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What Goes Up Might Come Down: the Spectacular Spread of an Endosymbiont Is Followed by Its Decline a Decade Later

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Abstract

Facultative, intracellular bacterial symbionts of arthropods may dramatically affect host biology and reproduction. The length of these symbiont-host associations may be thousands to millions of years, and while symbiont loss is predicted, there have been very few observations of a decline of symbiont infection rates. In a population of the sweet potato whitefly species (*Bemisia tabaci* MEAM1) in Arizona, USA, we documented the frequency decline of a strain of *Rickettsia* in the *Rickettsia bellii* clade from near-fixation in 2011 to 36% of whiteflies infected in 2017. In previous studies, *Rickettsia* had been shown to increase from 1 to 97% from 2000 to 2006 and remained at high frequency for at least five years. At that time, *Rickettsia* infection was associated with both fitness benefits and female bias. In the current study, we established matrilines of whiteflies from the field (2016, *Rickettsia* infection frequency = 58%) and studied (a) *Rickettsia* vertical transmission, (b) fitness and sex ratios associated with *Rickettsia* infection, (c) symbiont titer, and (d) bacterial communities within whiteflies. The vertical transmission rate was high, approximately 98%. *Rickettsia* infection in the matrilines was not associated with fitness benefits or sex ratio bias and appeared to be slightly costly, as more *Rickettsia*-infected individuals produced non-hatching eggs. Overall, the titer of *Rickettsia* in the matrilines was lower in 2016 than in the whiteflies collected in 2011, but the titer distribution appeared bimodal, with high- and low-titer lines, and constancy of the average titer within lines over three generations. We found neither association between *Rickettsia* titer and fitness benefits or sex ratio bias nor evidence that *Rickettsia* was replaced by another secondary symbiont. The change in the interaction between symbiont and host in 2016 whiteflies may explain the drop in symbiont frequency we observed.

Keywords Symbiont dynamics · Symbiosis · *Wolbachia* · Microbiome · *Bemisia tabaci* · *Rickettsia*

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Introduction

Bacterial endosymbionts of arthropods are widespread and may influence many aspects of host biology and ecology, including nutrient use, abiotic tolerances [1, 2], defense against natural enemies [3–6], host-plant interactions [7, 8], and longevity or reproduction [9, 10]. Host-symbiont interactions exist across a broad and dynamic spectrum of integration with the host and duration of the association. On the one end, obligate nutritional symbionts are generally intracellular and faithfully maternally transmitted and may have associations with their hosts that span hundreds of millions of years [11, 12]. These *primary* symbionts provide obligate functions for their hosts [13] (e.g., amino acid provisioning by *Buchnera aphidicola* in aphids [14], or B vitamin synthesis by *Wigglesworthia* in tsetse flies [15, 16]). In contrast, facultative or *secondary* intracellular symbionts are not required by the host and have relationships with their hosts that are more variable, and their generally larger genomes suggest shorter association times with particular hosts [17]. The secondary symbiont *Wolbachia* has been estimated to be associated with hosts for an average of seven million years [18].

Facultative symbionts may spread through a host population by acting as mutualists that provide fitness benefits to their host. Conversely, they may act selfishly by manipulating host reproduction, increasing transmission to, or fitness of, the infected females. Reproductive manipulation phenotypes include male-killing, in which males die in early development, potentially releasing resources to infected females, feminization, in which genetic males develop as functional females, parthenogenesis induction, in which genetic males are converted to genetic females, or via cytoplasmic incompatibility (CI), a crossing incompatibility that favors infected females [10]. Reproductive manipulators may also do their host both harm and good. For example, CI-inducing *Wolbachia* may also cause viral pathogen blocking, in which viral titers in the host are substantially reduced, relative to uninfected hosts [19, 20], and may assist in iron metabolism [21] in fruit flies. Symbionts that show both costs and benefits could be either beneficial or parasitic in different contexts. Fitness benefits associated with facultative symbionts that mediate ecological interactions are expected to be heterogeneous and especially dynamic in the environment, as their benefits are commonly conditional on the presence of the stress [22]. In the absence of the stress (e.g., natural enemies or temperature extremes), these symbionts may be costly to the host [23], leading to variation in infection frequency in host populations. For these reasons, we expect symbiont phenotypes that are conditional on the environment or confer both host fitness benefits and reproductive manipulation to be especially dynamic [18].

We might expect a change in the equilibrium infection frequency of beneficial symbionts in host populations to be influenced by or associated with a few factors [24]. First, a change in the net benefit or reproductive manipulation of the symbiont (perhaps because of a change of environment or host genetic background) will have selective consequences for the symbiont frequency. Second, when the strength of the symbiont phenotype is dependent on bacterial titer within hosts, something that is often, if not always true [24–29], a decline in bacterial titer might reflect a reduction in the penetrance of the phenotype and be associated with a decline in symbiont frequency. Third, a reduced maternal transmission rate of the symbiont will deterministically drive decline in symbiont frequency. Lastly, symbiont infection frequency can be influenced by the presence of other symbionts. Knowing how symbiont interactions develop, change, and break down over time will increase our understanding of the linked population genetics and ecologies of host and symbiont [24, 29].

Rickettsia is a facultative, maternally transmitted endosymbiont that swept rapidly into the US population of invasive sweet potato whiteflies (*Bemisia tabaci* (Hemiptera: Aleyrodidae), provisional species designation "Middle East-Asia Minor 1 or MEAM1") [30]. This endosymbiont strain is in the *Rickettsia bellii* clade and is closely related to the sequenced strain *Rickettsia* sp. strain MEAM1 from whiteflies in China and Israel [31, 32]. In Arizona whitefly populations, *Rickettsia* rose from an infection frequency of 1% to near-fixation (97%) in a six-year period (2000–2006) [9]. In 2011, high frequencies of *Rickettsia* were observed throughout its host range in the Southern United States [32]. This dramatic rise in infection frequency was accompanied by evidence from the laboratory of both reproductive manipulation and fitness benefits to *Rickettsia*-infected Arizona *B. tabaci* [9]. In a line where the uninfected whitefly background was introgressed into *Rickettsia*-infected (R^+) whiteflies to create a homogenous nuclear background, R^+ mothers produced more female offspring than uninfected (R^-) mothers, a type of reproductive manipulation. At the same time, *Rickettsia* increased fitness and performance of infected female whiteflies; R^+ whiteflies showed higher survivorship to adulthood, increased fecundity, faster development time [9], and increased resistance to pathogens [33]. In further studies, we demonstrated that the benefits of infection were reduced in a second introgressed laboratory line, although the female bias persisted [34]. Further, the differences in the effects of *Rickettsia* in the two lines were shown to be explained by host nuclear genotype [35]. All of this suggests that selection can act on heritable variation in the relationship between Rickettsia and its whitefly host.

Given this context, the population dynamics of *Rickettsia* in whitefly populations may be labile and affected by the coevolutionary interactions between the symbiont and its host. Indeed, this study was motivated by preliminary data showing the field infection frequency of *Rickettsia* had dropped from its previous near-fixation levels. Here, we addressed the question: "Has the infection frequency of *Rickettsia* declined in its

whitefly host population?" We further explored factors predicted to affect interactions between symbiont and host that could influence the infection frequency of *Rickettsia* in whitefly populations in the field:

1. *Maternal transmission frequency*: A decrease in symbiont transmission from mother to offspring would decrease *Rickettsia* frequency, possibly leading to the eventual elimination of the symbiont from the population.
2. *Titer of infection*: A decreased titer of infection within hosts (for example, following selection for host nucleotypes that dampen infection titer) could alter the host-symbiont phenotype. If the low-titer symbiont phenotype is then neutral or costly in fitness value to the host, we would predict an eventual drop in infection frequency in the population.
3. *Fitness effects of infection*: Whether changes in symbiont titer are involved in changing the relationship between symbiont and host or not, heritable differences in the fitness effects of the symbiont will drive changes in symbiont frequency in the host population. A decrease in net benefits to hosts or a loss of the reproductive manipulation of hosts could allow the frequency of the symbiont in the population to be reduced by selection (if the fitness consequences were negative) or drift (if the fitness consequences were near neutral).
4. *Presence of other secondary symbionts*: We determined whether the decline in *Rickettsia* frequency was accompanied by the rise in infection frequency of another symbiont. Competitive interactions between symbionts can lead to replacement of one lineage by another [36, 37].

Methods

Field Frequency of Infection

Sampling

In the large species complex that is collectively denoted by the name *Bemisia tabaci*, one invasive species, the "B" or "MEAM1," has dominated US field populations since its introduction [38]. In addition to *Rickettsia*, two other symbionts occur and have been shown to be present in every individual in this species: *Portiera aleyrodidarum* is the primary nutritional symbiont of all whiteflies [39], while *Hamiltonella defensa* is also fixed in *B. tabaci* MEAM1 and is presumed to also have a nutritional role [40, 41]. We collected this species of whitefly from plots of field cotton at the University of Arizona Maricopa Agricultural Center (Maricopa, AZ) across the three successive years of 2015–2017. Adult whiteflies were collected from 10–20 plants, each at least 10 m apart. No more than 10 whiteflies were collected from any one plant. All whiteflies were preserved in 97% ethanol, transported on ice, and stored at − 20 °C.

DNA Extraction and PCR

For each collection date, we randomly sampled at least 50 female whiteflies for determination of infection status (August and September 2015: $n = 218$; November 2016: $n = 85$; October 2017: $n = 50$). We extracted DNA from individual female whiteflies using a Chelex extraction protocol [42], 10 μL final volume. We also extracted DNA from randomly sampled female whiteflies collected from melons at the University of Arizona Yuma Agricultural Center (Yuma, AZ, 270 km west of Maricopa; September 2017: $n = 50$). PCR for *Rickettsia* was performed using *Rickettsia*-specific 16S rDNA primers 528F [5-ACTAATCTAGAGTGTAGTAGGGGATG ATGG-3] and 1044R [5-GTTTTCTTATAGTTCCTGGC ATTACCC-3] with 4 μL samples of DNA template [43]. All samples were run with a positive (confirmed R^+ whitefly extraction) and negative (no DNA template) control, and an additional PCR was performed to confirm the species status of the whiteflies using primers that amplify different product sizes [44]. All whiteflies were confirmed to be MEAM1.

In addition, to determine the sensitivity of our PCR protocol for detecting *Rickettsia*, we conducted an assay to determine limits of detection [32] in which DNA extractions from the field that were positive for *Rickettsia* in a PCR reaction were diluted in a tenfold dilution series. Standard PCR protocols and gel visualization techniques were applied to these full-strength extractions and dilutions. With this method, we found that 8 of 17 samples could be diluted tenfold and still be detected, while 9 could not be diluted. This suggests that titers of *Rickettsia* had dropped in the field, and we were closer to the limits of detection than in a previous study [32]. To increase the likelihood of detecting low-titer infections, all *Rickettsia* negative samples were rechecked via PCR or qPCR (see qPCR methods) at least two additional times to confirm the initial findings.

Establish Lab Matrilines

We collected live adult whiteflies from field cotton at the Maricopa Agricultural Center in August 2016. Groups of at least 50 males and 50 females each were reared on caged cowpea plants (*Vigna unguiculata* var. California blackeye; $n = 12$) for one generation in the lab. Cowpeas were used for experiments because they are fast growing and able to tolerate high densities of whiteflies. We isolated female late fourth instar larval whitefly progeny (*pupae*) immediately prior to eclosion to assure they were unmated, and used these to established matrilines. For each matriline, one newly eclosed virgin female was paired with one adult male (randomly

selected from a different plant cage). Each pair was introduced to a separate cowpea leaf disk experimental arena [9] and placed in plant growth chambers (16:8 light:dark, 27 °C, 35% humidity). Whitefly pairs were allowed to mate and lay eggs for 3 days, and then parents were collected from the leaf disks into ethanol. DNA was extracted from mothers, and diagnostic PCR was used to determine the infection status of each line using the methods described above. The F1 offspring were reared on leaf disks until adult eclosion, the newly eclosed adult offspring were removed every 48 h, and the collected offspring were preserved in ethanol and stored at − 20 °C. To propagate the matrilines, sibling cohorts of 2–5 male and 2–5 female newly eclosed F1 offspring from each leaf disk were placed on small cowpea seedlings in ventilated plexiglass boxes (12.5 cm × 8 cm × 2 cm) with the bottom edge of the boxes submerged in trays of water. The whiteflies were allowed to mate and lay eggs for five days before being removed. All leaf boxes were incubated in grow rooms at (16:8 light:dark, 27 °C) and used to establish subsequent generations in the same manner.

For subsequent analyses, we used 72 matrilines. We excluded other lines in which one or more of the parents died or were not recollected after oviposition (and therefore infection status could not be determined), or in which fungi or leaf arena deterioration interfered with offspring development. We also excluded any lines that did not produce at least one daughter, as whiteflies are haplodiploid and can produce sons without mating. Therefore, we could not confirm that the mother had mated when only sons were produced. The group of 59 matrilines we excluded showed no significant differences in frequency of infection or in offspring performance compared to the matrilines used in transmission, titer, and performance assays.

Vertical Transmission Frequency

Rickettsia transmission frequency was determined by sampling 5–10 female F1 offspring from each of 12 R^+ matrilines, randomly selected from the lines established above. We extracted DNA from individual females and performed diagnostic PCR as previously described. Also, in order to determine if transmission frequency was consistent across generations, we sampled 5–10 female F2 and F3 offspring from each of 10 of these R^+ matrilines, extracted DNA, and performed diagnostic PCR to determine infection status.

Titer of Infection

Quantitative PCR

We amplified *Rickettsia gltA* (*citrate synthase*) from total DNA extracted from 5–10 individual female F1 offspring, each 1–2 days old, from each of 20 R^+ matrilines [45]. *Bemisia tabaci actin* DNA (*β-actin*) was amplified as an internal standard to assess *Rickettsia* titer relative to host DNA. Each gene was amplified in triplicate for each biological replicate (individual female whiteflies). Extractions from known R^+ and R^- whitefly samples were included as controls. An ABI Prism® 7000 Sequence Detection System (Applied Biosystems) and accompanying software were used to quantify the real-time quantitative PCR data. Titer (relative density) of *Rickettsia* was calculated by comparing the cycle quantification value (C_t) of the *Rickettsia* gene to the C_t of the whitefly endogenous control gene using the $2^{-\Delta\Delta Ct}$ method [46].

We assessed titer in two previously established *Rickettsia*-infected lab lineages (MAC1 est. 2006 and MAC2 est. 2009) and in whiteflies collected from the same field site in 2011 [32] using the methods described above. Uninfected field whiteflies were excluded from the analysis. To determine if *Rickettsia* titer was heritable, we also extracted DNA from 5–10 individual female F2 and F3 offspring from each of 10 of the R^+ matrilines sampled above, and used qPCR to determine the matriline titers in the F2 and F3 generations as described above.

Fitness Assays to Determine Effects of R^+ Infection

To assess the current fitness effects of *Rickettsia* infection on whiteflies, we assayed the performance and reproductive manipulation of each of our field-collected matrilines, established above, during their first (F1) generation on cowpea leaf disks. To measure performance, we counted the number of eggs laid by each mother and then incubated the disks and, once progeny started to eclose, counted and sexed adult offspring every 48 h. We measured the percentage of offspring surviving to adulthood and the development time (days to eclosion). The percentage of unhatched eggs was determined at the conclusion of the experiment by counting unhatched eggs remaining on the leaf disk. To assess the reproductive manipulation of the whitefly host by *Rickettsia*, we also determined the percentage of adult female offspring produced by each mother.

Bacterial Community Characterization of Sample Whiteflies

We extracted DNA from single whiteflies sampled from laboratory lines, field-collected whiteflies (2011), and whitefly matrilines established from the field in 2016, as described above. We also performed an extraction without a whitefly to serve as a negative control. We then used the universal bacterial primer set 341f (5′-CCTACGGGNGGCWGCAG-3′) and 785r (5′-GACTACHVGGGTATCTAATCC-3′) to amplify the V3–V4 hypervariable regions of the 16S rRNA [47]. Library preparation and indexing followed the protocol outlined by Illumina [48]. The library was bidirectionally

sequenced on an Illumina MiSeq platform at the University of Arizona Genetics Core using 2 × 300 chemistry.

Sequences were first processed with the program *cutadapt* [49] to remove priming sites at the ends of sequences. Next, we truncated the sequences at the first instance of a quality score less than two and discarded sequences that contained any unassigned bases (*N*s) or had an overall expected error score higher than two. We used the DADA2 algorithm to infer which bacterial strains were present in the samples [50]. After de novo chimera checking and removal, taxonomy was assigned using the RDP classifier and the SILVA nr v123 training set [51, 52]. We detected seven bacterial strains in the negative extraction blank. Only one of these, a *Propionibacterium* species, was also detected in a sample. We removed all seven of these contaminant strains from the dataset prior to analysis. To control for differences among samples in sequencing depth, we discarded samples with low numbers of reads and rarefied sequences to 12,465 reads per sample. We generated a bar plot of strain composition within individual insects using the R package "phyloseq" [53].

Analysis

We used ANOVA to test for significant differences in *Rickettsia* titer among groups, and Tukey-Kramer pairwise comparisons were performed to relate infection titers of laboratory lines and 2016 field matrilines to titers of whiteflies previously collected from the same site in 2011. We similarly used ANOVA to test for significant differences in performance and reproductive effects between R^+ and R^- whiteflies as well as high R^+ titer, low R^+ titer, and R^- whiteflies. Count data was square root adjusted, and percentage data was arcsine transformed. A post hoc Bonferroni correction was used for multiple comparisons. The symbiont titers across three generations within lines were compared using Pearson's correlations. Results were analyzed using SAS 9.3.

Results

Field Frequency of Infection

We observed a drop of almost 50% in *Rickettsia* infection frequency of whiteflies in 2015 and 2016 (Maricopa 2015, 58%, n = 218; 2016, 58%, n = 85) compared to the high (93%) frequencies measured five years earlier, in 2011 [32] (Fig. 1). The decline in *Rickettsia* frequencies was also shown to continue in the sixth year (2017, 36%, n = 50; Fig. 1). Since our previous study of whiteflies collected in areas around Arizona and New Mexico in field cotton found that *Rickettsia* infection frequency rose rapidly from 1% in 2000 to near-fixation in 2006, the observations taken together show symbiont spread over six years, a high frequency for at least five years, and a gradual decline over 3-6 years (Fig. 1). The decrease in *Rickettsia* infection frequencies observed in Maricopa in 2017 were also comparable to those observed in whiteflies collected from melons in Yuma, AZ, in the same year, 270 km away (Yuma 2017, 34%, n = 50), suggesting that the frequency drop was regional and not limited to a specific location.

Transmission Frequency

Himler et al. [9] determined the rate of maternal transmission of *Rickettsia* to whitefly daughters in a laboratory line established from the field in 2006 to be 99.2% (n = 1 uninfected out of 120 whitefly females). In the current study in 2016, we found similarly high rates of transmission. In 12 *Rickettsia*-infected matrilines, whiteflies transmitted the infection to 98.0% of the female offspring sampled (n = 2 uninfected out of 100 female offspring in two of 12 matrilines). In addition, the two matrilines that had uninfected offspring in the F1 generation were not the same matrilines that had uninfected offspring in either the F2 or F3 generation (F2 uninfected: n = 0 out of 79 offspring, 0 of 10 matrilines; F3 uninfected: n = 2 out of 74 offspring, two of 10 matrilines), providing no evidence of heritable variation in symbiont transmission rates.

Titer

Titers of *Rickettsia* in whiteflies collected in the field in 2011 were comparable to the titers of whiteflies from two introgressed laboratory lines established in 2006 and 2009 [9, 34] and resampled in 2016 for the current study (Fig. 2). In contrast, the matrilines collected in the field in 2016 displayed strongly bimodal *Rickettsia* titer. Titer within matrilines either showed a significantly lower titer of infection (70% of the time, n = 14 of 20 lines, 6–12 whiteflies/line; Tukey-Kramer pairwise comparisons, $p < 0.002$) or were comparable to, or in one case, significantly higher than the 2011 infection titer (30% of the time, n = 6 of 20 lines, 6–12 whiteflies/line; Tukey-Kramer pairwise comparisons; Fig. 2). Laboratory lineages established in 2006 and 2009 ("MAC1 and MAC2" of [34, 35]) showed no significant difference in infection titer compared with 2011 field-collected whiteflies (Fig. 2).

Rickettsia titer appeared heritable. Among 10 matrilines, three of three high-density matrilines had high titers in F2 and F3 offspring sampled, and seven of seven low-density matrilines had low titers in F2 and F3 offspring sampled (Pearson's correlation: $p < 0.01$, all generations; F2: n = 6–10 whiteflies/line, F3: n = 6–11 whiteflies/line; Fig. 3).

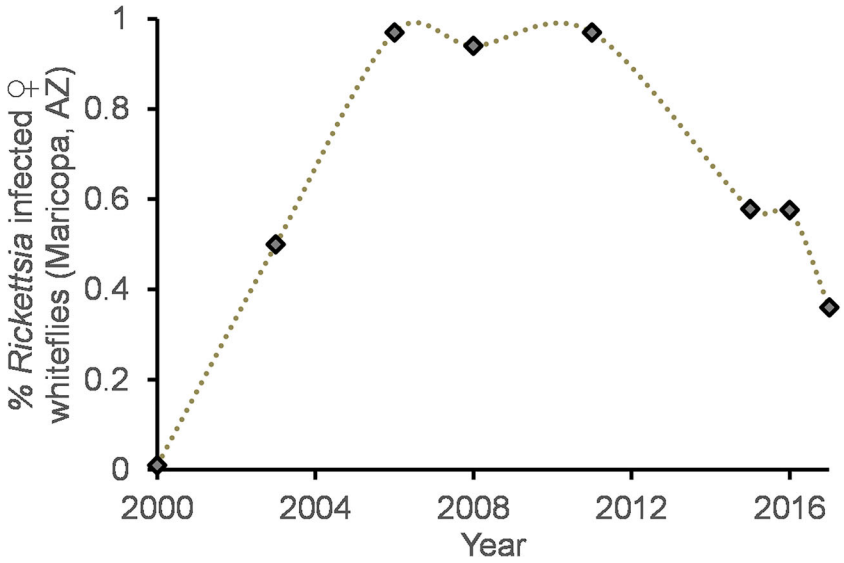

Fig. 1 Percentage of *Rickettsia*-infected field-collected female whiteflies from Maricopa, AZ, field site. Data for the samples from 2000 to 2011 were published previously [9, 32]

Fitness Effects

Rickettsia infection was not significantly associated with improvements in whitefly performance (Fig. 4). We found no significant difference in the number of eggs laid ($F_{1,51}$ = 2.05, p = 0.1579; Fig. 4a), percent of offspring surviving to adulthood ($F_{1,51}$ = 1.40, p = 0.2426; Fig. 4b), or speed of development to adulthood (female: $F_{1,51}$ = 1.05, p = 0.3111; male: $F_{1,39}$ = 0.30, p = 0.5833; Fig. 4c, d). In fact, the one statistically significant difference suggested a cost; *Rickettsia*-infected whiteflies had significantly lower rates of egg hatching than did uninfected whiteflies ($F_{1,51}$ = 6.14, p = 0.0165; Fig. 4e). *Rickettsia* infection also did not significantly affect the percent of female offspring produced compared to uninfected whiteflies ($F_{1,51}$ = 0.50, p = 0.4807; Fig. 4f).

Rickettsia titer did not clearly influence whitefly performance. There were no statistically significant differences in performance among the high-titer, low-titer, and uninfected lines (Fig. 5; eggs laid: $F_{2,37}$ = 1.16, p = 0.3249; survival: $F_{2,37}$ = 2.74, p = 0.0775; female development time: $F_{2,37}$ = 0.57, p = 0.5685; male development time: $F_{2,36}$ = 1.52, p = 0.2323; percent female: $F_{2,37}$ = 0.10, p = 0.9030), except in the case of egg hatch, where an average of 8.4% of eggs from low-titer lines failed to hatch compared to 3.6% from high-titer lines and 2.9% from R^- lines (Fig. 5; non-hatching: $F_{2,37}$ = 3.36, p = 0.0455).

Bacterial Community Characterization of Sample Whiteflies

The bacterial communities of all individual whiteflies assessed (laboratory, field, and matrilines established from the field) were dominated by the three lineages we expected to find: *Portiera aleyrodidarum*, the primary nutritional symbiont; *Hamiltonella defensa*, a symbiont that is also fixed in the MEAM1 whitefly species; and the *Rickettsia* that is the

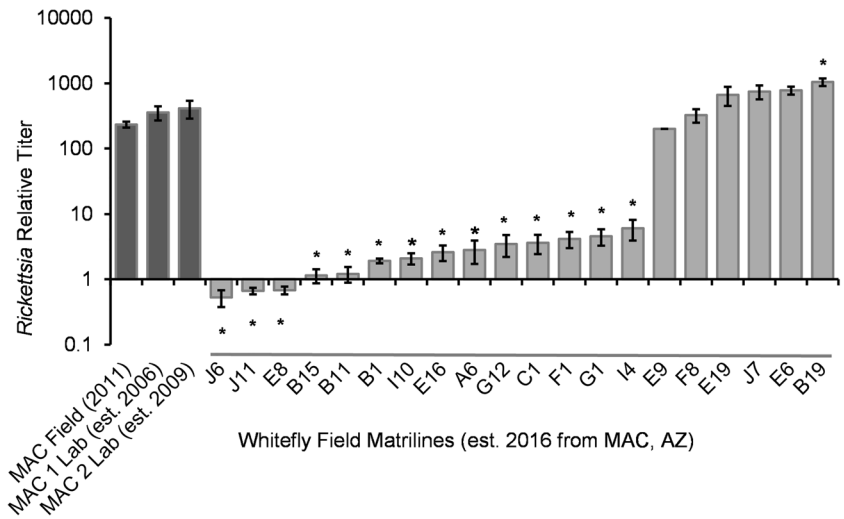

Fig. 2 The average relative titer of the *Rickettsia gltA* genes in adult female whiteflies from each lineage relative to host DNA (*β-actin*), plotted on a logarithmic scale. Asterisks show groups with least-square means that were statistically significantly different from the 2011 field infection level (first bar) using Tukey-Kramer pairwise comparisons with Bonferroni correction for multiple comparisons. Error bars show standard error. n = 6–12 individuals for each of the samples

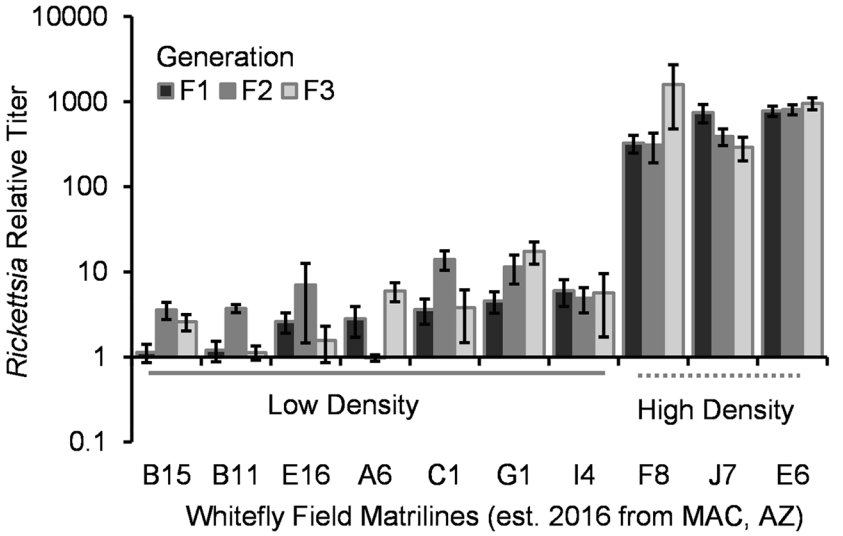

Fig. 3 The average relative titer of *Rickettsia* in adult female whiteflies from each of three generations of 10 matrilines compared to host DNA (*actin*). Matrilines were categorized as "low density" or "high density" based on estimates of F1 whitefly *Rickettsia* titer. Error bars show standard error (n = 6–12 whiteflies per line in each generation)

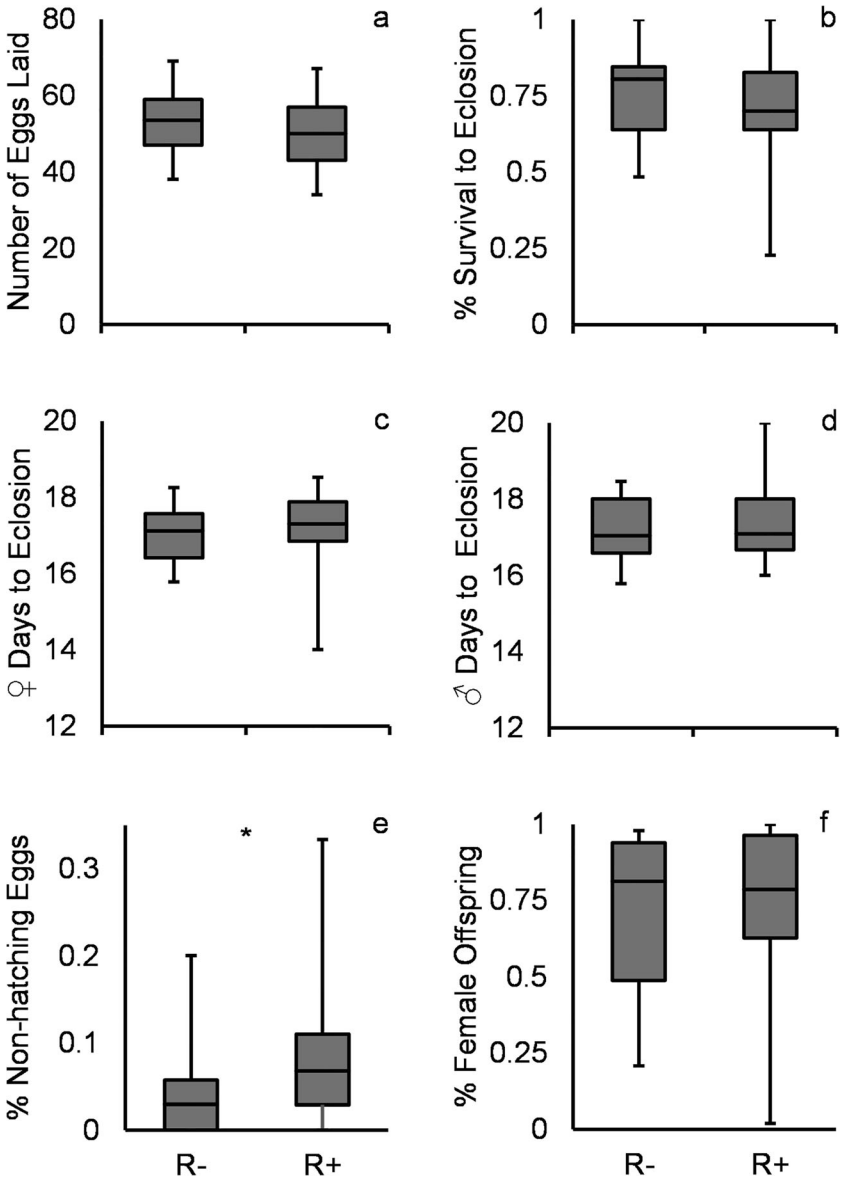

Fig. 4 Boxplots of outcomes for F1 offspring of uninfected whitefly mothers (R^-, n = 22) vs. infected whitefly mothers (R^+, n = 31). The dividing line in each box refers to the median value, and the bottom and top of the box enclose the first and third quartile, respectively. The lines indicate the range of the data. The asterisk indicates a significant difference between R^- and R^+ whiteflies ($p < 0.05$)

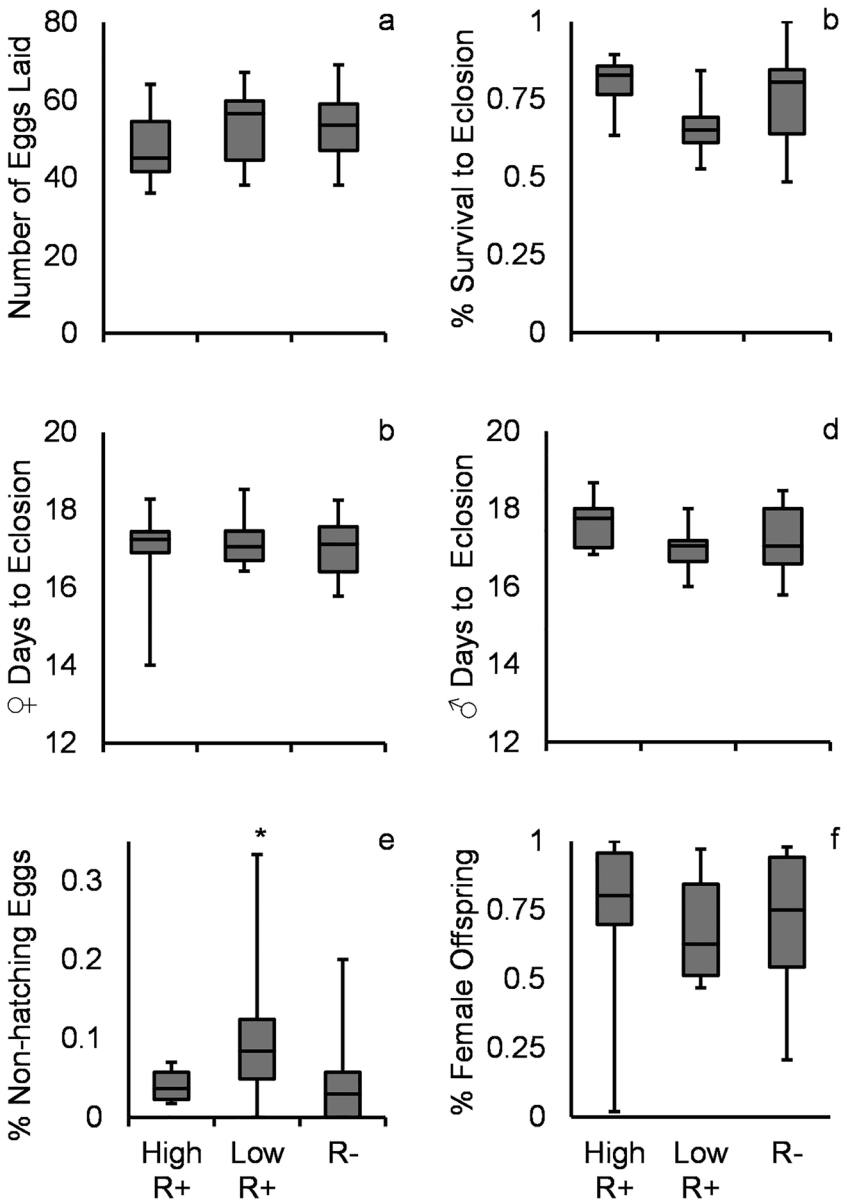

Fig. 5 Boxplots of outcomes for F1 offspring of uninfected whitefly mothers (R^-, $n = 22$) vs. infected whitefly mothers (R^+, $n = 31$; whiteflies collected in 2016). Infected whiteflies were categorized as "high R^+" if their infection titer was found to be as high or higher than 2011 R^+ whiteflies ($n = 6$) or as "low R^+" if their infection titer was found to be significantly lower than 2011 R^+ whiteflies ($n = 12$). The dividing line in each box refers to the median value, and the bottom and top of the box enclose the first and third quartile, respectively. The lines indicate the range of the data. Asterisks indicate significant differences among groups ($p < 0.05$)

focus of the current study (Fig. 6). While the number of *Rickettsia* reads was higher in the "high-density matrilines" than in the "low-density matrilines" as expected, and absent in the R^- matrilines, there was no other apparent pattern to the relative abundance of the three dominant bacteria (Fig. 6).

We detected a few other bacterial taxa: six strains belonging to the genera *Sphingobium*, *Bacillus*, *Paracoccus*, *Rhizobium*, *Chryseobacterium*, and *Pseudomonas* as well as one unidentified Enterobacteriaceae strain. All seven strains were present at extremely low abundances and represented < 0.1% of the total reads. There was no pattern to suggest these rare taxa were associated differentially with high- vs. low-density individuals or between R^+ vs. R^- whiteflies; in fact, each of the seven strains was only detected in a single whitefly individual. The negative extraction blank did not show any overlap with whitefly sample sequences, suggesting negligible sample contamination.

Discussion

After the dramatic six-year sweep of *Rickettsia* infection of its whitefly host in the Southwestern United States, followed by years of near-fixation frequencies, our results indicate that the frequency of *Rickettsia* infection of whiteflies is now undergoing an almost equally dramatic and rapid decline in frequency in Arizona populations, dropping from ~ 95% in 2011 [32] to as low as 36% a mere six years later. Previous studies have documented the rapid spread of symbionts through

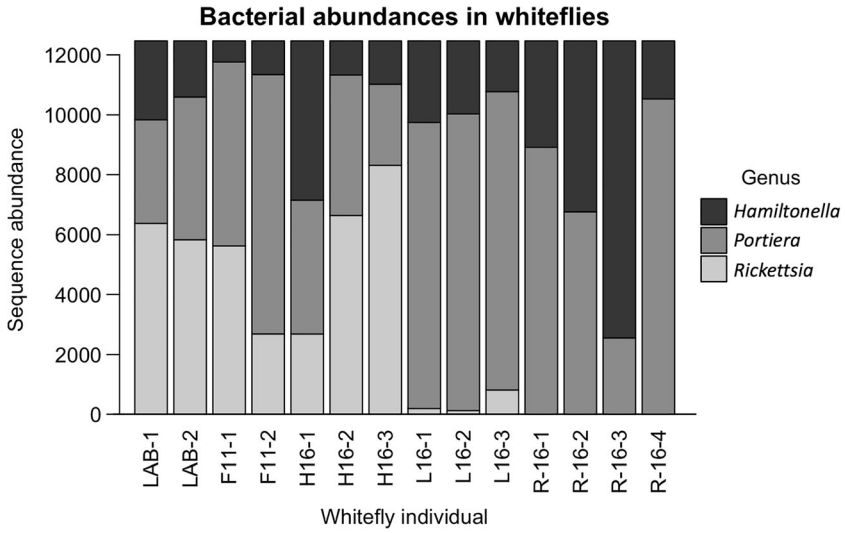

Fig. 6 Each bar represents 16S rRNA bacterial sequence abundances within a whitefly individual, with all samples rarified to 12,465 sequences. The taxonomic identity of the sequences is indicated by shade. The three bacteria shown (*Portiera*, *Hamiltonella*, and *Rickettsia*) represent > 99.9% of all bacterial sequences present. From L to R, the whitefly samples shown are LAB (whiteflies from the MAC1 laboratory line), F11 (whiteflies collected from the field in 2011), H16 (whiteflies from the high-density matrilines established in 2016), L16 (whiteflies from the low-density matrilines established in 2016), and R-16 (whiteflies collected from the field in 2016 that had been determined with PCR to be *Rickettsia*-free)

populations, generally facilitated by either fitness benefits to the host or reproductive manipulation or a combination of both factors [5, 36, 54]. Such rapid within-species invasions fit within a broader picture of symbiont-associated diversification, speciation, and the spread of symbionts between species over (relatively) rapid evolutionary time [55]. A decline in the frequency of symbionts within host populations has been less well documented, however, perhaps because some of the factors that we expect to cause decline (e.g., evolution of host resistance, incomplete vertical transmission, drift) may occur over a longer time scale that is difficult to observe [56]. An important exception to this pattern is the example of the facultative symbionts of pea aphids, in which symbiont frequencies fluctuate in response to environmental variables, even within a season [22]. Here, we explored four proximate factors that could have driven the steady decline in *Rickettsia* infection frequency.

We found no evidence of a change in vertical transmission frequency of infection. *Rickettsia* transmission by female whiteflies remained very high (98%, comparable to the 99% observed 7 years earlier) [9]. This rate is similar to that of many secondary endosymbionts which exhibit near-perfect vertical transmission [57, 58]. We cannot rule out the possibility that a decline in vertical transmission rate could have had a role, however. Our vertical transmission results are from a laboratory setting, and factors such as high temperatures or exposure to natural (or manmade) antibiotic substances have been shown to lower symbiont titer and transmission and may be relevant in the field [59–61].

Within-host density, or symbiont titer, has been linked to transmission rates as well as to the penetrance of symbiont-related effects on host phenotype and sex ratio [26, 28, 29]. We predicted that the observed decline in infection frequency might be driven by a drop in within-host titer of infection, coupled with an associated decrease in fitness benefits of infection. We found marginal support for this prediction. *Rickettsia* titers in whiteflies were, on average, lower than that at the time of peak infection frequency, and performance and sex ratio effects of infection also appeared to be lower or absent. However, about 30% of lines retained high *Rickettsia* titers, comparable to previous observations, and we found no evidence that differences in titer were associated with differences in performance relative to uninfected whiteflies (Fig. 5). While the apparently bimodal pattern of *Rickettsia* titer we observed is intriguing, a targeted study with very high replication might be necessary to discern if any subtle titer-dependent performance costs or benefits exist. Titer differences also did not relate to the presence of another secondary symbiont (Fig. 6). The presence of particular symbiont lineages may cause an increase or reduction in the titer of other taxa in the same host relative to when that symbiont is absent [62, 63], but the heritable bimodal pattern of *Rickettsia* titer in whiteflies we observed occurred in a uniform background of the two other symbionts: *Portiera* and *Hamiltonella*.

Our results instead support the idea that a decrease in the benefits of infection may have driven the decline in symbiont frequency. By examining matrilines established from the field in 2016, we found no association between *Rickettsia* infection in whitefly fecundity, development time, survival to adulthood, or sex ratio. In fact, *Rickettsia*-infected whitefly eggs were slightly less likely to hatch, suggesting a net cost to infection. These data stand in stark contrast to the findings

of Himler et al. [9], in which *Rickettsia*-infected individuals had higher fecundity, shorter development times, higher developmental success, and female-biased sex ratios, relative to uninfected individuals with the same genetic background. The current study results are more similar to *Rickettsia* effects in a second ("MAC2") background, established a few years later. In MAC2, *Rickettsia* had moderate effects, and a one-generation population cage experiment suggested the symbiont was effectively neutral [34].

One limitation to our ability to compare the current results to those of previous studies is that the role of *Rickettsia* was examined here in matrilines established from field-collected mated pairs, and not, as in previous studies, in introgressed laboratory lines. Our approach for the current study allows a much broader sampling of field genotypes and provides more insight into the full phenotypic range of the host-symbiont interaction in the field. However, due to the variation in genetic background of hosts, we expect this type of analysis to be less sensitive to the effects of *Rickettsia* than when the genetic background has been homogenized via introgression [64]. In spite of this, our data represent a reasonably large sample size, and we found no trends in *Rickettsia*-associated differences in sex ratio or benefits to performance.

Given the apparent benefits of *Rickettsia* infection documented at the time of its spread, we might ask: why would this symbiont—or any beneficial symbiont—decline in a host population? We speculate that the observed decline is driven by changes in context-dependent fitness effects of hosting the bacteria. One way in which selection could act is via differences in the interaction of the symbiont with the host nuclear background. While both the fitness benefits and female bias observed in the first laboratory whitefly line (MAC1) should promote spread of the symbiont, the second introgressed line MAC2, as mentioned, showed fewer fitness benefits, but a persistent female sex ratio bias [34]. Female bias will promote symbiont spread, but manipulation of sex ratio should be resisted by the host. We found that the difference in the phenotypic effects of infection in the two lines could be largely explained by the host nuclear genotype, rather than maternally inherited symbiont or mitochondrial lineages [35]. Thus, we predict a difference in the way hosts should respond to selection in the two lines, and in the different genotypes of the population as a whole when the symbiont has variable benefits. If a host developed resistance to infection (either through exerting control of titer or directly on the sex ratio phenotype), we would expect resistance to spread in genotypes like the MAC2 line where infection is costly. If the MAC2-type host genotypes are selectively competitive, we would predict a decline in *Rickettsia* infection across the population. Such shifts in host genotypes might be driven by environmental change or by frequency- or titer-dependent effects (e.g., a nucleotype with a novel fitness strategy may become advantageous only when *Rickettsia* infection is widespread) [59]. We cannot say what caused the decline with certainty, and any number of environmental variables could have also influenced the whitefly-*Rickettsia* relationship. However, the variable benefits of infection among host genotypes that we observed could have been the starting point for the development of host resistance to infection among some genotypes. Should resistant genotypes become dominant, *Rickettsia* infection frequency would decline. This explanation alone presents a plausible hypothesis for the decline we observed.

The Life Cycle of a Symbiont-Host Relationship

While our understanding of how symbionts spread is supported by a growing body of evidence, the decline and loss of a symbiont partner has rarely been documented, and then only because of replacement by another microbial partner [36, 37] or by environmental effects on the symbiont rather than the host. For example, coral that hosts less thermal-tolerant protist symbiont strains is more prone to climate change–induced symbiont death and subsequent coral bleaching [65]. Our study provides a unique image of the full *life cycle* of a host-symbiont partnership, one in which a secondary symbiont declines without replacement from another strain or species (Fig. 6). Although some host-symbiont life cycles may occur over millions of years [18], the current study results raise the possibility that the rapid formation and breakdown of host-symbiont partnerships may also be common and part of a largely unobserved phenomenon [66].

The evolution of populations can be fundamentally altered by relationships with symbionts that can develop and break down again within decades, relationships which we might never know had existed if we sampled after they had disappeared. While gaining a symbiont may fundamentally change the biology and genome of a host, losing it will not return the host population to its original state and is likely to be no less of a transformation. For example, symbiont sweeps will drag all other maternally inherited DNA— mitochondrial haplotypes and other symbiont genotypes—with them, reducing variation in mtDNA, creating new cytonuclear conflicts and skewing reconstructions of historical patterns of population demographics and speciation [67, 68].

The life cycle of invasion and decline of *Rickettsia* in whiteflies that we observed could be one example of a large, replicated natural experiment around the world. The invasive *B. tabaci* MEAM1 species was introduced to countries around the world in the last several decades and is infected with *Rickettsia* at varying frequencies [69–72]. In Israel, although a rise in infection was not documented, we saw a similar decline of *Rickettsia* infection in MEAM1 over a decade [32]. In Asia, *Rickettsia* has climbed to near-fixation in whiteflies in Japan [71] and has been documented at high frequency in China [69, 72]. It would be very interesting to follow

Rickettsia frequency in these areas to determine whether it will also drop after a time.

Lastly, documenting and understanding symbiont declines is likely to be relevant for the application of symbiont-based control strategies. Particular CI *Wolbachia* have been shown to cause mosquitoes to become refractory to some vector-borne pathogens of humans [73–75]. The introduction and subsequent drive of these symbionts into mosquito populations in the field has been successfully associated with a decline in human arboviruses [76, 77]. While the exciting promise of this approach is a stable transformation of mosquito populations and long-term decline of disease, the current study points to the possibility of secondary symbiont-host interactions being labile, and frequencies dynamic, underlining the recognized importance of long-term monitoring of symbiont frequencies in these programs [78].

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