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A rapidly spreading deleterious aphid endosymbiont that uses horizontal as well as vertical transmission

Xinyue Gu[a,1,2], Perran A. Ross[a,b,1,2], Alex Gill[a], Qiong Yang[a], Eloïse Ansermin[a], Sonia Sharma[a], Safieh Soleimannejad[a], Kanav Sharma[a], Ashley Callahan[a], Courtney Brown[a], Paul A. Umina[a,c], Torsten N. Kristensen[b], and Ary A. Hoffmann[a,b,2]

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Endosymbiotic bacteria that live inside the cells of insects are typically only transmitted maternally and can spread by increasing host fitness and/or modifying reproduction in sexual hosts. Transinfections of *Wolbachia* endosymbionts are now being used to introduce useful phenotypes into sexual host populations, but there has been limited progress on applications using other endosymbionts and in asexual populations. Here, we develop a unique pathway to application in aphids by transferring the endosymbiont *Rickettsiella viridis* to the major crop pest *Myzus persicae*. *Rickettsiella* infection greatly reduced aphid fecundity, decreased heat tolerance, and modified aphid body color, from light to dark green. Despite inducing host fitness costs, *Rickettsiella* spread rapidly through caged aphid populations via plant-mediated horizontal transmission. The phenotypic effects of *Rickettsiella* were sensitive to temperature, with spread only occurring at 19 °C and not 25 °C. Body color modification was also lost at high temperatures despite *Rickettsiella* maintaining a high density. *Rickettsiella* shows the potential to spread through natural *M. persicae* populations by horizontal transmission and subsequent vertical transmission. Establishment of *Rickettsiella* in natural populations could reduce crop damage by modifying population age structure, reducing population growth and providing context-dependent effects on host fitness. Our results highlight the importance of plant-mediated horizontal transmission and interactions with temperature as drivers of endosymbiont spread in asexual insect populations.

aphid | *Rickettsiella* | horizontal transmission | endosymbiont

Significance

Endosymbiotic bacteria usually spread through insect populations by maternal transmission and by providing reproductive advantages to their host. Here, we describe a *Rickettsiella* endosymbiont which spreads rapidly in an asexual aphid host despite causing substantial fitness costs.
We show that the rapid spread of *Rickettsiella*is driven by plant-mediated horizontal transmission and modulated by temperature. This work provides insights into the spread of endosymbionts in asexual insect populations. It also has significant applications for agricultural pest control through the spread of endosymbionts with deleterious effects and seasonal suppression of populations.

Aphids are important pests that cause substantial losses in yield and/or quality in most arable, horticultural and fruit crops (1). Aphids damage host plants in diverse ways, including phloem-feeding, secretion of phytotoxic saliva, the transmission of viruses, and promoting the production of sooty molds (1, 2). The green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is one of the most economically important aphid crop pests (3) due to its worldwide distribution, a host range of more than 400 plant species (4), multiple routes of plant damage (1), and widespread resistance to insecticides (2, 5). Associations between symbiotic bacteria and aphids are pervasive and can profoundly influence the ecology and evolution of their hosts (6). Almost all aphids carry the obligate symbiont *Buchnera aphidicola,* which provides essential amino acids that aphids cannot obtain from the phloem of plants themselves (7, 8). Many aphids also harbor facultative endosymbionts which provide benefits under specific ecological conditions, including heat shock resistance (9–11), host plant specialization (12–14), and resistance to fungi and parasitoid wasps (15–17). Vertical transmission (from mother to offspring) is the main pathway for endosymbionts to spread within a population, but horizontal transmission of facultative endosymbionts between aphid lineages can also occur via parasitism (18) and via the plant tissue (19).

Novel endosymbiont infections can be generated through microinjection of hemolymph or cytoplasm between insects (10, 20–22). Transfers of *Wolbachia* endosymbionts have been adopted to control arbovirus transmission by mosquitoes (23–25). However, there are challenges to implementing this approach in asexual populations where the endosymbiont provides no reproductive advantage. Endosymbiont transfers in aphids have largely been undertaken to shed light on their function, but there are many potential agricultural applications (26–29). Releases of aphids carrying novel endosymbionts could suppress populations through the introduction of susceptibility to insecticides or natural enemies (30–32), vulnerability to high temperatures (33), or reduced reproduction (34). Endosymbionts could also reduce plant virus transmission by aphids, either directly through virus blocking (35, 36) or indirectly through changes in host fitness, age structure, or behavior (37). Populations of *M. persicae* rarely carry secondary endosymbionts (38–40) and no *M. persicae* strains carrying novel endosymbionts have been created, raising the potential to use endosymbionts to modify populations of this important pest.

Author affiliations: [a]Pest and Environmental Adaptation Research Group, School of BioSciences, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, VIC 3052, Australia; [b]Section for Bioscience and Engineering, Department of Chemistry and Bioscience, Aalborg University, Aalborg 9220, Denmark; and [c]Cesar Australia, Brunswick, VIC 3052, Australia

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[1]X.G. and P.A.R. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: xigu2@unimelb.edu.au, perran.ross@unimelb.edu.au, or ary@unimelb.edu.au.

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Bacteria in the genus *Rickettsiella* infect a wide variety of arthropods including aphids (41). In *Acyrthosiphon pisum* (Harris), it is a facultative endosymbiont, but is also known to be pathogenic (42) and can induce cytoplasmic incompatibility in spiders (43). *Rickettsiella* is most notable for its effects on *A. pisum* body color, where infection shifts aphid color from red to green (44, 45) and may modify predator–prey interactions (46). While *Rickettsiella* induces physiological costs to *A. pisum* (47), it can also provide advantages such as protection against entomopathogenic fungi (16). While its phenotypic effects in *A. pisum* are well characterized, no interspecific transfers of *Rickettsiella* have been performed and its effects in other aphid species are unknown.

In this study, we generated a *Rickettsiella* infection in *M. persicae* and characterized its phenotypic effects in the novel host. Our study focused on measuring traits under different thermal conditions given that aphid endosymbionts are often vulnerable to high temperatures (48) and strong interactions between temperature and endosymbiont prevalence occur in natural populations (49–51). We show that *Rickettsiella* infection modified *M. persicae* body color and induced costs to fecundity and heat tolerance. The infection also spread rapidly despite the obvious cost in caged populations through plant-mediated horizontal transmission, with both spread and phenotypic effects modulated by temperature. Our work highlights the potential for endosymbionts to spread through asexual aphid populations under thermally variable conditions. We also highlight plant-mediated horizontal transmission as a key driver of rapid endosymbiont spread through aphid populations, particularly for those that provide no obvious fitness benefit to their host. We discuss the potential applications of *Rickettsiella* to manipulate and suppress pest aphid populations and help protect crops.

Results

***Rickettsiella* Transinfection and Molecular Analysis.** We transferred *Rickettsiella viridis* from a donor line of *A. pisum* to *M. persicae* through microinjection of hemolymph (Fig. 1*A*). We injected 85 aphids, with 15/21 surviving aphids that produced nymphs testing positive for *Rickettsiella* and two of these passing *Rickettsiella* to their offspring. We selected one lineage with the highest *Rickettsiella* density at F_1 and F_2, then offspring were pooled and maintained without selection. Although the *A. pisum* donor carried both *Serratia symbiotica* and *Rickettsiella* infections, the *Serratia* infection was lost in the F_1 generation. In contrast, the *Rickettsiella* infection was stable and has remained at a frequency of 100% for over 30 generations following selection at F_0-F_2 (*SI Appendix*, Table S1).

We observed a dramatic effect of *Rickettsiella* infection on body color at 19 °C, where *M. persicae* carrying *Rickettsiella* [*Rickettsiella* (+)] were dark green, while wild-type aphids [*Rickettsiella* (–)] were light green (Fig. 1*B*). This effect has persisted across long-term laboratory culture. Bacterial 16S rRNA gene sequencing and phylogenetic analysis of the *A. pisum* donor and *Rickettsiella* (+) *M. persicae* lines identified *Rickettsiella* sequences that were identical to each other and grouped with the reference *R. viridis* sequence (Fig. 1*C*). Dissection and qPCR of tissues from the *Rickettsiella* (+) line revealed a broad distribution of *Rickettsiella* within *M. persicae* individuals, with all tissues testing positive for *Rickettsiella*, except

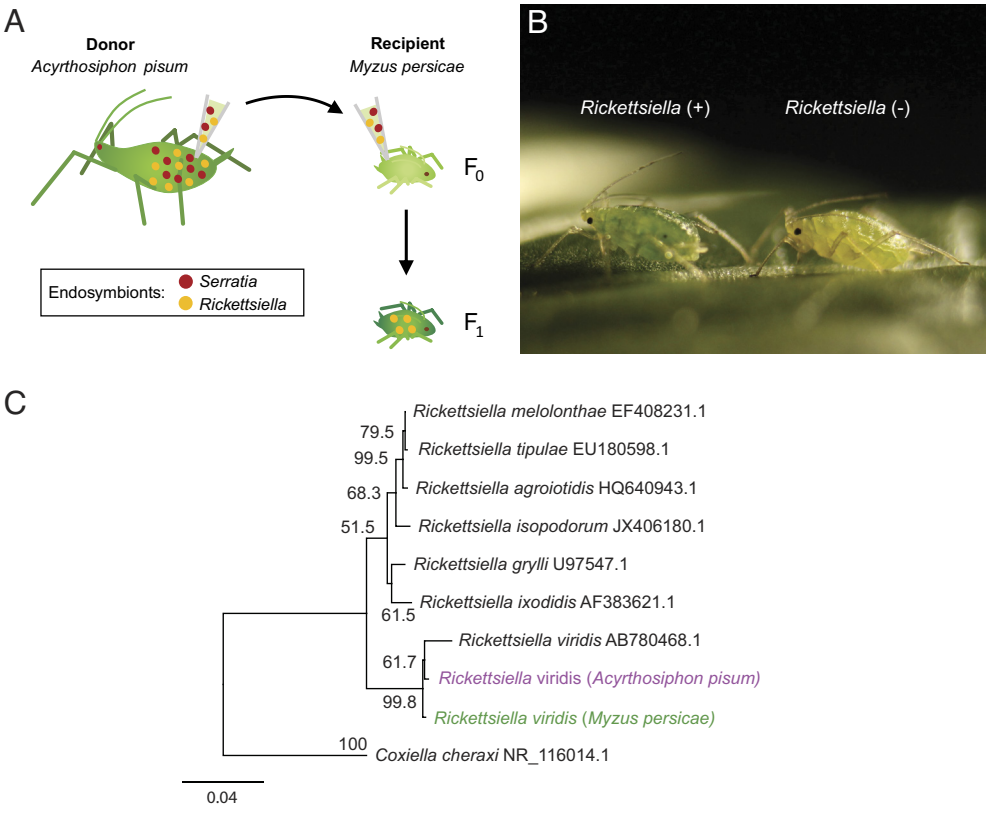

Fig. 1. Generation of a *Rickettsiella* transinfection in *M. persicae* and molecular analysis. (*A*) Schematic of the microinjection procedure showing the transfer of hemolymph containing *Rickettsiella* from *A. pisum* to *M. persicae* and transmission of *Rickettsiella* to the F_1 generation. (*B*) Body color phenotype in *Rickettsiella* (+) (dark green) and *Rickettsiella* (−) (light green) *M. persicae* adults. (*C*) Phylogenetic analysis of *R. viridis* in the *A. pisum* donor (purple) and *M. persicae* recipient (green) based on 16S rRNA gene variation. Neighbor-joining trees were constructed via the Kimura-2 parameter model using MEGA. Numbers at branches represent bootstrap values of 1,000 replicates (values >50 are shown). Sequences from different species of the genus *Rickettsiella* were selected from GenBank for comparison, with *Coxiella cheraxi* included as an outgroup.

for the head and proboscis where endosymbionts were absent or infrequently detected (*SI Appendix*, Fig. S1). We detected *Buchnera* in both the legs and wings (*SI Appendix*, Fig. S1) which was surprising given that this endosymbiont is thought to be restricted to bacteriocytes in the abdomens of aphids (e.g., ref. 52). We confirmed this in a repeat experiment with leg dissections (6/7 pools positive, median Cp of 35.4, range 31.1 to 37.0, n = 6). In contrast, *Buchnera* was not detected in the legs of *A. pisum* (n = 4 pools), consistent with expectations.

***Rickettsiella* Infection Modifies Body Color and Reduces Aphid Fecundity.** We measured life history traits, body color, and endosymbiont densities in *Rickettsiella* (–) and *Rickettsiella* (+) *M. persicae* of the same clone under standard laboratory culture conditions (19 °C) or following exposure to a heat shock of 35 °C for 4 h (Fig. 2). *Rickettsiella* infection had a dramatic effect on body color in apterous adults (GLM: hue: $F_{1,71} = 201.053$, $P < 0.001$, saturation: $F_{1,71} = 98.001$, $P < 0.001$, lightness: $F_{1,71} = 181.546$, $P < 0.001$). *Rickettsiella* (+) aphids were darker, were less saturated, and had a different hue compared to *Rickettsiella* (–) aphids (Fig. 2 *A*–*C*). Differences in body color due to *Rickettsiella* infection were consistent throughout development (*SI Appendix*, Fig. S2). While there was no overall effect of heat shock on body color (all $P > 0.120$), we found a significant interaction between *Rickettsiella* infection and heat shock treatment for two of the three color components when considering all developmental stages of aphids (hue: $F_{1,266} = 14.485$, $P < 0.001$, saturation: $F_{1,266} = 3.120$, $P = 0.079$, lightness: $F_{1,266} = 11.534$, $P = 0.001$), where differences in body color between *Rickettsiella* (+) and *Rickettsiella* (–) individuals decreased following a heat shock.

We then measured life history parameters in *Rickettsiella* (+) and *Rickettsiella* (–) aphids reared individually in Petri dishes containing agar and fresh bok choy (*Brassica rapa* subsp. *chinensis*) leaf discs at 19 °C, with or without a heat shock. *Rickettsiella* infection had a substantial effect on fecundity (GLM: $F_{1,48} = 23.264$, $P < 0.001$), with a ~50% decrease in total offspring produced in *Rickettsiella* (+) aphids compared with *Rickettsiella* (–) aphids (Fig. 2*E*). Early-life fecundity was similar between *Rickettsiella* (+) and *Rickettsiella* (–) aphids but declined more rapidly in *Rickettsiella* (+) aphids as they developed (*SI Appendix*, Fig. S3), leading to an earlier peak of reproduction (average age when nymphs were produced: $F_{1,48} = 6.422$, $P = 0.015$). *Rickettsiella* infection also reduced longevity (Cox regression: females: $\chi^2 = 4.832$, d.f. = 1, $P = 0.028$, Fig. 2*F*) but did not influence development time (GLM: $F_{1,48} = 0.167$, $P = 0.685$, Fig. 2*D*) or body length ($F_{1,71} = 0.282$, $P = 0.597$, Fig. 2*G*). We found no significant effect of the 35 °C heat shock on any life history trait (all $P > 0.345$). However, we did notice that quite a few *Rickettsiella* (+) aphids died at an early age when many would not have yet produced offspring (Fig. 2*F*). We examined this further by looking at the total reproductive output when all aphids are included, using a zero inflation Poisson regression to account

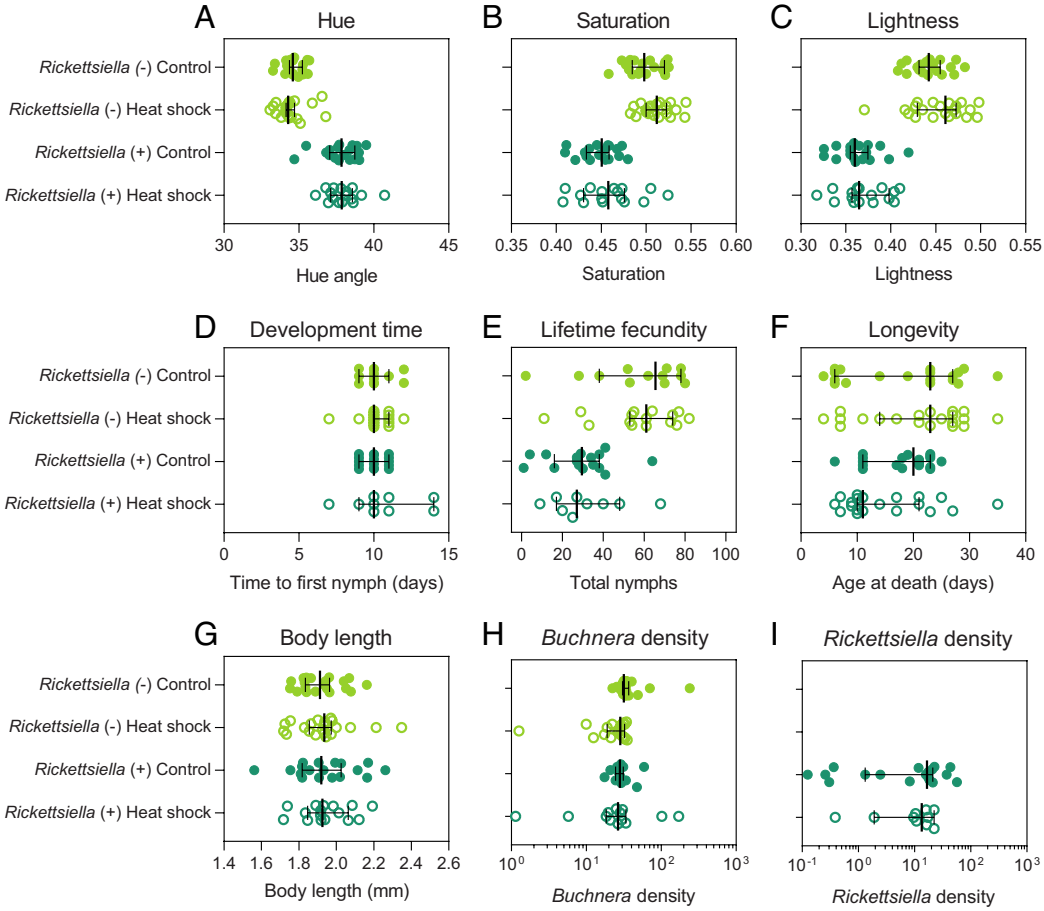

Fig. 2. Body color, life history parameters, and endosymbiont densities of *Rickettsiella* (+) and *Rickettsiella* (–) *M. persicae* following a heat shock. Aphids were exposed to a single heat shock of 35 °C or left untreated (Control). Aphids were measured for (*A*–*C*) body color, (*D*) development time, (*E*) lifetime fecundity, (*F*) longevity, and (*G*) body length. We also quantified (*H*) *Buchnera* and (*I*) *Rickettsiella* density relative to actin, where numbers represent the average difference in Cp values between endosymbiont and actin markers, transformed by $2^{\Delta Cp}$. Body color was separated into three components: (*A*) hue, (*B*) saturation, and (*C*) lightness. Dots represent data from individual aphids, while vertical black lines are medians, and error bars are 95% CIs.

for the large number of zero values. While there was no difference between the temperature treatments on the total production of nymphs (z = −0.961, $P = 0.336$), there was a treatment difference for the *Rickettsiella* (+) aphids (z = 2.146, $P = 0.032$), partly reflecting the high zero counts under the heat shock treatment.

We measured *Buchnera* and *Rickettsiella* densities in three generations of aphids following exposure of the parental generation to a heat shock (Fig. 2 *H* and *I* and *SI Appendix*, Fig. S4). There was no effect of heat shock on *Buchnera* density in the parental generation (GLM: $F_{1,62} = 1.595$, $P = 0.212$, Fig. 2*H*). However, we observed a reduction in the F_1 generation (*SI Appendix*, Fig. S4*A*), where the offspring of parents exposed to a heat shock had reduced densities compared with the controls ($F_{1,75} = 15.412$, $P < 0.001$). By the F_2 generation, we again found no significant effect of heat shock on density ($F_{1,71} = 2.382$, $P = 0.127$, *SI Appendix*, Fig. S4*B*), suggesting a recovery of *Buchnera*. We found no effect of *Rickettsiella* infection on *Buchnera* density, except for a marginally significant effect in the F_1 generation ($F_{1,75} = 4.123$, $P = 0.046$), suggesting that the addition of a novel *Rickettsiella* infection does generally not perturb *Buchnera*. *Rickettsiella* density was not affected by the heat shock in any generation (all $P > 0.627$, Fig. 2*I* and *SI Appendix*, Fig. S4 *C* and *D*).

Temperature-Dependent Phenotypic Effects of *Rickettsiella* Infection. Due to the limited effects of short-term heat shocks observed in our experiments, we then tested whether the effects of *Rickettsiella* were influenced by developmental temperature. We reared *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* of the same clone in groups at four constant temperatures (10, 19, 25, and 30 °C) and then measured body color, fecundity, and endosymbiont densities. We found significant effects of both *Rickettsiella* infection (GLM: hue: $F_{1,225} = 7.463$, $P = 0.007$, saturation: $F_{1,225} = 168.986$, $P < 0.001$, lightness: $F_{1,225} = 127.394$, $P < 0.001$) and developmental temperature (hue: $F_{3,225} = 26.566$, $P < 0.001$, saturation: $F_{3,225} = 189.393$, $P < 0.001$, lightness: $F_{3,225} = 190.810$, $P < 0.001$) on the three color components (Fig. 3 *A*–*C*). There were also interactions between developmental temperature and *Rickettsiella* infection (hue: $F_{3,225} = 9.858$, $P < 0.001$, saturation: $F_{3,225} = 24.394$, $P < 0.001$, lightness: $F_{3,225} = 10.852$, $P < 0.001$). Body colors of *Rickettsiella* (+) and *Rickettsiella* (−) lines were distinct at lower culturing temperatures, but these differences were diminished at higher temperatures. At 30 °C, there was no significant effect of *Rickettsiella* infection on any color component (all $P > 0.062$).

Aphid fecundity was influenced by temperature (GLM: $F_{2,53} = 39.162$, $P < 0.001$) and there was a marginally significant effect of *Rickettsiella* infection ($F_{1,53} = 5.030$, $P = 0.029$), where *Rickettsiella* (−) aphids produced more nymphs than *Rickettsiella* (+) aphids during this period (Fig. 3*D*). This pattern was consistent across all temperatures, with no significant interaction between temperature and *Rickettsiella* infection ($F_{2,53} = 0.503$, $P = 0.607$). Body length decreased at higher temperatures ($F_{3,224} = 156.391$, $P < 0.001$), but there was no effect of *Rickettsiella* infection on this trait ($F_{1,224} = 0.165$, $P = 0.685$).

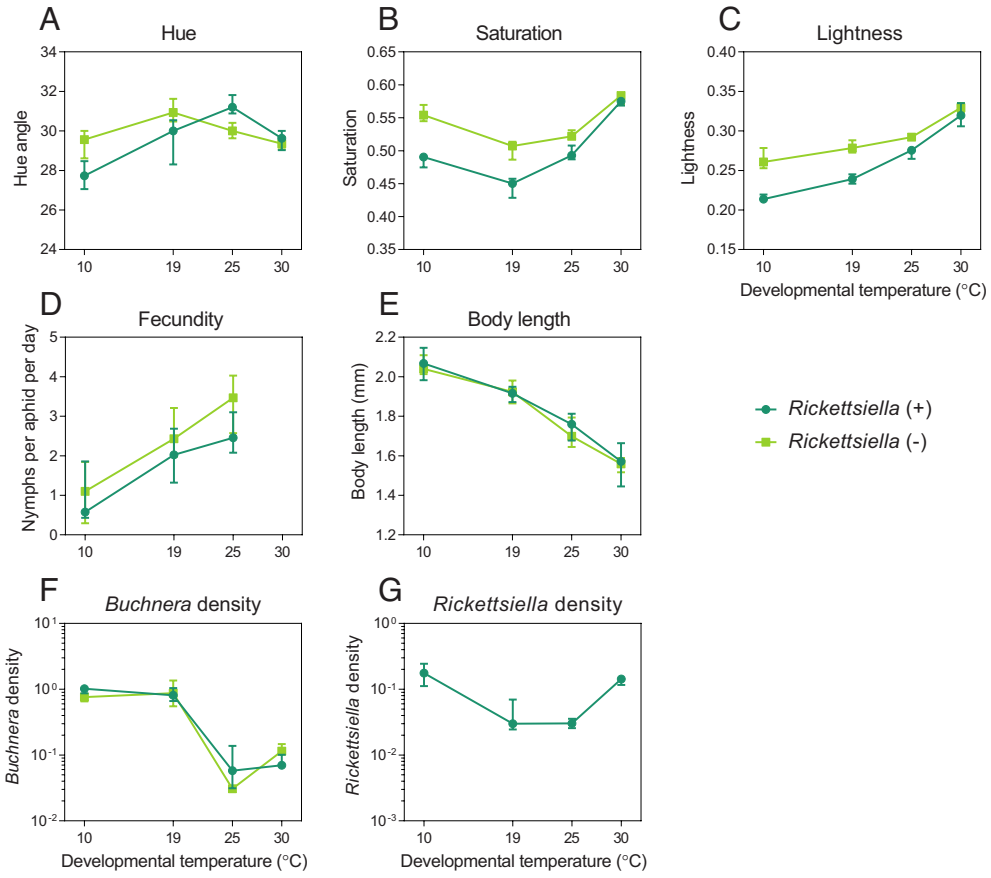

Fig. 3. Body color, life history parameters, and endosymbiont densities in *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* at different developmental temperatures. Aphids were reared in groups within Petri dishes and then assessed for (*A*–*C*) body color, (*D*) average fecundity, and (*E*) body length. We also quantified (*F*) *Buchnera* and (*G*) *Rickettsiella* density relative to actin, where numbers represent the average difference in Cp values between endosymbiont and actin markers, transformed by $2^{\Delta Cp}$. Body color was separated into three components: (*A*) hue, (*B*) saturation, and (*C*) lightness. Fecundity was measured on groups of up to 10 aphids, while the other traits were measured on 20 to 30 adults per treatment. We measured average fecundity by counting the total number of nymphs produced per group of aphids every 2 d for 6 d. Aphids were not scored for fecundity at 30 °C due to low viability at this temperature. Symbols represent medians and error bars are 95% CIs.

Endosymbiont densities were also influenced by temperature, with significant effects of temperature on both *Buchnera* (GLM: $F_{3,167}$ = 214.507, P < 0.001, Fig. 3*F*) and *Rickettsiella* ($F_{3,81}$ = 39.256, P < 0.001, Fig. 3*G*) densities. The two endosymbionts showed different patterns with respect to density, with *Buchnera* density being higher at the two lower temperatures and *Rickettsiella* density being higher at the two extremes. There was a marginally significant effect of *Rickettsiella* infection on *Buchnera* density ($F_{1,167}$ = 4.047, P = 0.046) and an interaction with temperature ($F_{3,167}$ = 5.697, P = 0.001), with *Rickettsiella* (+) and *Rickettsiella* (–) aphids showing slightly different patterns in *Buchnera* density with respect to temperature.

Rapid Spread of *Rickettsiella* in Population Cages. We established mixed populations of *Rickettsiella* (+) and *Rickettsiella* (–) *M.persicae* of the same clone on whole bok choy plants to investigate *Rickettsiella* infection dynamics across time in populations with overlapping generations (Fig. 4*A*). Starting at an initial frequency of 1:1 [*Rickettsiella* (+): *Rickettsiella* (–)], the infection spread rapidly at 19 °C, with a frequency above 80% in all replicate populations by week 3 (Fig. 4*B*). A high prevalence of *Rickettsiella* was maintained until the end of the experiment at week 12. At 25 °C, the infection also spread initially, but then decreased in frequency from weeks 4 to 12 in all replicate cages (Fig. 4*C*). *Rickettsiella* density was stable at 19 °C, with no effect of week (GLM: $F_{2,210}$ = 1.828, P = 0.163, *SI Appendix*, Fig. S5) but declined from week 4 to 8 at 25 °C ($F_{1,83}$ = 8.055, P = 0.006, *SI Appendix*, Fig. S5), indicating potential transmission instability across generations at higher temperatures. We also assessed separate subsets of aphids from each time point for body color and found that the proportion of dark green individuals increased at 19 °C and decreased at 25 °C (*SI Appendix*, Fig. S5). However, the gradual increase at 19 °C did not match the rapid increase in *Rickettsiella* prevalence at this temperature (cf. *SI Appendix*, Fig. S5 with Fig. 4*B*), indicating a potential decoupling between *Rickettsiella* infection status and expected body color [dark green for *Rickettsiella* (+) and light green for *Rickettsiella* (–)] in mixed populations.

Plant-Mediated Horizontal Transmission of *Rickettsiella*. In the previous experiment, *Rickettsiella* spread rapidly in caged populations despite clear costs of infection identified in our earlier experiments. Horizontal transmission via the host plant is one potential explanation for this spread. We performed experiments in *M. persicae* to test for horizontal transmission of *Rickettsiella* from a *Rickettsiella* (+) line to a *Rickettsiella* (–) line of a different clone (Fig. 5). In mixed populations maintained on the same plant, *Rickettsiella* spread to a frequency of 100% from a starting ratio of 1:1 in four replicate cages within 9 wk (Fig. 5*A*). Microsatellite analysis demonstrated that 20/60 positive *M. persicae* individuals were from the clone that was originally uninfected, indicating that *Rickettsiella* had spread to these aphids via horizontal transmission.

We then performed experiments where *Rickettsiella* (+) and *Rickettsiella* (–) *M. persicae* were reared on the same plant but

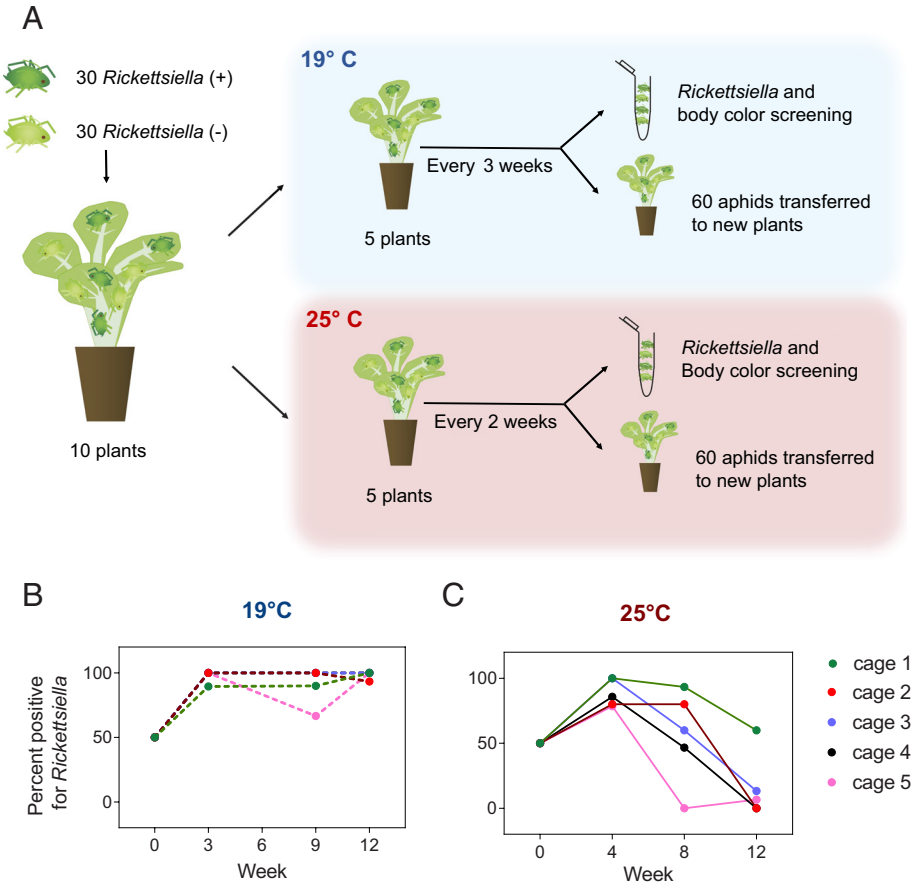

Fig. 4. *Rickettsiella* infection dynamics in mixed population cages at 19 °C and 25 °C. (*A*) Experimental design. Populations were initiated with 30 *Rickettsiella* (+) individuals and 30 *Rickettsiella* (–) *M. persicae* on caged bok choy plants. Five replicates were set up and maintained at each temperature (19 °C or 25 °C). Random samples of 60 aphids were transferred to new plants every 3 wk at 19 °C and every 2 wk at 25 °C. The remaining aphids were stored for *Rickettsiella* and body color measurements. (*B* and *C*) *Rickettsiella* infection dynamics at 19 °C (*B*) and 25 °C (*C*). Symbols represent the proportion of individuals testing positive for *Rickettsiella* from 15 aphids per time point, per replicate cage. Infection frequency data are also shown in *SI Appendix*, Table S2.

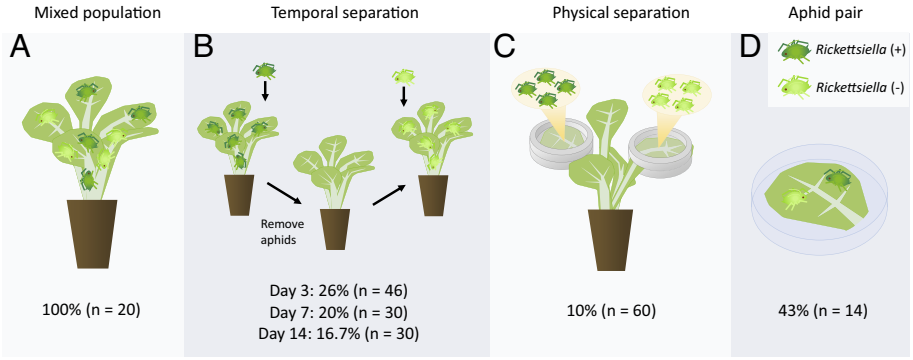

Fig. 5. Experimental design and outcomes of plant-mediated horizontal transmission experiments. (*A*) *Rickettsiella* (+) and *Rickettsiella* (−) aphids were placed on whole bok choy plants at a 1:1 frequency and maintained for 9 wk before screening. (*B*) *Rickettsiella* (+) aphids were placed on bok choy plants for 1 wk, removed, and then replaced with *Rickettsiella* (−) aphids which were screened after 3, 7, and 14 d. (*C*) 10 *Rickettsiella* (+) and *Rickettsiella* (−) aphids were placed on different leaves of the same host plant and separated physically with clip cages, and then screened after 7 d. (*D*) Single *Rickettsiella* (+) and *Rickettsiella* (−) aphids were placed together on leaf discs and screened after 7 d. In all experiments, aphids were screened for *Rickettsiella* infection and clonal type with qPCR assays and microsatellite analysis, respectively. Numbers below each panel indicate the percentage of light green aphids in the experimental groups testing positive for *Rickettsiella*.

separated temporally (Fig. 5*B*) or physically (Fig. 5*C*). In both cases, a small percentage of aphids from the uninfected clone tested positive for *Rickettsiella*, indicating that direct contact between aphids is not required for horizontal transmission. We also found evidence of horizontal transmission between pairs of aphids on leaf discs within Petri dishes (Fig. 5*D*), demonstrating that whole intact plants are not necessary for horizontal transmission, nor are high densities of aphids required. While horizontal transmission appears to be rapid (within 3 d), aphids testing positive for *Rickettsiella* in the experimental treatments typically had low endosymbiont densities (median *Rickettsiella* Cp of 32.49, range: 24.65 to 38.79, n = 55, *Rickettsiella* (+) controls: median: 19.23, range: 15.04 to 25.97, n = 65) and did not display a dark green body color. In all experiments, there was no amplification in negative controls which were *Rickettsiella* (–).

We then tested whether plant-mediated horizontal transmission of *Rickettsiella* also occurs in its native host *A. pisum* by placing pairs of aphids on broad bean leaf discs. In two separate experiments with nymphs and adults, respectively, 5.3% (1/19, Cp of 19.89) and 3.5% (1/29, Cp of 37.05) of aphids in the experimental groups tested positive for *Rickettsiella* (median Cp of *Rickettsiella* (+) controls: 18.84, n = 48). This suggests relatively low horizontal transmission.

Vertical Transmission of *Rickettsiella* Following Horizontal Transmission. We tested whether horizontally acquired *Rickettsiella* could infect the ovaries and be transmitted to the next generation. We detected *Rickettsiella* in the dissected ovaries of 2/51 (3.9%) *Rickettsiella* (–) aphids when placed together with a *Rickettsiella* (+) aphid on bok choy leaf discs (*SI Appendix*, Table S3), indicating vertical transmission. In two additional experiments, we found vertical transmission of *Rickettsiella* by aphids that acquired the infection by horizontal transmission (*SI Appendix*, Table S3). In both experiments which involved a low aphid density, the frequency of transmission was low and *Rickettsiella* density was also low and variable (*SI Appendix*, Table S3). In one of the experiments, we also tracked transmission to the second generation at a low frequency (2/9) and density (median 35.85, n = 2, positive control: 18.02, n = 5).

Stable Transmission of *Rickettsiella* at High Temperatures. We previously demonstrated that the spread of *Rickettsiella* in mixed populations of *M. persicae* is strongly temperature dependent, with potential transmission failure at higher temperatures. We therefore tested the stability of *Rickettsiella* across generations at 25 °C when the infection was fixed in the population. When aphids were maintained on whole bok choy plants, the infection was maintained at a frequency of 100% in three replicate cages after 12 wk (n = 20 per replicate), indicating efficient transmission of *Rickettsiella* at 25 °C. Stable transmission of *Rickettsiella* was also observed across 4 generations when aphids were maintained as single individuals in Petri dishes (n = 20 aphid lines). Given that the vertical transmission of *Rickettsiella* is stable at 25 °C, its loss in mixed populations at 25 °C (Fig. 4*C*) is likely explained by temperature-dependent fitness costs or decreased horizontal transmission efficiency at this temperature.

***Rickettsiella* Infection Reduces Aphid Heat Tolerance.** Facultative endosymbionts are known to modulate host thermal tolerance (53). Given the strong temperature-dependent effects of *Rickettsiella* found in this study, we were interested in testing its effect on host heat tolerance. We measured the heat tolerance of *Rickettsiella* (+) and *Rickettsiella* (–) *M. persicae* of the same clone in two assays: heat knockdown time and Critical Thermal Maxima (CTmax (Fig. 6). In the heat knockdown assay, *Rickettsiella* (+) aphids had a significantly shorter time to knockdown compared with *Rickettsiella* (–) aphids (GLM: $F_{1,140} = 150.259$, $P < 0.001$, Fig. 6*A*). We also found a significant effect of run ($F_{1,140} = 12.864$, $P = 0.015$), with overall knockdown times varying between each run (*SI Appendix*, Fig. S6), though *Rickettsiella* infection significantly reduced time to knockdown in all the five replicate runs (GLM: all $P < 0.001$). Effects of *Rickettsiella* infection on CTmax were less clear-cut, with no significant effect of *Rickettsiella* infection ($F_{1,84} = 1.570$, $P = 0.377$) or run ($F_{2,84} = 1.967$, $P = 0.377$) overall (Fig. 6*B*).

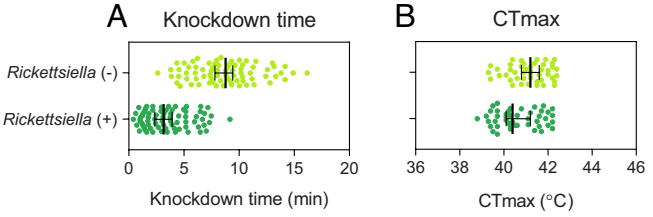

Fig. 6. Effects of *Rickettsiella* infection on *M. persicae* heat tolerance. *Rickettsiella* (+) and *Rickettsiella* (−) aphids were measured for (*A*) heat knockdown time at a constant 41.5 °C and (*B*) CTmax at a ramping rate of 0.1 °C/min. Dots represent data from individual aphids, while black vertical lines are medians and error bars are 95% CIs. Data are presented separately for each heat knockdown and CTmax run in *SI Appendix*, Fig. S6.

However, we found a significant interaction between *Rickettsiella* infection and run ($F_{2,84}$ = 5.334, P = 0.007), with *Rickettsiella* infection reducing CTmax in runs 2 ($F_{1,28}$ = 8.012, P = 0.009) and 3 ($F_{1,28}$ = 8.876, P = 0.006) but not run 1 ($F_{1,28}$ = 1.057, P = 0.313) when each of the three CTmax runs were analyzed separately. Overall, these results suggest that *Rickettsiella* infection reduces aphid heat tolerance, with the strength of the effect varying depending on the type of assay.

Discussion

Here, we describe an interspecific transfer of *R. viridis* and its spread through populations of a novel aphid host and important agricultural pest, *M. persicae*. The infection was transmitted both maternally and to other aphids sharing the same host plant (thus horizontally). Furthermore, *Rickettsiella* enacted substantial phenotypic effects on *M. persicae*, including a shift in body color and a ~50% reduction in fecundity. These features demonstrate the potential for *Rickettsiella* infections to spread through and modify pest populations of aphids, and thus reduce the risk of damage caused to crops.

Previous work in the native host *A. pisum* showed that *Rickettsiella* modifies body color and reduces fecundity (44, 45), and we showed here that these key characteristics have persisted even in a novel host where it does not occur naturally. The *Rickettsiella* infection was stable for more than 30 generations, in contrast to the *Serratia* endosymbiont that was also transferred but did not persist. Our subsequent attempts to transfer *Serratia* have also failed to generate stable infections in *M. persicae*, highlighting the likely endosymbiont-specific nature of success in cross-species transfers in aphids.

The spread or decline of endosymbionts through aphid populations is constrained by host fitness effects (54). Unlike in species that normally reproduce sexually, endosymbionts in aphids cannot spread by mechanisms such as cytoplasmic incompatibility, which can drive an invasion by the endosymbiont despite fitness costs (55). Facultative endosymbionts typically have physiological costs (34, 56, 57), which makes invasion difficult in species that predominantly reproduce via parthenogenesis, unless there are context-specific fitness benefits (17, 58).

Despite fitness costs, *Rickettsiella* did spread into populations under cool conditions and we show here that horizontal transmission via plants seemed to be a critical driver of this spread. Plant-based horizontal transmission has previously been demonstrated for other endosymbionts including *Rickettsia* (59), *Wolbachia* (60), *Cardinium* (61), *Asaia* (62), *Hamiltonella* (63), and *Serratia* (19) in their original hosts. While plants may be a common route by which endosymbionts spread in sap-feeding herbivores, this has been rarely explored and observed in natural populations (64). Our experiments suggest that horizontal transmission can be rapid, with a proportion of horizontally acquired infections being passed vertically. Combined horizontal and vertical transmission will be crucial for driving the spread of *Rickettsiella* in the absence of beneficial host effects and future work should aim to determine the frequency of vertical transmission in nature. It will also be interesting to test whether transmission varies with host plants and whether it also occurs across aphid species. While we show horizontal transmission through several experimental designs, we acknowledge that detections of *Rickettsiella* in experiments lacking physical separation could result from direct or indirect contact (such as through honeydew) rather than plant-based transmission.

While it is unclear whether plant-based transmission depends on temperature, we note that the spread and subsequent decline of *Rickettsiella* at 25 °C despite stable vertical transmission suggests that the efficiency of horizontal transmission may be reduced at higher temperatures and/or that there are temperature-specific fitness effects. It is worth noting that although *A. pisum* and *M. persicae* share several host plants (4), natural *Rickettsiella* infections have not been detected in *M. persicae* (38, 39). This suggests that the efficiency of horizontal transmission may differ between host plant or aphid species, and that interactions with temperature (and potentially other abiotic conditions) may also reduce spread in natural populations, particularly given the low total reproductive output of *Rickettsiella* (+) following a heat shock.

Aphids and their bacterial symbionts are exposed to a variety of environmental stresses which can influence their fitness (9). While aphid endosymbionts are often vulnerable to high temperatures (33), some can also provide protection to their hosts and other endosymbionts that increase aphid survival (15, 53, 65). Previous research has shown that the costs and benefits of endosymbionts are highly temperature dependent (11, 48), helping to explain why their prevalence fluctuates in natural populations (49, 51, 66, 67). Here, we found that transinfected *Rickettsiella* reduced aphid heat tolerance, which is likely to decrease its rate of spread through aphid populations but also decrease host fitness during the summer period. However, both *Buchnera* and *Rickettsiella* appear to be resilient to high rearing temperatures and heat shocks in *M. persicae*. In pilot experiments, heat shocks above 35 °C caused high aphid mortality but had little impact on *Buchnera*, suggesting that this endosymbiont may not be limiting the thermal tolerance of its *M. persicae* host unlike in some other aphid species (33). Our results suggest that *Rickettsiella* would be most harmful to aphids in the hot season, but this might limit its potential agricultural use if it cannot spread under warm conditions. From a biocontrol perspective, the seasonal timing of potential releases and conditions at release sites should be considered carefully.

Our study describes an insect endosymbiont modifying body color following an interspecific transfer. The change in color from light to dark green is consistent with the production of green pigments in *A. pisum* (44) and suggests that this mechanism is retained even in a novel host. In earlier experiments, we noted aphids with pink, yellow, and dark brown color morphs 1 to 2 generations after microinjection. These lines were infected with *Rickettsiella*, but were unstable with variable *Rickettsiella* levels and the colors may have been due to a stress response (68). In contrast, the dark green color change has persisted across more than 30 generations of culture at 11 °C and 19 °C. The stability and discreteness of this color change highlights the potential use of *Rickettsiella* as a phenotypic marker; however, this is complicated by horizontal transmission and the fact that the extent of body color variation is strongly temperature dependent. It is also possible that other environmental factors, such as light intensity and host plant which are known to influence body color (69), will also modify endosymbiont-induced color changes. More research is needed to understand whether body color changes caused by *Rickettsiella* infection in *M. persicae* have any effect on host plant preferences, interactions with parasitoids, or camouflage against predators that use vision to locate aphids. Body color changes may also have implications for thermal responses, given that darker body colors can provide benefits under cold conditions (70).

Our characterization of *Rickettsiella* in *M. persicae* raises the question of whether this endosymbiont could be used in alternative ways to control aphid pests. Releases of *Aedes* mosquitoes carrying *Wolbachia* endosymbionts have achieved suppression of target populations and disease incidence (23, 25, 71). Previous literature has speculated on the potential uses of endosymbionts in pest control (26–29), but so far, there have been few direct

applications in the field despite the development of transinfections that cause cytoplasmic incompatibility in tephritid fruit fly pests (22) and plant virus blockage in planthoppers (35). Our findings suggest that *Rickettsiella* can rapidly spread through aphid populations despite asexuality under cool conditions, and this can happen through both vertical and horizontal transmission. Given aphids in winter grain crops often build up from a low population density (72), deliberate releases of transinfected aphids early in the crop development stage and their subsequent rapid spread could lead to the collapse of the aphid population later in the crop stage when conditions become warmer due to the fecundity costs of *Rickettsiella* and their impact on heat resistance. In this way, *Rickettsiella* could impact aphid population dynamics in novel ways and persistent *Rickettsiella* infections could even have impacts across multiple years, increasing in frequency as conditions become cooler during the establishment phase of winter crops. The endosymbiont could also have an impact on pest control in closed systems such as greenhouses where *M. persicae* can be problematic (73, 74).

Obviously, considerable research is still required to test the feasibility of such applications and dynamics under relevant conditions. For example, release experiments in greenhouse and field plots are needed to evaluate the potential of *Rickettsiella* to spread in natural aphid populations. In addition, modeling and experimental work are required to assess optimal release rates, release patterns, and timing of releases. Our experiments were limited to a single clone of *M. persicae* due to challenges with interspecific endosymbiont transfers, but it will be crucial for future work to investigate the effects of *Rickettsiella* and its transmission in multiple genotypes, given that the effects of endosymbionts can depend on genotype (15, 75). Nevertheless, with chemical options rapidly dwindling following environmental concerns around the use of pesticides (76) and the rapid evolution of insecticide resistance (2), it is important to explore such environmentally friendly approaches, particularly as they can be self-sustaining.

Although such applications may appear "blue sky" at this stage, we point to the rapid progress made in using transinfected *Wolbachia*-based strategies for the control of mosquito vectors and their transmitted pathogens in recent years, with laboratory work indicating the potential of this approach in mid-2000 (77, 78), the first field releases in 2010 to 11 (24), and widespread adoption of the approach soon afterward for disease control and population suppression (23, 25, 71). Prior to the first *Wolbachia* releases, a thorough risk assessment was completed (79) and the endosymbiont-based approach is now regarded as a biological method of control which has proven to be safe and with an absence of nontarget effects. We suspect that this past experience will help in developing a pathway to adoption of other arthropod-specific endosymbionts in pest control, particularly given that these endosymbiotic bacteria are already part of the natural environment and that transfers of these bacteria already occur naturally but at a very low rate across arthropod species (e.g., ref. 80). In the case of *M. persicae*, secondary endosymbionts could be particularly useful because they are rare in natural populations in the invaded range of this species (40). While another endosymbiont, *Regiella*, has been found in a population in Australia (81), recent field surveys across Australia and from other invaded regions have failed to detect this secondary endosymbiont in *M. persicae* (40) and in their native range where it does occur, it only does so at a low relative abundance (39).

In summary, we have performed an experimental transfer of an endosymbiont into a naturally uninfected host and documented its spread through an asexual insect population. Our work demonstrates the possibility of developing endosymbiont-based population replacement and/or suppression strategies for major agricultural pests. While much more work is needed to understand spread potential in natural populations and effects on crop production (26), our findings point to an approach for using endosymbionts in pest control even in asexual species.

Materials and Methods

Aphid Lines and Maintenance. Aphids were cultured in controlled temperature cabinets (PG50 Plant Growth Chambers, Labec Laboratory Equipment, Marrickville, NSW, Australia) at 10 ± 1 °C with a 16:8 light:dark photoperiod. *M. persicae* were cultured on bok choy (*Brassica rapa* subsp. Chinensis) leaf discs (35 mm) and *A. pisum* were cultured on lucerne (*Medicago sativa*, cv. Sequel) stems and trifoliate leaves. Leaf discs or stems were placed in or on top of a layer of 1% agar in Petri dishes (60 mm × 15 mm), with aphids transferred to a fresh Petri dish and plant material weekly. Plants were grown in a shade house supported with plant growth lights (40W Grow Saber light-emitting diode (LED) 6,500 K, 1,200 mm length) set to a 16:8 light:dark photoperiod, specifically to target vegetative growth. Bok choy plants were grown for ~9 wk before use, while lucerne plants were ~4.5 wk old at the time of use.

For microinjection experiments, we used a single clone of *A. pisum* collected from Tintinara, South Australia (GPS: −35.9, 140.1), which carried both *S. symbiotica* and *R. viridis*. For horizontal transmission experiments, we also used a clone of *A. pisum* carrying *Regiella insecticola* but lacking *Rickettsiella* collected from Noradjuha, Victoria (−36.9, 141.9). We used a single clone of *M. persicae* in all experiments (multilocus genotype 188), except for the horizontal transmission experiment where we used an additional clone (multilocus genotype 37) to check for *Rickettsiella* transmission via the host plant ("Plant-Mediated Horizontal Transmission of *Rickettsiella* in *M. persicae*"). Multilocus genotype 188 was provided by the Grains Innovation Park (Horsham, Victoria), while multilocus genotype 37 was collected from Toongabbie, Victoria (GPS: −37.785, 144.982). Multilocus genotype 188 from super clone B is resistant to carbonate, synthetic pyrethroids, and organophosphates and has some resistance to neonicotinoids. Multilocus genotype 37 has an unknown resistance status. These genotypes (and others) can be separated using a set of microsatellite loci (82, 83). Both *M. persicae* clones were naturally uninfected with secondary endosymbionts [*Rickettsiella* (−)], but we generated a line of multilocus genotype 188 which carried a stable *R. viridis* infection [*Rickettsiella* (+)] derived from *A. pisum* ("*Endosymbiont Transfer through Microinjection*"). Endosymbiont infection and the clonal status of aphids were screened routinely (approximately every three generations) and before experiments commenced through qPCR ("*Endosymbiont Detection and Quantification*") and microsatellite analysis ("*Microsatellite Analysis*"), respectively.

Endosymbiont Transfer through Microinjection. We introduced *R. viridis* from *A. pisum* into adult *M. persicae* through microinjection. To transfer endosymbionts, hemolymph was withdrawn from the donor aphid and immediately injected into the recipient (10) using a MINJ-1000 microinjection system (Tritech Research, Los Angeles, CA, USA). Individual aphids were immobilized on an insect holder (a single 10 μL XL pipette tip attached to a vacuum) under a stereo microscope. We injected 85 *M. persicae* with hemolymph (approximately 0.075 μL to 0.1 μL per aphid) from around twenty donor *A. pisum*. Injected aphids were maintained at 10 °C in groups on Petri dishes (60 mm × 15 mm) with a bok choy leaf disc placed on 1% agar. After 10 d, surviving aphids were placed individually on fresh bok choy leaf discs in Petri dishes. After producing more than four nymphs, parents were stored in 100% ethanol and screened for *Rickettsiella* infection using qPCR ("*Endosymbiont Detection and Quantification*"). Only the nymphs from adults with the highest *Rickettsiella* density were selected for the next generation. This process was repeated until we observed a 100% infection rate and high density in F_2, after which we maintained the *Rickettsiella* (+) line as a population. Individuals from the *Rickettsiella* (+) line were screened routinely to ensure that the infection was maintained at a high frequency (*SI Appendix*, Table S1). *M. persicae* lines were at least 14 generations postinjection before experiments started.

Dissection and qPCR of Aphid Tissues. Adult apterous *Rickettsiella* (+) *M.persicae* were placed in 100% ethanol and different tissues were subsequently dissected and separated, including heads (excluding the proboscis), proboscis, legs, bacteriocytes, and embryos. Wings were also dissected from separate groups of alate *Rickettsiella* (+) *M. persicae*. Moults from the preadult stages were also

collected. Tissues from groups of 4 aphids were stored in 100% ethanol with 6 to 8 replicate tubes per group. All samples were screened for *Rickettsiella* and *Buchnera* using qPCR ("*Endosymbiont Detection and Quantification*"). Samples which did not amplify for the actin marker were excluded from the analysis.

In a second experiment, we performed dissections of adult *Rickettsiella* (+) *M. persicae* as well as *Rickettsiella* (+) *A. pisum* as described above with tissues from groups of four aphids per tube and four replicate tubes per group. Aphid legs and the remaining carcasses were separated and screened for *Buchnera* using qPCR.

Population Amplification and Age Matching. To produce large numbers of aphids of a similar age for experiments, *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* were introduced to whole bok choy plants (~9 wk old) in insect rearing cages (30 × 30 × 62 cm, mesh 160 μm aperture to prevent aphids escaping) 3 wk prior to experiments. To avoid crowding while maintaining large population sizes, 30 aphids were transferred to fresh whole plants every 3 wk. Prior to experiments, adult apterous aphids from these populations were used to set up age-matching dishes, which consisted of a 60-mm Petri dish with a layer of 1% agar and a 35-mm bok choy leaf disc. Thirty adults were transferred to each dish and nymphs were collected every 24 h for use in experiments. Both caged populations and age-matching dishes were maintained in controlled temperature cabinets or controlled temperature rooms at 19 °C and a photoperiod of 16L:8D.

Effects of Heat Shock and *Rickettsiella* Infection on Life History, Body Color, and Endosymbiont Density. We exposed *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* to a heat shock treatment and measured life history traits in individual aphids. Three-day-old nymphs from age-matching dishes were placed in sealed (Parafilm) Petri dishes (35 mm × 10 mm) with a layer of 1% agar and a 20-mm bok choy leaf disc, and then transferred to zip-lock bags and sealed. We set up five replicate dishes for each temperature and aphid line, with 20 individuals on each dish. Bags were then submerged in water baths set to a constant 19 °C (control) or a temperature cycle that ramped up from 19 °C to 35 °C over 2 h, maintained 35 °C for 4 h, then ramped down to 19 °C over 2 h (heat shock).

Surviving aphids were transferred individually to 35 mm Petri dishes with fresh bok choy leaf discs (20 mm) and returned to 19 °C for life history measurements, with 20 replicate dishes per aphid line, per temperature treatment. Aphids were checked daily and scored for time to produce their first nymph (as a proxy for development time), daily fecundity, and longevity (time to death), with plant material refreshed twice weekly. Aphids that died before producing nymphs were excluded from the fecundity and development time analyses.

The remaining surviving aphids were transferred to larger Petri dishes (60 × 15 mm) with bok choy leaf discs (35 mm) in groups of 10 and maintained at 19 °C. Subsets of these aphids were measured for body color and length ("*Body Color and Length Measurements*") and *Rickettsiella* and *Buchnera* density ("*Endosymbiont Detection and Quantification*"). For body color and length measurements, 10 aphids were selected at random from each heat shock and *Rickettsiella* infection treatment on days 1, 2, 3, 4, and 5 post heat shock. These were photographed ("*Body Color and Length Measurements*") and then returned to Petri dishes for measurements on subsequent days. On day 7 post heat shock (when aphids were 10 d old), 20 adult apterous aphids from each treatment were measured for body color and body length, and then stored in 100% ethanol for endosymbiont quantification. The remaining adults were allowed to produce nymphs which were maintained at 19 °C for endosymbiont density measurements in the F_1 and F_2 generations post heat shock. The subsequent generations were stored in 100% ethanol once they became adults (measured by first nymphal production).

Effects of Developmental Temperature and *Rickettsiella* Infection on Life History, Body Color, and Endosymbiont Density. *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* were reared at constant temperatures of 10, 19, 25, and 30 °C to measure the impact of rearing temperature on *Rickettsiella* phenotypic effects. Nymphs (<24 h old) were transferred to Petri dishes (60 × 15 mm) with bok choy leaf discs (35 mm) in groups of 10 and maintained at each temperature in controlled temperature cabinets. Aphids reaching adulthood were subsequently transferred to fresh bok choy leaf discs in groups of 10. We then measured the average fecundity for 10 replicate groups per temperature and aphid line by counting the total number of nymphs produced per group of aphids every 2 d for 6 d. The number of nymphs produced was adjusted to the number of adult aphids alive and averaged across the three measurement periods for each replicate dish. Fecundity was not measured in aphids at 30 °C due to low viability of both the *Rickettsiella* (+) and *Rickettsiella* (−) lines at this temperature. Subsets of aphids from each treatment were measured for body color and body length ("*Body Color and Length Measurements*"), with 30 replicates per treatment, and *Rickettsiella* and *Buchnera* density ("*Endosymbiont Detection and Quantification*"), with 20 replicates per treatment.

***Rickettsiella* Population Dynamics.** We tracked the spread of *Rickettsiella* in mixed populations of *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* at 19 °C and 25 °C. Thirty *Rickettsiella* (+) and 30 *Rickettsiella* (−) *M. persicae* of mixed ages were placed at random on the leaves of 9-wk-old bok choy plants and maintained within insect-rearing cages (30 × 30 × 62 cm, mesh 160 μm aperture) and placed under plant growth lights (16L:8D light cycle, 40W LED 6,500 K, 1,200 mm length), with five replicate cages initiated at each temperature. To prevent overcrowding of aphids and maintain plant health, aphids were transferred to new plants every 3 wk at 19 °C and every 2 wk at 25 °C. When changing plants, leaves from the old plant were cut off and cleared of aphids by tapping them into a plastic tray and lightly mixed. Cages were sprayed with 100% ethanol to kill aphids that remained in the cage, and 60 aphids from the tray were placed at random on leaves of the new plant. The remaining aphids were stored in 1.7 mL tubes with 100% ethanol. This process was repeated for 12 wk, at which point the experiment was terminated and all aphids were removed from plants and stored in 100% ethanol. To measure *Rickettsiella* infection frequencies across time, we screened 15 aphids per replicate cage at multiple time points throughout the experiment (weeks 3, 9, and 12 at 19 °C and weeks 4, 8, and 12 at 25 °C) with a qPCR assay ("*Endosymbiont Detection and Quantification*"). We also estimated the proportion of aphids with a light green or dark green body color by phenotyping 27 to 70 aphids from each time point and replicate cage.

Plant-Mediated Horizontal Transmission of *Rickettsiella* in *M. persicae*. We performed four experiments with different designs to test for horizontal transmission of *Rickettsiella* between aphids via the host plant. In each experiment, the *Rickettsiella* (+) and *Rickettsiella* (−) lines were different *M. persicae* clones that were distinguishable via microsatellite markers to ensure that positive detections were not due to contamination with aphids from the *Rickettsiella* (+) line. We also included *Rickettsiella* (+) and *Rickettsiella* (−)-only controls in each experiment where all individuals were expected to test positive and negative for *Rickettsiella* infection, respectively. All experiments were performed at 19 °C where the *Rickettsiella* infection spread readily in caged populations.

In the first experiment (Fig. 5*A*), we set up mixed populations of *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* at a 1:1 frequency on bok choy plants, with an identical setup to the previous experiment ("*Rickettsiella Population Dynamics*") except for the use of different clones. We set up four replicate cages, with 20 aphids per clone. At week 9, we screened 15 aphids per replicate cage for *Rickettsiella* and clonal type using microsatellite markers ("*Endosymbiont Detection and Quantification*" and "*Microsatellite Analysis*").

In the second experiment (Fig. 5*B*), we placed 50 *Rickettsiella* (+) *M. persicae* on bok choy plants in three replicates and allowed them to colonize the host plant for 7 d. Aphids were then removed from plants and killed by wiping the plant surface with a paper towel and spraying the soil, cage, and plant with 100% ethanol. Fifty *Rickettsiella* (−) aphids were then placed on the plant and a subset of individuals with a light green body color were later removed at 3, 7, and 14 d and screened for *Rickettsiella* and clonal type, with 10 to 15 individuals screened per cage and time point. At each time point, plants were checked for nymphs with a dark green body color and these were also screened with microsatellite analysis to confirm that there was no contamination.

In the third experiment (Fig. 5*C*), *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* were placed on the same plant with physical separation. Ten adult aphids of each line were placed on separate leaves of a bok choy plant which were individually enclosed within clip cages [foam discs with mesh coverings that are sealed around the leaf with insect pins (84)] to prevent aphids escaping. We set up six replicates for each treatment, and 10 aphids from the clip cage containing *Rickettsiella* (−) aphids were screened for *Rickettsiella* infection and microsatellite analysis after 7 d on the host plant.

In the fourth experiment (Fig. 5*D*), we placed a single *Rickettsiella* (+) and a single *Rickettsiella* (−) aphid together on a 20 mm bok choy leaf disc in a 35 mm ×

10 mm Petri dish with agar. This was replicated 20 times. Aphids were left 7 d before screening all individuals for *Rickettsiella* infection and microsatellite markers. Dishes where one or both aphids died were excluded from the analysis.

Vertical Transmission of *Rickettsiella* Following Horizontal Transmission in *M. persicae*. We tested the potential for vertical transmission of *Rickettsiella* acquired through the host plant at 19 °C. In the first experiment, a single *Rickettsiella* (+) and *Rickettsiella* (−) nymph (2 to 3 d old) were placed together on a 20-mm bok choy leaf disk in a 35 mm × 10 mm Petri dish with agar (replicated 60×). Five positive [two *Rickettsiella* (+) aphids] and negative [two *Rickettsiella* (−) aphids] dishes were set up as control groups. Aphids were left for 7 d until *Rickettsiella* (−) aphids (distinguishable by their light green color) were removed and dissected ("*Dissection and qPCR of Aphid Tissues*"). Dissected ovaries and the remaining carcasses of individuals were screened for *Rickettsiella* ("*Endosymbiont Detection and Quantification*"). *Rickettsiella* (+) aphids were also screened to confirm infection status. Dishes where one or both aphid(s) died were excluded from the analysis.

We then performed two additional experiments to test for vertical transmission more directly by screening offspring of positive individuals using the above protocol, each with 50 replicates. *Rickettsiella* (+) and *Rickettsiella* (−) aphids were left for 7 d and then *Rickettsiella* (−) aphids were moved to a new Petri dish to produce offspring. After 3 to 4 d, the *Rickettsiella* (−) aphids that produced more than five nymphs were stored and screened for *Rickettsiella* ("*Endosymbiont Detection and Quantification*"). The F_1 nymphs of parents testing positive for *Rickettsiella* were maintained individually on bok choy leaf discs. After producing more than five F_2 nymphs, the F_1 aphids were stored for *Rickettsiella* screening. In the first additional experiment, we also screened the F_2 offspring from positive F_1 individuals. We refreshed the plant material twice a week in the first additional experiment and once a week in the second one.

Plant-Mediated Horizontal Transmission of *Rickettsiella* in *A. pisum*. To test whether a high propensity for horizontal spread of *Rickettsiella* was also observed in the native host *A. pisum*, we set up two experiments using either nymphs (20 replicates) or adults (30 replicates). Methods followed the Petri dish horizontal transmission experiments described above, but with a single *Rickettsiella* (+) individual (from the clone used in microinjection) and a *Rickettsiella* (−) aphid (from a clone lacking *Rickettsiella* but naturally infected with *Regiella*) *A. pisum* placed together on 20 mm broad bean leaf discs for 7 d.

***Rickettsiella* Transmission at 25 °C.** We performed two experiments to test the stability of *Rickettsiella* transmission at 25 °C. In the first experiment, we initiated three replicate population cages with 30 *Rickettsiella* (+) *M. persicae* on bok choy plants. These populations were maintained at a constant temperature of 25 °C by transferring aphids to new plants every 14 d according to the above procedure ("*Rickettsiella Population Dynamics*"). We then measured *Rickettsiella* infection frequencies at week 12 by screening 20 aphids per replicate cage with a qPCR assay ("*Endosymbiont Detection and Quantification*").

In the second experiment, 20 adult *Rickettsiella* (+) aphids were placed individually on 20 mm bok choy leaf discs in Petri dishes (35 mm × 10 mm) and maintained at 25 °C. Adults were left to produce offspring for 3 d, after which all nymphs were removed and new nymphs were collected within 7 h of birth to reduce the potential for transmission of *Rickettsiella* via the host plant. A single nymph from each replicate line was transferred to a new bok choy leaf disc and parents were stored in 100% ethanol. This process was repeated until the F_4 generation, where F_4 nymphs were stored in 100% ethanol when less than 72 h old. We then screened all adults from the F_1, F_2, and F_3 generations for *Rickettsiella* ("*Endosymbiont Detection and Quantification*") to test for the loss of infection in each lineage.

Heat Tolerance. We measured the tolerance of *Rickettsiella* (+) and *Rickettsiella* (−) aphids to high temperatures using static and dynamic heat knockdown assays (85). In each experiment, 7-d-old aphids were placed individually in 5 mL glass vials that were sealed with a plastic screw-top lid. Vials were randomized in order, clipped to a plastic rack, and submerged in a water tank. To measure heat knockdown time, vials were placed in a water bath set to a constant 41.5 °C. We then recorded the time that aphids became incapacitated (i.e., unable to right themselves).

To measure CTmax, vials were established in the same manner and placed in a water bath which increased in temperature from 25 °C at a rate of 0.1 °C per minute until all aphids became incapacitated. We then recorded the temperature where aphids became incapacitated to the nearest 0.1 °C. Water temperatures were monitored in real time with a custom probe and recorded with ibuttons (Thermochron; 1-Wire, iButton.com, Dallas Semiconductors, Sunnyvale, CA, USA) placed in a glass vial in the water tank. We performed five separate runs for the heat knockdown assay and three runs for CTmax, with 10 to 15 individuals from each line measured in each run.

Endosymbiont Detection and Quantification. qPCR assays were used to confirm the presence or absence of *Rickettsiella* and *Buchnera* infection and measure their density relative to a host gene. DNA was extracted using 150 μL 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) according to methods described previously (86). Endosymbiont detection and quantification was performed using a LightCycler® 480 High Resolution Melting Master (HRMM) kit (Roche; Cat. No. 04909631001, Roche Diagnostics Australia Pty. Ltd., Castle Hill New South Wales, Australia) and IMMOLASETM DNA polymerase (5 U/μL) (Bioline; Cat. No. BIO-21047) as described by Lee et al. (86). Each run included at least three *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* of a known infection status, plus wells with primers but no DNA. Three primer sets were used to amplify markers specific to *M. persicae* (actin, *Rickettsiella,* and *Buchnera*). Relative *Rickettsiella* and *Buchnera* densities were determined by subtracting the Cp value of the *Rickettsiella*- and *Buchnera*-specific marker from the Cp value of the aphid–actin-specific marker. Differences in Cp were averaged across 2 to 3 consistent replicate runs, then transformed by $2^{\Delta Cp}$. Units presented in the figures are the transformed values shown on a log scale.

Body Color and Length Measurements. We measured body length and relative differences in body color by placing live aphids on filter paper in a Petri dish under a dissecting microscope (Leica MS5, 40× magnification). Photographs of each aphid were acquired with a microscope camera (Leica IC80 HD) and LAS EZ software under consistent lighting conditions, magnification, and camera settings. Photographs were analyzed with ImageJ, with linear measurements taken from the front of the head to the rear of the abdomen (excluding the cauda) as an estimate of body length. To measure relative body color, we selected 150 × 150 pixel circles on the abdomen and obtained average Red Green Blue (RGB) values using the RGB measure plugin (https://imagej.nih.gov/ij/plugins/rgb-measure.html). RGB values were then converted to HSL (hue, saturation, lightness) in Microsoft Excel. Images were measured twice each for body length and body color, with the two measurements averaged for analysis. Alate (winged) aphids, damaged aphids, and photographs that were out of focus were excluded from the analysis.

Microsatellite Analysis. All *M. persicae* lines used in the experiments and all individuals from the horizontal transmission experiments that tested positive for *Rickettsiella* were genotyped to confirm their clonal type using 10 DNA microsatellite loci (82, 83). DNA extractions were centrifuged for 2 min at 20,800 g (Eppendorf Centrifuge 5417 C, Hamburg, Germany) and 2 μL of the supernatant was used as template in polymerase chain reactions. Loci were pooled into three groups, labeled with unique fluorophores (FAM, NED, VIC, and PET), and coamplified by multiplex PCR using a Qiagen multiplex kit and an Eppendorf Mastercycler S gradient PCR machine according to the protocol described by (87). Genotyping was subsequently performed using a 3730 capillary analyzer (Applied Biosystems, Melbourne, Australia) and product lengths were scored manually using GeneMapper version 4.0 (Applied Biosystems).

Statistical Analysis. Phenotypic data were analyzed with IBM Statistical Package for Social Sciences (SPSS) Statistics 29.0 for Windows. Data for development time, fecundity, body length, endosymbiont densities, and body color components were analyzed with general linear models, with temperature (developmental temperature or heat shock treatment), *Rickettsiella* infection status, and their interaction included as factors. *P* values were adjusted with Bonferroni correction if multiple traits were measured in the same individual or groups of aphids. By including aphids that died before they produced offspring, we also computed the total reproductive output of the aphids. These data had many zero values, so we tested differences among the treatments within the *Rickettsiella* (+) and *Rickettsiella*- lines with zero inflation regression using the zeroinf function in the R package pscl run as a plug in within IBM SPSS Statistics. For thermal tolerance traits (heat knockdown time and CTmax), we included run as a random factor and also performed analyses on each run separately due to significant interactions between *Rickettsiella* infection status and run. We used Cox regression to assess the impact of *Rickettsiella* infection on aphid longevity.

Developmental time was recorded based on the time to produce their first nymph. Fecundity was determined by counting the total nymphs produced during an individual's lifetime. Aphids that died before reaching adulthood were excluded from the analyses of development time and fecundity. To assess the timing of peak reproduction, we calculated the parental age when each nymph was produced and averaged this value across all nymphs for each parent. For body color, we analyzed each component (hue, saturation, lightness) separately. Endosymbiont densities were analyzed separately for each generation; it was not possible to perform direct comparisons between generations because densities were quantified in separate runs.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*. *Rickettsiella viridis* 16S rRNA gene sequences are available on Figshare at doi:10.26188/22541608 (88).

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