sublines (Fig. 2A; $F_{1,69} = 0.8042$, P= 0.37). Dose was statistically significant, with lower doses resulting in lower mortality (Fig. 2A; $F_{1,68} = 354.8398$, P= <2.2e-16), as was the interaction between dose and block (Fig. 2A; $F_{1,66} = 27.558$, P= 1.85e-06). In contrast, block was not significant as a main effect (Fig. 2A; $F_{1,70} = 2.5878$, P= 0.113) and there were no significant differences in nymphal mortality rates between the R^- and R^+sublines of the water-dipped control treatment (Fig 2B, $F_{1,20} = 0.0174$, P= 0.90).

In the third assay, MAC1 and MAC2 lines were outcrossed with outbred whiteflies from the field for two generations to produce the MAC1-O and MAC2-O lines. Here, both *Rickettsia*-infected sublines of MAC1-O and MAC2-O showed significantly reduced mortality relative to the R^- sublines (Fig. 3A; $F_{1,93} = 78.11$, P= 8.13e-14). Block was also statistically significant (Fig. 3A; $F_{1,95} = 19.01$, P= 3.48e-05) as was host line (Fig. 3A; $F_{1,94} = 20.85$, P= 1.59e-05), with a median of 38% dead in the MAC1-O and 47% dead in the MAC2-O lines. Nymphal mortality of the water-dipped control treatments was not significantly different among lines (Fig. 3B; $F_{1,90} = 1.371$, P= 0.245) or with respect to *Rickettsia* infection (Fig. 3B; $F_{1,91} = 0.03$, P= 0.86). Control mortality rates differed significantly between blocks, however, with a median of 3.6% dead in the first block and 6.6% in the second (Fig. 3B; $F_{1,92} = 28.59$, P= 6.716e-07).

Figures:

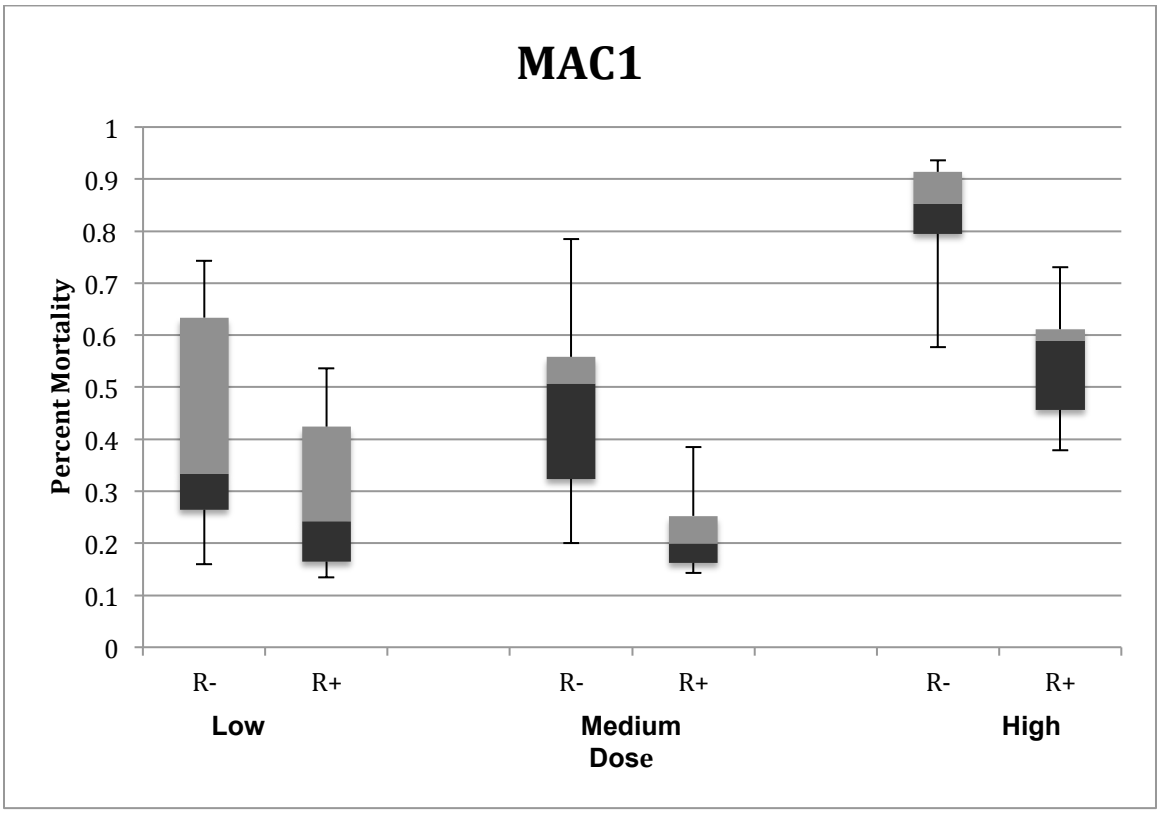

Fig. 1A. The percent of MAC1 whitefly nymphs that died at different doses of *B. bassiana*, Mycotrol™. R^- whiteflies are *Rickettsia*-free, R^+ are *Rickettsia*-infected. The "high" dose is half the recommended rate (2.5 ul/ml), the "medium" dose is 25% of the recommended rate, and the "low" dose is 10% of the recommended rate,. The spore equivalents for the doses were approximately 1.25×10^7 spores/ml (high), 6.25×10^6 spores/ml (medium) and 3×10^6 spores/ml (low). Both fungal dose and *Rickettsia* infection significantly influenced nymphal mortality (see text for details).

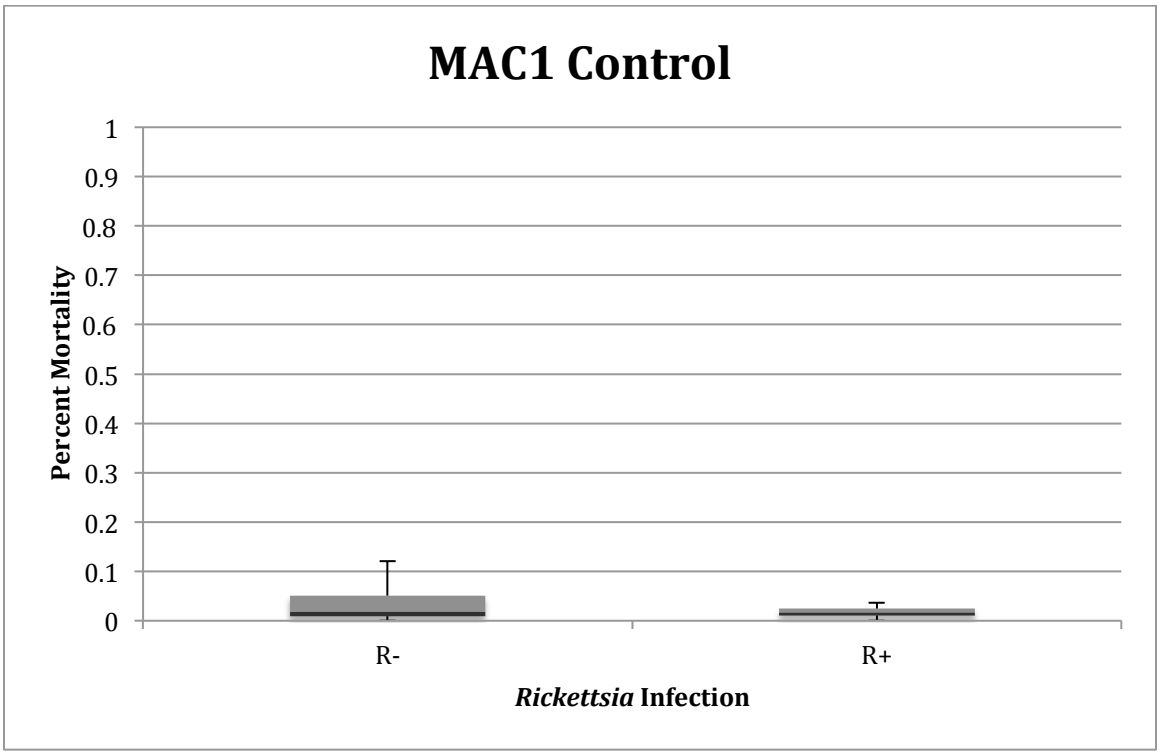

Fig. 1B. The percent of MAC1 whitefly nymphs that died in the water-dipped control. R^- whiteflies are *Rickettsia*-free, R^+ are *Rickettsia*-infected. Although mortality was low in both sublines, a significantly greater percent of R^- whiteflies died than R^+ whiteflies (see text for details).

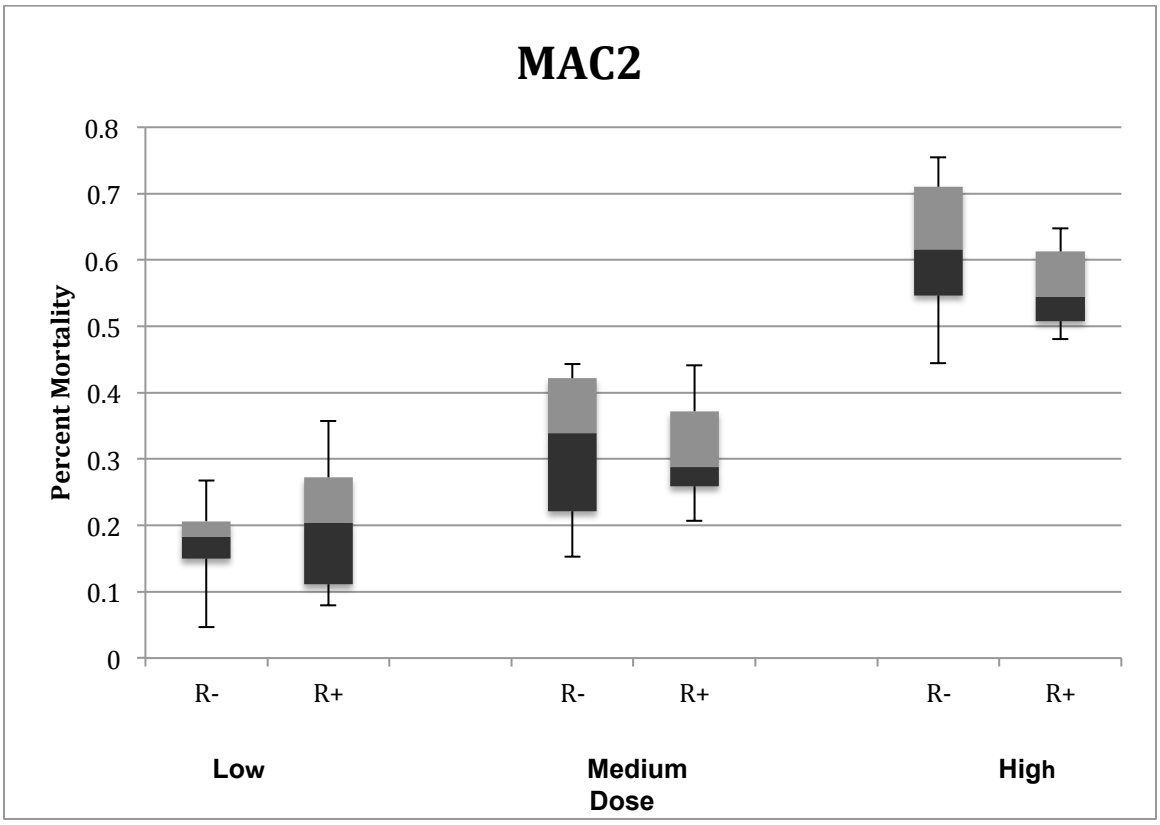

Fig. 2A. The percent of MAC2 whitefly nymphs that died at different doses of *B. bassiana*, Mycotrol™. R^- whiteflies are *Rickettsia*-free, R^+ are *Rickettsia*-infected. The "high" dose is half the recommended rate (2.5 ul/ml), the "medium" dose is 25% of the recommended rate, and the "low" dose is 10% of the recommended rate,. The spore equivalents for the doses were approximately 1.25 X 10^7 spores/ml (high), 6.25 X 10^6 spores/ml (medium) and 3 X 10^6 spores/ml (low). In the MAC2 genetic line, fungal dose significantly influenced nymphal mortality but *Rickettsia* did not (see text for details).

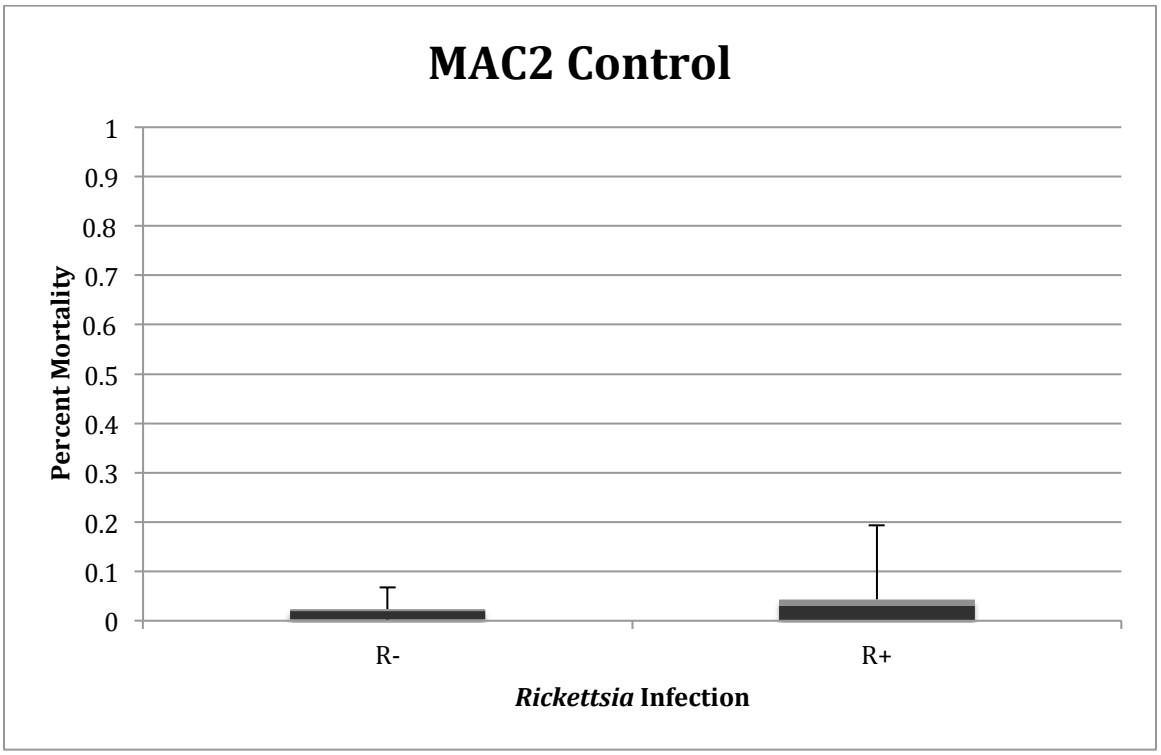

Fig. 2B. The percent of MAC2 whitefly nymphs that died in the water-dipped control. R^- whiteflies are *Rickettsia*-free, R^+ are *Rickettsia*-infected. Mortality was not significantly different between the sublines (see text for details).

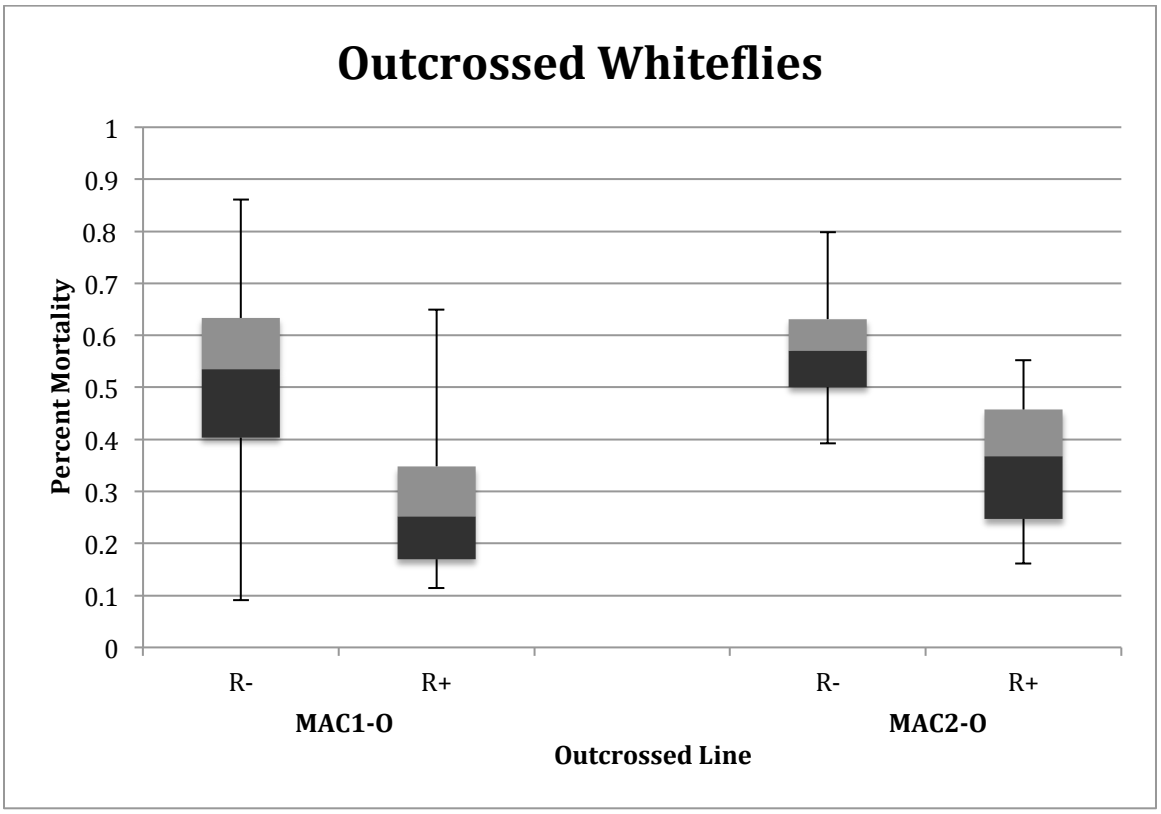

Fig. 3A. Percent mortality of outcrossed lines of whitefly nymphs after exposure to our "High" dose (half the recommended rate (2.5 ul/ml)) of Mycotrol™ (approximately 1.25 X 10^7 spores/ml. R^- whiteflies are *Rickettsia*-free, R^+ are *Rickettsia*-infected. *Rickettsia* infection significantly decreased percent mortality, as did line, with the MAC2-O line dying at a greater rate than the MAC1-O. Block also significantly influenced nymphal mortality (see text for details).

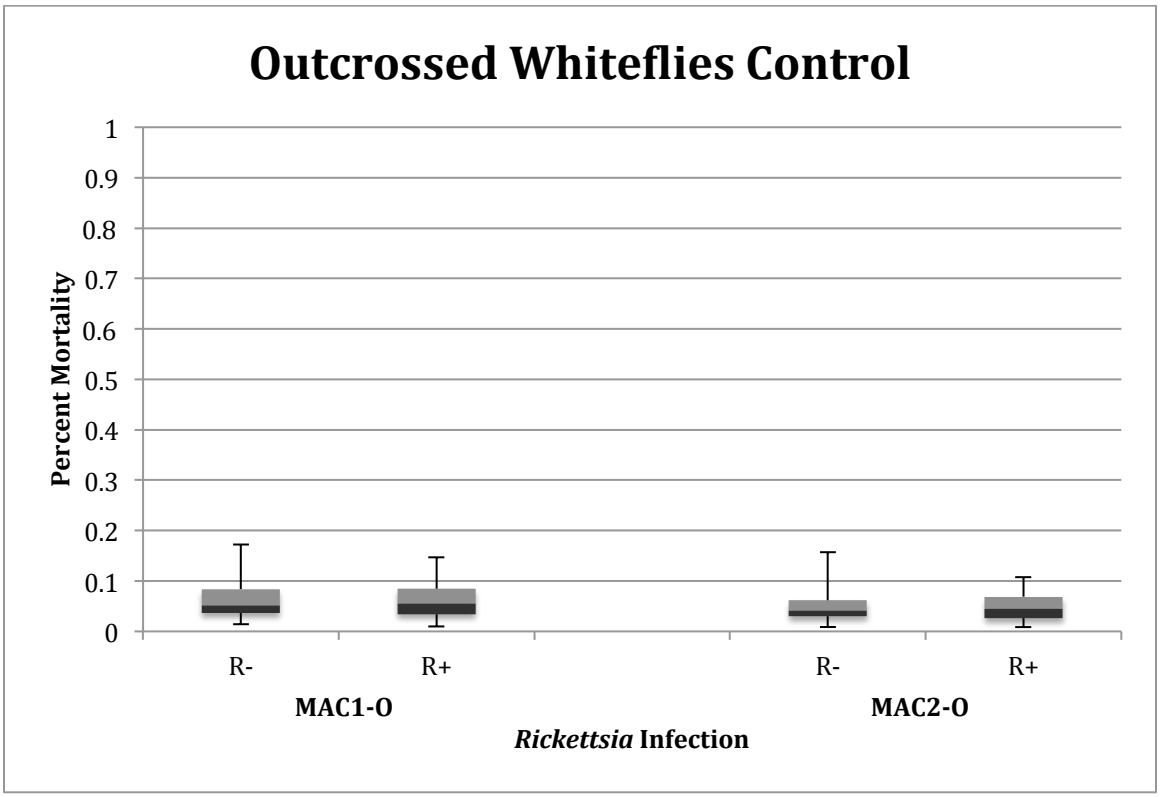

Fig. 3B. The percent of outcrossed whitefly nymphs that died in the water-dipped control. R^- whiteflies are *Rickettsia*-free, R^+ are *Rickettsia*-infected. Neither *Rickettsia* nor line had a significant influence on control mortality, although block was statistically significant (see text for details).

Discussion:

The bacterial endosymbiont *Rickettsia* reduced whitefly mortality in the presence of generalist entomopathogenic fungus, *B. bassiana*. The protection this symbiont offers is linked to the host genotype, with *Rickettsia* conferring protection in the MAC1 line but not in the MAC2 line. These lines differ in their interaction with *Rickettsia* in other respects, including the finding of fewer

fitness benefits associated with *Rickettsia* in the MAC2 line (Cass et al. 2016). The differences between them are associated with host nuclear genes rather than *Rickettsia* differences (Hunter et al. 2016). Our assay of fungal mortality of the original lines outcrossed with an outbred culture of whiteflies showed a more general protective effect of *Rickettsia.* These results suggest that *Rickettsia* may benefit a wider range of whitefly genotypes than are present in the two laboratory lines. Whether *Rickettsia* protects whiteflies in the field from fungal pathogens in the southwestern USA, where this study took place, is unclear. In general, the aridity of this region is likely to limit the frequency and extent of fungal pathogen epizootics (Braga et al. 2007). It would be very interesting to know, however, whether *Rickettsia* protects whiteflies against *B. bassiana* or other whitefly fungal pathogens in the same family such as *Verticillium lecanii* and *Paecilomyces fumosoroseus* in the more mesic climate of the southeastern USA, and many other parts of the world where the *Rickettsia*-infected MEAM1 *B. tabaci* species has invaded (e.g. Hu et al. 2011).

Some fungal pathogen protection mediated by *Rickettsia* could perhaps have been predicted given the potent protection of pea aphids by related *Rickettsia* strain against *Pandora neoaphidis* (Lukasik 2012). However, unlike *P. neoaphidis*, *B. bassiana* is a generalized fungal pathogen, and to our knowledge no study has shown symbiont-conferred protection against this species. *Beaveria bassiana* is often used for biological control efforts. This fungus is effective

against a broad group of insect taxa (Devi 2008, Ibrahim et. al 1993, Kaufman et. al 2005), is resistant to some commonly used fungicides (Loria et. al 1983), and can survive persistently under greenhouse UV radiation levels (Costa et. al 2001). In a previous study, a symbiont that protects pea aphids from *P. neoaphidis* (Scarborough et al. 2005), *Regiella insecticola,* was also protective against another aphid-specific fungal pathogen *Zoopthora occidentalis,* but not against *B. bassiana* (Parker et. al 2013). Although it has been a known entomopathogen for decades, it is not exactly clear why the interactions between *B. bassiana* and known defensive symbionts have not been better studied, especially since defensive symbionts could limit effectiveness of this pathogen against target pests. *Beauvaria bassiana* has also been known to be taken up as defensive symbionts by plants, and when associated as an endophyte can protect its host plant from insects (Ollecka 2008, Alvarez 2006) and bacteria (Ownley 2008). Whether the *Rickettsia* could protect whiteflies that encounter *B. bassiana* as an endophyte is unclear and would be interesting to test.

The mechanism of how *Rickettsia* protects whiteflies is unknown. However, there are multiple examples of symbiont-conferred protection varying with host genotype (Lukasik et al 2013, McLean & Godfray 2015). Mechanisms of protection can include general differences in vigor leading to symbiont-associated performance benefits and resistance to stress (Burke et al. 2009). In the MAC1 line, R^+ whiteflies do generally perform better than R^- whiteflies

(Himler et al. 2011). In the current study, there was a statistically significant, if slight, increase in control mortality in R^- whiteflies relative to R^+. The difference in rates of mortality of R^+ and R^- in controls did not extend to the experiment in which both outcrossed lines showed *Rickettsia*-conferred protection to *B. bassiana*, however. Other mechanisms for symbiont protection include host immune priming (Moreira et al. 2009), competition for limiting host resources (Kambris et al 2009), or a symbiont toxin that differentially targets a particular enemy (Degnan et al 2007, Oliver et al. 2009). Although any of these mechanisms are possible, evidence that *Rickettsi*a protects against both a generalized entomopathogenic fungus as well as entomopathogenic strains of *Pseudomonas syringae* (Hendry et al. 2014) is less compatible with the idea that *Rickettsia* produces a toxin for a specialized target. In contrast, the genome of *Rickettsia* in whiteflies encodes a variety of effectors, which could have a role in immune priming or generalized defense (Zhu et al. 2016).

Future experiments with a range of other fungal and bacterial symbionts would help determine the range of protection conferred by *Rickettsia* and perhaps shed light on the mechanism of *Rickettsia* protection. Similarly, testing the generality of protection by *Rickettsia* sp. nr. *bellii* strains in other taxa of insects to *B. bassiana,* could give us more insight into the whether protection against this pathogen is a common phenotype of this bacterial symbiont lineage. Microscopic approaches could also provide insight into fungal infection and

growth in R+ and R- whiteflies, perhaps suggesting whether the symbiont inhibits penetration of the host by hyphae, or growth within the host (Pekrul et al 1979).

We found that the intracellular symbiont *Rickettsia* can confer protection to whiteflies against a generalist entomopathogenic fungus, *B. bassiana*. This conferred protection is integrally linked to host genotype and is not shown in all genetic backgrounds. In an applied context, symbiont mediated defense, especially against a virulent pathogen like *B. bassiana,* could make biological control efforts and integrated pest management less predictable and effective (Ghanim & Kontsedalov 2009). We might predict as well that strong selection on the pest population in the form of repeated exposure to a entomopathogen would lead to an increase in the frequencies of the symbiont-host genotype combinations that are most resistant, potentially leading to a decrease in pathogen efficacy over time. In contrast, defensive symbionts could benefit biological control agents, for example to protect herbivores employed against weeds, or natural enemies of arthropod pests from predators or hyperparasitoids (Russell et al 2006).

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