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Title:
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Date:
2025-11-01

Citation:
Ansermin, E., Hoffmann, A. A. & Ross, P. A. (2025). Changes in the behavior of Myzus persicae (Hemiptera: Aphididae) in response to predators after transinfection with Rickettsiella (Legionellales: Coxiellaceae) endosymbionts. Annals of the Entomological Society of America, 118 (6), pp.502-512. https://doi.org/10.1093/aesa/saaf034.

Persistent Link:
https://hdl.handle.net/11343/367888

Annals of the Entomological Society of America, 2025, 118(6), 502–512
https://doi.org/10.1093/aesa/saaf034
Advance access publication 25 September 2025
Research

Changes in the behavior of *Myzus persicae* (Hemiptera: Aphididae) in response to predators after transinfection with *Rickettsiella* (Legionellales: Coxiellaceae) endosymbionts

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Subject Editor: David Onstad

Infection with endosymbionts can cause changes in the phenotype of their insect hosts, particularly for life-history traits such as fecundity and temperature sensitivity. Economically important pests such as aphids carry different species of endosymbiotic bacteria, some of which have potential as biocontrol agents. The impact of newly transinfected bacteria on behavioral responses of aphids (Hemiptera: Aphididae) has rarely been explored; however, traits such as microhabitat selection and response to predators could disrupt or reinforce the effectiveness of biocontrol. In this study we compared the behavior of green peach aphids, *Myzus persicae* (Sulzer, Hemiptera: Aphididae), with or without a transinfection of the facultative endosymbiont *Candidatus Rickettsiella viridis* (Legionellales: Coxiellaceae). In whole plants assays, we tested differences in microhabitat selection, leaf surface productivity and response to the predatory ladybird *Hippodamia variegata* (Goeze, Coleoptera: Coccinellidae). The transinfection affected the variance but not the mean of leaf surface preference, with the infected line aphids being more consistent across days. Aphid productivity did not differ between surfaces nor was it affected by the infection. Choice tests suggested that ladybird preference was not affected by *Rickettsiella.* However, the aphid lines responded differently to the predator in that the infected aphids were relatively more common on the adaxial surface in the presence of the predator whereas uninfected aphids showed no difference. Our results suggest that transinfected *Rickettsiella* endosymbionts can change their hosts' behavior in subtle ways but their immediate impacts on the effectiveness of predator-based biocontrol are not necessarily clear.

Keywords: endosymbiont, pest, predation, sustainability, aphid

Introduction

Herbivorous insects detect and respond to numerous cues to select microhabitats, including plant suitability and leaf quality, and also seek protection against extreme temperatures, rainfall, natural enemies, and sometimes pesticides (Rosenzweig 1991, Nalam et al. 2021, Vives-Ingla et al. 2023). Responses to multiple components of habitat properties could lead to contradictory outcomes which might then result in fitness costs overall. An example is the foraging-predation trade-off, where the prey compromise between gaining energy through nutrition or avoiding predation (Houston et al. 1997, Lima 1998). The impacts on overall fitness could then depend on microhabitats where organisms are found and their quality in terms of nutritional resources versus refuges against predators (Rosenzweig 1991, Houston et al. 1997, Krebs 2002, Nalam et al. 2021).

For aphid species, trade-offs in habitat selection can shift according to habitat conditions and intrinsic factors of the aphids. When selecting between 2 microhabitats that are both suboptimal, competing species can change their behavior based on a range of factors. In particular, Yin et al. (2021b) showed that *Rhopalosiphum padi* and *Sitobion avenae* had different habitat responses, with the former selecting a more favorable microclimate of a wheat plant over an optimal diet and the latter showing the reverse response. This species difference resulted in *R. padi* being common lower in the plant whereas *S. avenae* was more common on upper leaves and ears. Nevertheless, this response led to high fitness for both species and likely reduced competition among them through "niche separation" (De Vita 1979). This may be a general phenomenon not only in animals but also in plants, fungi, and protists, that

Received: 28 March 2025. **Revised:** 23 August 2025.

in turn produces functional redundancy in ecosystems (Gunderson 2000, Silvertown 2004, Nolte et al. 2010, Cabrera-Mora et al. 2019).

In aphids, leaf orientation may also impact fitness, depending on the response of aphids to gravity (Wu et al. 2022). Yin et al. (2021a) showed that the wheat aphid, *Metopolophium dirhodum*, had a clear preference for the upper (adaxial) surface of leaves when ovipositing, and *Myzus persicae* migrated toward the abaxial leaf surface of radish plants (Calabrese and Edwards 1976). Abiotic factors like temperature may differ between the 2 sides of a leaf, as well as sugar or amino acids availability (Keiser et al. 2013). Aphids may respond to leaf quality in foraging and oviposition (Yin et al. 2021a).

From an applied perspective, microhabitat selection and predator–prey interactions of aphid pests can be economically important. Aphids, like the green peach aphid, *M. persicae*, are major pests distributed worldwide and feed on a large spectrum of crops and ornamental plants, causing important economic losses every year both through direct feeding and through plant virus transmission (van Emden et al. 1969, Guerrieri and Digilio 2008, Valenzuela and Hoffmann 2015, Machado-Assefh and Alvarez 2018). Pesticides have been heavily used to control aphids in crops but often represent an environmental hazard to nontarget species (Mata et al. 2024). Chemical control is becoming less effective due to the evolution of resistance, in particular in *M. persicae* (Foster et al. 2000). Additionally, pesticide impact can be countered by pest behavior, and specifically by their microhabitat selection. Contact pesticides will be less effective if pests are protected by the architecture of plants and their surroundings.

Natural enemies can help to sustainably regulate aphid pest populations through predation or parasitism, but they are affected by abiotic and biotic factors that are hard to predict (Brodeur and McNeil 1989, Pervez and Omkar 2011, Wang et al. 2013, Lumbierres et al. 2021, Ge et al. 2023), as well as microhabitat selection by pests which can make it harder for natural enemies to find preys. Nevertheless, insects such as ladybirds can be important biocontrol agents against aphids (Dixon 1959, Hodek 1973, Guerrieri and Digilio 2008). Ladybirds can be deliberately released into environments following culture in insectaries and they can be conserved in natural environments by reducing applications of pesticides. Predatory ladybirds like *Hippodamia variegata* (Goeze) have historically been one of the most efficient aphid predators of aphids (Hodek and Michaud 2008, Cabral et al. 2009, Madadi et al. 2011). Their foraging behavior starts by locating their preys' habitat through olfactory cues that allow ladybirds to detect and recognize volatile allelochemicals emitted by plants infested with aphids (Khan et al. 2017). Following arrival in the preys' habitat, ladybirds search for aphid silhouettes and coloring in addition to aphid secreted honeydew (Evans and Richards 1997) and alarm pheromones (Francis et al. 2004, 2005). As already noted architecture of the plant itself can influence the predators' success (Pervez and Yadav 2018).

Apart from natural enemies, there is increasing interest in using microbes as biocontrol agents, including bacteria living inside aphids known as endosymbionts. These are symbiotic intracellular bacteria found in aphids as well as most other insects, and their presence influences a variety of their host's traits (Batra and Buchner 1968, Chen and Purcell 1997, Feldhaar 2011). The application of endosymbiotic infections in pest control has been explored most extensively for *Wolbachia* endosymbionts, which are already used widely to reduce the impact of mosquito borne diseases through both suppressing *Aedes* mosquito populations and the suppress arbovirus transmission into *Aedes* populations via introduction of *Wolbachia* strains originating from *Drosophila* or other *Aedes* mosquitoes (Hoffmann et al. 2011, Zheng et al. 2014, 2021, Hu et al. 2019, Nazni et al. 2019, Ryan et al. 2019).

An aphid endosymbiont that may be useful for pest control is *Candidatus Rickettsiella viridis* (referred to hereafter as *Rickettsiella*). The natural infection of this endosymbiont in the pea aphid, *Acyrthosiphon pisum*, causes color change in its host to dark green (Tsuchida et al. 2010). *Rickettsiella* has been transinfected into *M. persicae*, where it also modifies their body color to dark green, and significantly decreases their fecundity (Gu et al. 2023). Its ability to spread vertically from mother to offspring and horizontally via plant tissues provides an opportunity to use targeted releases to suppress *M. persicae* populations (Gu et al. 2023, Slavenko et al. 2024). However *Rickettsiella* may have undesirable properties on their transinfected host which need to be tested; in particular, the effect of endosymbionts on behavioral traits such as microhabitat selection is often neglected in favor of common life-history traits like fecundity (Feldhaar 2011). Furthermore endosymbionts sometimes have effects on higher trophic levels (Clark et al. 2010), which include parasitoids and ladybirds. While transinfected *Rickettsiella* may not influence parasitism rates (Soleimannejad et al. 2023), the infection could have an impact on predation susceptibility particularly given the body color changes that have been observed. Previous studies have shown that *Rickettsiella* can influence predation susceptibility in a context-dependent manner (Polin et al. 2015, Bitsch et al. 2025), but these studies have not elucidated whether effects are due to predator preferences or differences aphid behavior, nor if plant architecture plays any role in them.

In this laboratory study, we investigated the effects of the endosymbiont *Rickettsiella* on aphid habitat selection and susceptibility to predation by the aphidophagous ladybird, *Hippodamia variegata*. Because behavior of both the predator and the prey may be influenced by plant architecture (Yin et al. 2021a), we performed assays on whole plants to better reflect the conditions in natural settings. Aphids were either infected or uninfected with *Rickettsiella*, which had been transinfected into *M. persicae*. We show an impact of the transinfection on the variance of aphid preferences for leaf surfaces and in behavioral changes in pure versus mixed cohorts but no clear connections to aphid fitness under predation.

Materials and Methods

Aphid Maintenance and Age Matching

We cultured *M. persicae* in controlled temperature chambers at 19 ± 1 °C with a 16:8 light:dark photoperiod, on bok choy (*Brassica rapa* var. *chinensis*) leaf discs kept in Petri dishes (60 × 15 mm) on a layer of 1% (w/v) agar gel. We changed plant material and Petri dishes weekly. The culture procedure followed methods described by Yang et al. (2023).

The 2 lines used in this study and hereafter referred to as RCL− and RCL+ both originated from a clone initially collected in Horsham (Victoria, Australia, GPS coordinates: −36.72263; 142.175415), on canola, *Brassica rapa*. The RCL− line was not modified, while the RCL+ line was transinfected with *Rickettsiella viridis* from a donor line of pea aphid, *Acyrthosiphon pisum* by microinjection (Gu et al. 2023), and maintained in the laboratory under the same conditions as the RCL– line. Because these aphid lines are both derived from the same clone recently, they should be almost entirely genetically identical for their nuclear genome.

To obtain groups of aphids of synchronized age, adults were separated from the mixed-age population and kept in Petri dishes with 1% agar gel and leaves of bok choy at 19 °C. After 24 h, the nymphs that were produced overnight were separated from the adults and kept in a separate Petri dish to be used in experiments at the appropriate age.

Host Plant Maintenance

We grew bok choy (*Brassica rapa* var. *chinensis*) in sterilized soil and in shade house conditions. Plant growth was supported with LED lights (40 W Grow Saber light-emitting diode [LED] 6,500 K, 1,200 mm length) with a photoperiod of 16:8 light:dark. Plants were watered 2 to 3 times a week.

Aphid Leaf Surface Preference

Aphid leaf preference was assessed by releasing 20 to 30 5-d-old individuals from the RCL– or RCL+ lines onto a 3-wk-old bok choy plant and counting the number of aphids on each surface daily. At the start of the experiment, aphids were haphazardly released on the adaxial surface of the plant's leaves, as it is difficult to release them on the abaxial surface without any support structure. Each plant was kept in a cage (23 cm × 23 cm × 60 cm, mesh size not exceeding 160 µM). We only focused on adaxial versus abaxial leaf surfaces and not on aphids feeding on the stem as almost no aphids were found there. The experiment was replicated in 4 blocks. Each replicate was evaluated daily for 5 d after leaving the insects on the plant for 3 d to acclimate, although in the first experimental block the replicates were evaluated every second day. We evaluated a total of 21 replicates for RCL+ and 16 for RCL–.

Leaf Surface Cumulative Fecundity

To estimate the relative fitness of RCL– and RCL+ *M. persicae* on the adaxial versus the abaxial surfaces, 1-d-old nymphs were placed in clip cages (4 cm diameter, mesh up to 160 µM) with a single nymph on both the adaxial and abaxial surface of a bok choy leaf. Leaves that were (haphazardly) selected held 2 clip cages (1 per surface). Support for the clip cages was provided by bamboo sticks, to ensure that the leaves were kept parallel to the ground throughout the experiment and that the weight of the clip cage did not bend over the plants. We used whole plants of 5 to 9 wk old, in their vegetative stage. Aphids were maintained in a temperature-controlled room at 19 °C and with a 16:8 light:dark photoperiod. After 10 d, the clip cages were opened and the number of offspring on each leaf surface was counted. The experiment involved 23 and 15 replicates, respectively, for RCL– and RCL+.

Aphid Response to Predators

To verify the correlation between aphid color and infection status which facilitates scoring in behavioral assays, we screened 19 green individuals and 21 yellow individuals with qPCR (described below). All these green aphids were infected with *Rickettsiella*, and all the yellow aphids were not infected; therefore, we determined infection status for this experiment by noting the color of each scored aphid. Note that we only used adult aphids where the color difference is more pronounced, as it is difficult to score in younger nymphs.

Adult *Hippodamia variegata* were sourced from a commercial provider (Biological services, Loxton, Australia). They were kept at 11 °C (with a photoperiod of 16:8 light:dark) upon arrival and were fed with RCL– *M. persicae* adults every 4 d after arrival prior to the experiments.

We measured aphid responses to predators by setting up single or mixed populations of RCL– and RCL+ aphids in the presence or absence of ladybirds (starved for 24 h and then added) and measuring leaf surface preferences and *Rickettsiella* frequencies. We set up 3 different treatments in the presence of ladybirds (RCL+: $n = 5$, RCL–: $n = 3$, mixed: $n = 18$), each paired with a control treatment (RCL+: $n = 5$, RCL–: $n = 6$, mixed: $n = 15$) where the ladybird was not added (Supplementary figures).

In mixed cohorts, we added 10 aphids from each of the 2 lines per plant. For the single line treatments, we used 20 aphids per plant from a single line. We placed 6-d-old aphids on a single 4-wk-old plant, contained in a clear plastic cup (9 cm diameter, 14 cm length) and sealed with fabric mesh (up to 160 µM). After 48 h since placing the aphids on the plants, we counted aphids on adaxial and abaxial surfaces and added a ladybird (starved as mentioned above). Aphids were scored every 2 h until all replicates with ladybirds had around 50% of their starting number of aphids left. Scoring was achieved by temporarily opening the cup and removing the ladybird to count aphids on the leaf surfaces. We gently removed the plant from the cup as it offered a clearer view of the actual aphid number. We rarely observed aphids on the stem or parts of the plant different than leaf surface; therefore, those aphids were excluded from the evaluation.

Predator Preference Assay

Adults of *Hippodamia variegata* were sourced and were kept as for the previous experiment. One day before the assay, the ladybirds were sexed by observing genitalia under a microscope according to methods described elsewhere (Stellwag and Losey 2014). Males were discarded and female ladybirds were starved for 24 h prior to assays.

We put age-matched *M. persicae* on either the adaxial or abaxial surface (2 aphids per side, 1 for each treatment: RCL– and RCL+). Their ages ranged from 5 to 7 d since emergence with all individuals used in a replicate having the same age. The clip cages were sealed with a rubber band. The leaves with clip cages were placed into plastic cylinder cups and held stable by sliding the stem into a hole at the base of the cup. The cups were kept in water-filled racks for 24 to 48 h at 19 °C.

To assess the predator's initial preference for an RCL+ or RCL– aphid, a female ladybird starved overnight was released into the base of the cup after clip cages were opened to allow access of the predator to the 4 aphids being tested (Supplementary figures). This was done sequentially. Infection status was based on aphid color (dark green or yellow)—this was confirmed for some aphids with quantitative polymerase chain reaction (qPCR). The arena was then closed with a plastic lid and Parafilm and the predator's behavior was observed until a choice of prey was made or 30 min had elapsed (for $n = 35$ arenas). The plastic cup was oriented with the abaxial surface of the leaf parallel and facing the ground.

The experiment was performed in blocks of 10 replicates and repeated until we reached a total of 35 "valid" replicates where the ladybird had made a choice.

DNA Extraction and Endosymbiont Detection by qPCR

For endosymbiont detection and quantification, we followed methods previously described by Gu et al. (2023). To extract DNA, insects were placed separately into Eppendorf tubes with 2 glass beads (2 mm), 150 µl of Chelex 100 resin solution 5% m/V (Bio-Rad Laboratories, Hercules, California), and 3 µl of proteinase K. To grind the insects' tissues, the samples were put in a TissueLyser (Qiagen TissueLyser II) for 1 min at 25 hz with 2 glass beads. The homogenized samples were then centrifuged (Eppendorf centrifuge 5425) at room temperature for 1 min at 21,300 x g and incubated in a water bath at 65 °C for 1 h and a further 10 min at 90 °C.

The supernatant was collected from DNA extractions after centrifuging for 5 min at 21,300 x g then diluted 1:3 with purified water (ELGA). We detected endosymbiont infection by 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). To 2 µl of diluted DNA ($n = 4$ per sample), we added 8 µl of master mix (for 1 sample): 5 µl of RRT buffer (3.27 µl purified water, 0.4 µl magnesium dichloride 50 mM, 1 µl ThermoPol reaction buffer Biolabs, 0.25 µl HRMM, 0.06 µl deoxynucleotide triphosphate), 2.92 µl of water, 0.08 µl of forward and reverse primers mix, and 0.01 µl of IMMOLASETM DNA polymerase. In each run, we included at least 3 *Rickettsiella*-infected *M. persicae* of known positive infection status, and negative control wells with water and master mix but no DNA.

We determined the relative *Rickettsiella* density from the difference between the Cp values of the *Rickettsiella*-specific marker and the aphid actin-specific marker as outlined in Gu et al. (2023).

Statistical Analysis

All statistical analyses were performed in R (version 4.3.0) except for the binomial confidence intervals for which we used an online calculator (see below). To test leaf surface preference, we transformed our proportional data with the logit link function (Douma and Weedon 2019). We then fitted a generalized linear mixed model with multivariate normal random effects using penalized quasi-likelihood (glmmPQL), with no maximum iterations. Logit-transformed proportions of aphids on adaxial surface were tested against the interaction between aphid line and day of evaluation (repeated measurements), while we treated the cage in which plants were kept as a random effect. The model was tested with an ANOVA Type II analysis.

Leaf surface cumulative fecundity was tested using the generalized linear model approach outlined above but adding nested random factors (leaf in plant in container in block). Where an aphid died on a leaf, data were excluded.

For leaf preference across days, we also considered the difference in variance between the 2 aphid lines in terms of the proportions found on different leaf surfaces across days (logit transformed). This because differences in variance could arise from transinfected aphids having altered movement rates and/or other behaviors such as olfactory responses to leaf surfaces. Two types of variance comparisons were carried out. In the first, we computed the variance in preference on a particular day and then compared these estimates between *RCL+ and RCL−* aphids with an *F*-test. We also looked for consistent patterns between days by combining probabilities from each test on a particular day using Fisher's approach (Power et al. 1970). In the second approach, we first computed the slope and variance in the change in preference across days for each replicate. We then compared these between RCL+ and RCL− aphids using a separate variance *t*-test and also tested whether the slopes were consistently different from zero.

For aphid responses to predators, the groups were tested with Student's *t*-tests and a Bonferroni corrected pairwise comparison.

The predator choice assay was analyzed by using a generalized linear mixed model with multivariate normal random effects using penalized quasi-likelihood (glmmPQL), no maximum iterations and assuming a binomial distribution. We nested as random factors the block and the ladybird. The model was tested with an ANOVA Type II analysis. We also computed binomial confidence intervals using the Wilson method in Epitools (https://epitools.ausvet.com.au/).

Results

Leaf Surface Preference

The main aim of this experiment was to test if *M. persicae* settling on a new plant, has a preference between the abaxial and the adaxial leaf surfaces, and if *Rickettsiella* changes the preference (Fig. 1). The proportion of adult aphids on the adaxial surface was affected by the day of evaluation (normal penalized quasi-likelihood GLM: $\chi^2 = 11.36$, df = 4, $P < 0.01$), but not by the aphid line (Figs 1 and 2, $\chi^2 = 0.29$, df = 1, $P = 0.59$).

Overall, there was a tendency for the percentage of aphids on the adaxial surface to decline across time. This is more easily seen when the patterns are averaged across replicates (Fig. 2). We tested the slope of the change in preference across days (signed) and found no difference in slope between the lines ($t = -1.118$, df = 22.39, $P = 0.138$). However, there was a consistent tendency for a negative slope across time (RCL−, $b = -3.76$; RCL+, $b = -2.14$) which differed from 0 by a *t*-test overall ($t = -4.164$, df = 36, $P < 0.001$).

We also noted that the RCL− aphids seemed to display a higher variance in the proportions on the adaxial surface within days and across days (Fig. 1). Comparing aphid line variance by day, we observed a significant difference at day 3, but not at any other timepoint (Table 1), although differences in variance were consistent. We used Fisher's method to determine the combined probability of the comparisons across days which resulted in a significant combined effect ($\chi^2 = 21.04$, df = 10, $P = 0.021$).

We also noted that the average variance of replicates across days was higher for the RCL− line (238.24) than the RCL+ line (127.36). The 2 lines' average replicate variances were marginally nonsignificantly different in a *t*-test with separate variances assumed ($t = 2.019$, df = 19.96, $P = 0.06$).

Finally, we correlated the variance of each replicate across days with their absolute values of their slopes for both aphid lines (Fig. 3). These 2 variables were positively correlated for RCL− (Pearson correlation test: $P < 0.001$, $r = 0.86$), but there

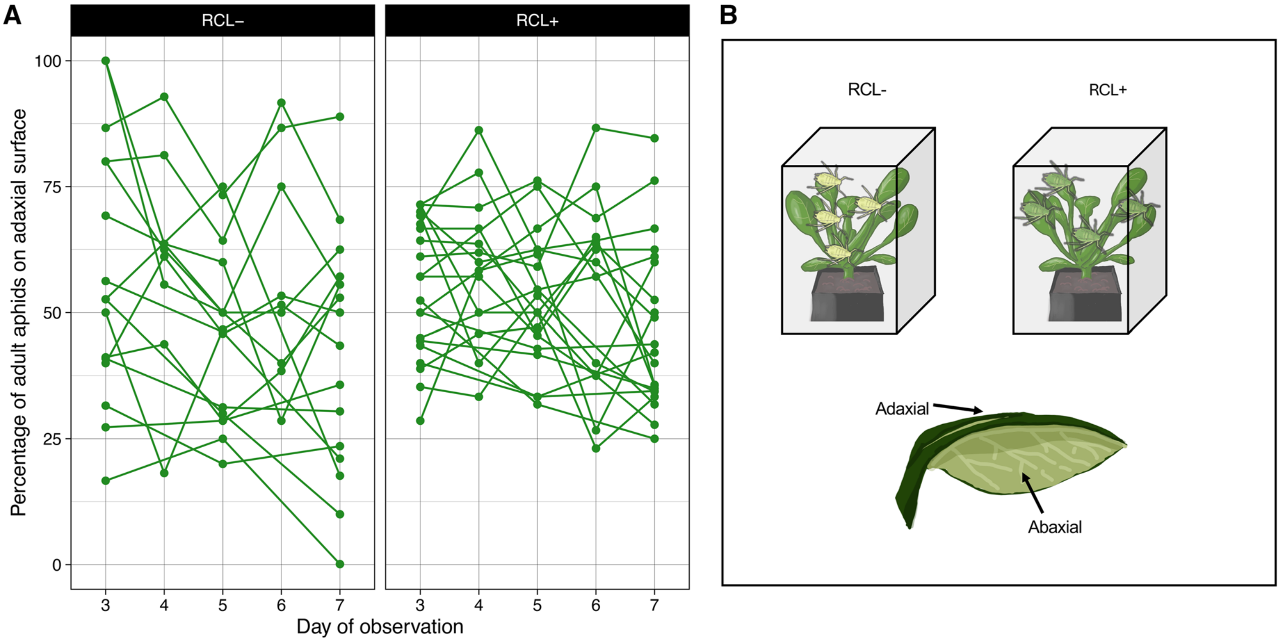

Fig. 1. (A) Percentage of adult aphids on the adaxial surface (*y* axis) over time for the RCL− and RCL+ lines. Dots and lines represent data from a replicate plant. (B) Graphical illustration of the experimental set up, aphids of each line were put on a plant in a cage and let acclimate for 3 d prior to the evaluations starting.

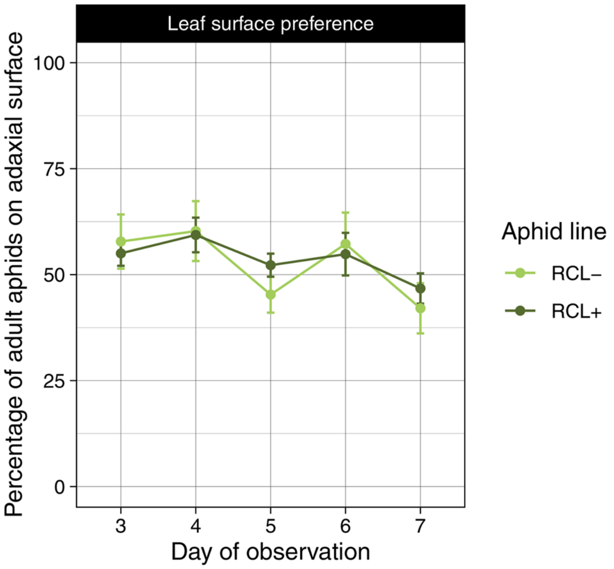

Fig. 2. Percentage of adult aphids on the adaxial surface (*y* axis) over time for the RCL− and RCL+ lines. Dots represent the mean, lines connect the means along timepoints. Vertical lines show the standard error.

was only a weak marginally nonsignificant correlation for the RCL+ line ($P = 0.087$, $r = 0.38$).

These data suggest that while the *Rickettsiella* infection did not influence the preference of aphids for the abaxial or adaxial surface, the RCL+ aphids seemed to behave more consistently within and across days. At a finer level, the RCL− aphids that showed a higher variation in preference across days tended to show a higher absolute change in their preference for the abaxial surface across time (Fig. 3).

Table 1. Variance homogeneity of the 2 aphid lines at each timepoint (day)

Day	Degrees of freedom	*P*-value	95% confidence interval	Ratio of variances
3	20, 15	**0.008**	0.101 to 0.715	0.278
4	12, 8	0.245	0.115 to 1.692	0.482
5	20, 15	0.194	0.195 to 1.383	0.537
6	13, 8	0.572	0.173 to 1.434	0.718
7	20, 15	0.124	0.174 to 1.232	0.479

We calculated the combined probability of the *P*-values with Fisher's method. Bold text highlights significant values. Combined probability (Fisher method): $P = \mathbf{0.021}$.

Leaf Surface Productivity

After 10 d, the number of nymphs produced was similar in both lines (GLM: $\chi^2 = 0.0005$, df = 1, $P = 0.98$), and surfaces ($\chi^2 = 1.19$, df = 1, $P = 0.27$) (Fig. 4). The 2 surfaces may therefore have had a similar nutritional value for both lines. Note that the RCL− line seemed to show a higher variance in nymph production across the leaf surfaces.

Aphid Response to Predators

As expected, ladybird presence reduced numbers in both RCL+ and RCL− treatments and in the mixed cohorts (Fig. 5B and C). Given the similar reductions in the mixed cultures of both RCL+ and RCL−, it is not surprising that the proportion of infected aphids was similar regardless of the presence of the predator and was around 50% (Fig. 5A). The proportion of infected aphids in mixed cohorts was tested with a Student's *t*-test ($t = 0.384$, df = 23.434, $P = 0.704$), and it suggests no preference of the ladybirds for either aphid line in the mixed cages

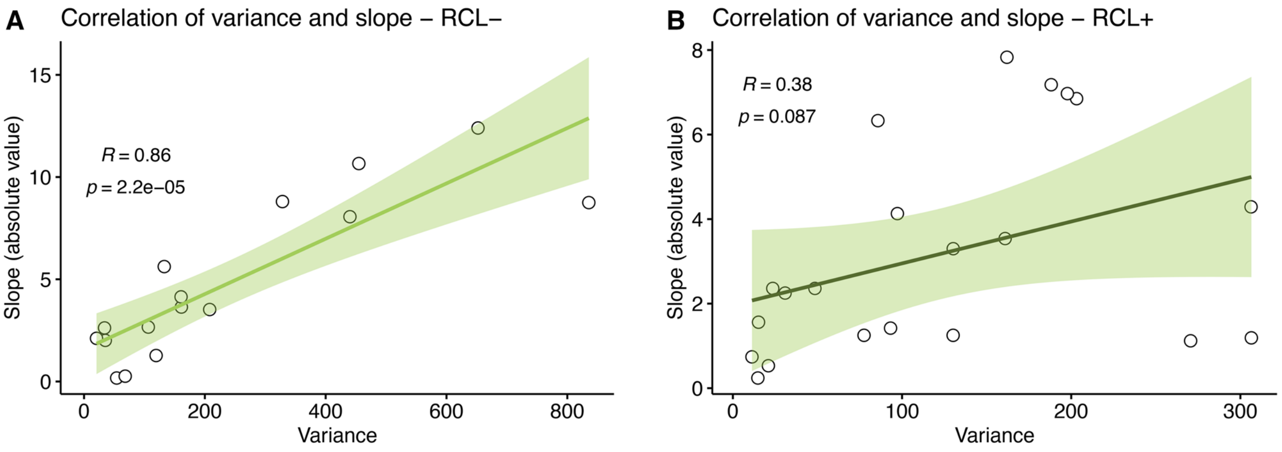

Fig. 3. Correlation between variance and slope of each replicate for RCL− (A), and RCL+ (B). Dots represent individual replicates, their position in the plot is determined by the variance of the evaluations during the experiment (*x* axis), and the absolute value of their slope (*y* axis). *R* and *P* are the correlation coefficient and the *P*-value of the Pearson correlation test, respectively. Lines are the linear association of the 2 variables tested, and the darkened area is the 95% confidence interval.

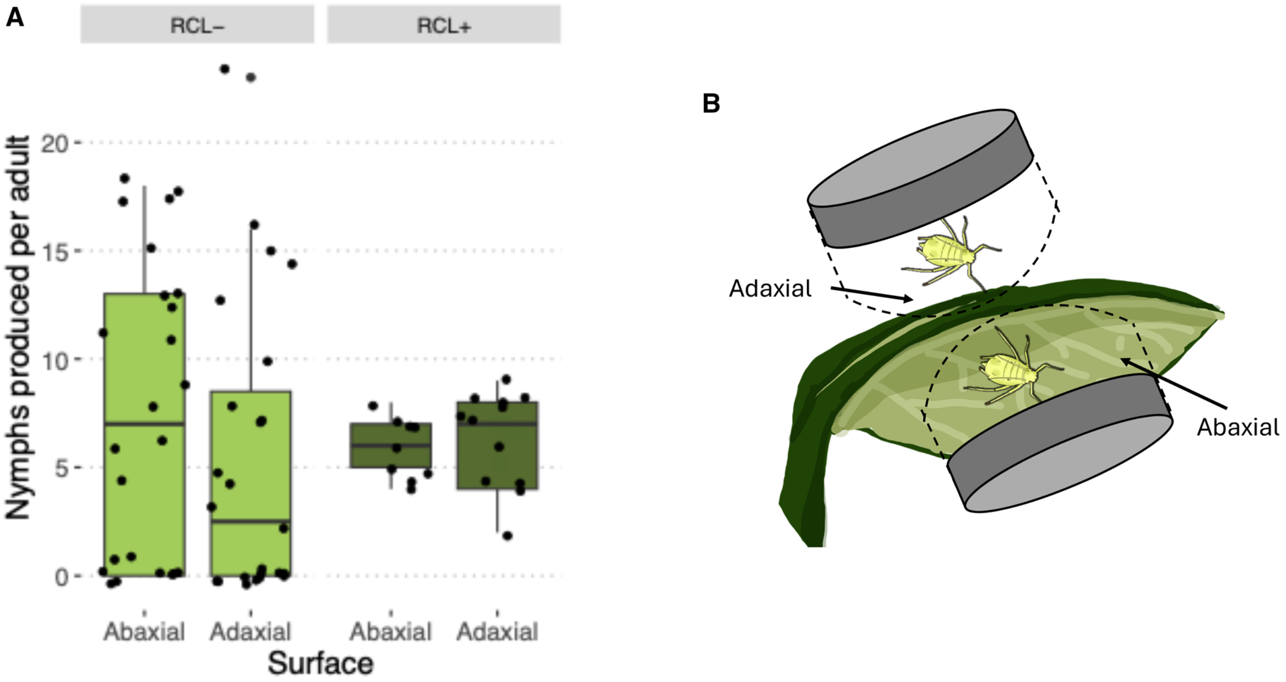

Fig. 4. Nymphs produced per adult. (A) Bold horizontal lines show medians; the lower and upper limits of the boxplot hinges correspond to the 25th and 75th percentiles. The upper and lower whiskers extend to highest and lower values, respectively. Values exceeding 1.5 times the interquartile range are not included in the whiskers' extent. Dots show values for single data points. (B) Graphical visualization of the experiment and what is represented in panel A.

(Fig. 5A). Patterns across the adaxial and abaxial surface were also similar in terms of the number of aphids present across treatments where the predator was present or absent (Fig. 5D and E).

However, there was a different response to the adaxial and abaxial surface in RCL+ and mixed cohorts. While the presence of the ladybird did not affect the relative distribution of aphids between the 2 surfaces (Fig. 6B and C) in the RCL− ($t = 0.469$, df = 2.953, $P = 0.672$) and mixed cohorts ($t = 1.501$, df = 29.999, $P = 0.144$), there was a difference in the RCL+ cohorts, where aphids were significantly more common on the adaxial surface (Fig. 6A, Student's *t*-test: $t = -3.705$, df = 6.302, $P = 0.009$).

The proportion of RCL+ aphids was similar on the 2 surfaces in the mixed cohorts (RCL−, Student's *t*-test: $t = 1.500$, df = 29.99, $P = 0.144$; RCL+, $t = -0.619$, df = 30.561, $P = 0.54$) as expected based on the distribution of green and yellow aphids and numbers on the 2 leaf surfaces (Fig. 5).

Predator Choice Assay

Ladybirds did not have a clear preference between RCL+ and RCL− aphids in the first 30 min (binomial GLM: $\chi^2 = 2.94$, df = 1, $P = 0.23$). However, we observed a marginally significant difference between leaf surfaces ($\chi^2 = 3.82$, df = 1, $P = 0.05$). The prey on the adaxial surface was more commonly chosen by the ladybirds (Table 2).

Discussion

Endosymbiont-driven behavioral modifications are poorly documented, although it is generally assumed that microbes

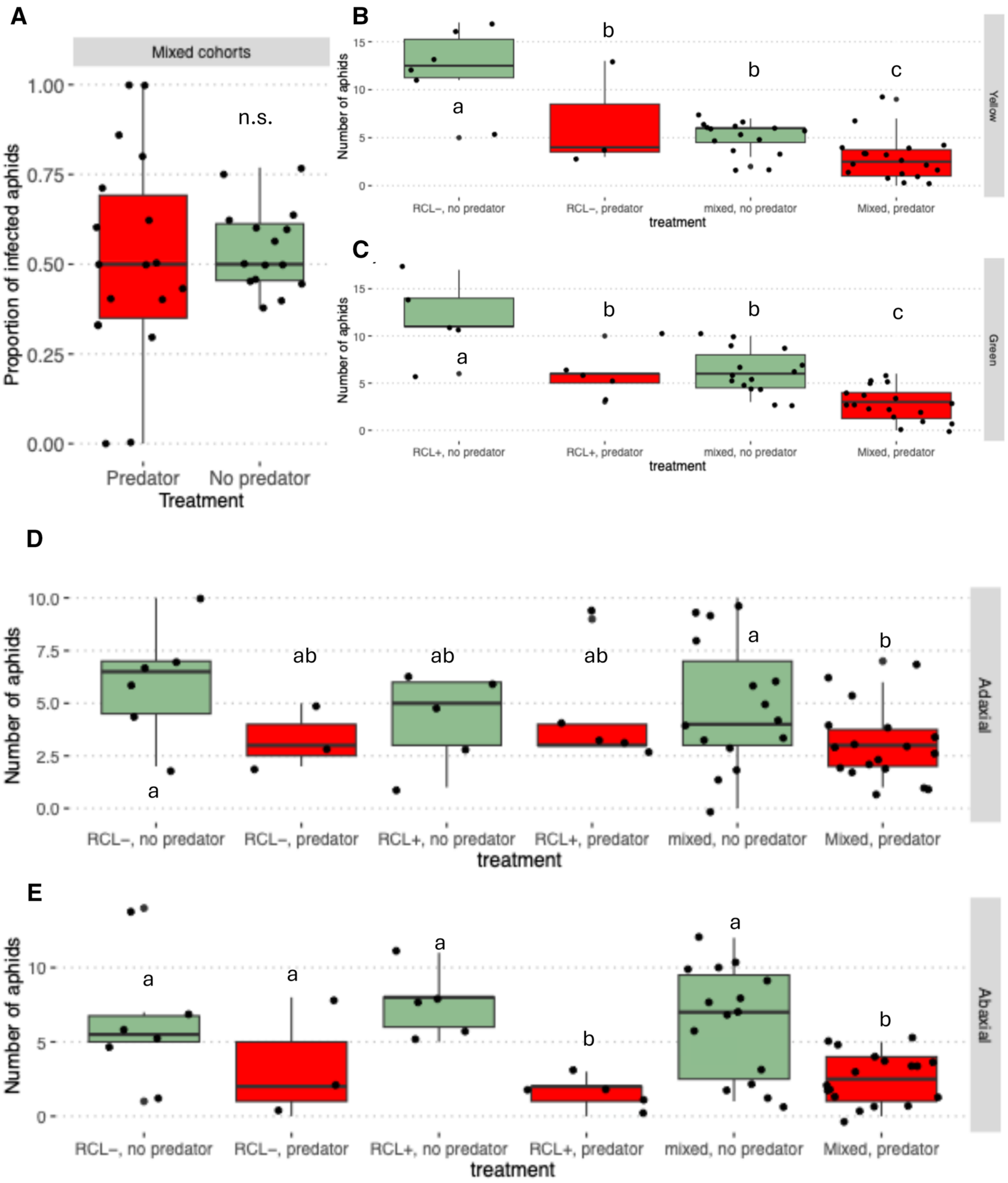

Fig. 5. (A) Proportion of aphids infected with Rickettsiella in mixed cohorts grouped by presence or absence of the ladybird (*x* axis), n.s. stands for nonsignificant (Student's *t*-test). (B) Number of uninfected (yellow) aphids (*y* axis) in each treatment (*x* axis). (C) Number of infected (green) aphids (*y* axis) in each treatment (*x* axis). (D) Number of aphids (*y* axis), in each treatment (*x* axis) on the adaxial surface, and the adaxial surface (E). Bold horizontal lines show medians, the lower and upper limits of the boxplot hinges correspond to the 25th and 75th percentiles. The upper and lower whiskers extend to highest and lower values, respectively. Values exceeding 1.5 times the interquartile range are not included in the whiskers' extent. Dots show values for single data points. Different letters atop or under the boxes determine statistical difference (Bonferroni-corrected pairwise comparison where there are more than 2 treatments), each pane (black header boxes) was analyzed independently from the others.

can modify their host's behavior to increase their chances to spread or their survival (Lewis and Lizé 2015). Foraging and oviposition behaviors seem to be the most commonly altered by these microbes (Jose et al. 2019, Sivakala et al. 2022). However, our results do not indicate a particular habitat preference of *M. persicae* for either leaf surface of bok choy plants after infection by a *Rickettsiella* endosymbiont (Figs 1 and 2) and our data do not show a fitness gain derived by feeding on inverted surfaces as previously described by Wu et al. (2022). Nevertheless, the RCL+ line exhibits lower variance regardless how this is analyzed, remaining more stable and consistent over time (Fig. 1A).

The higher variance of the RCL− line might indicate increased mobility, a more responsive nature to relevant cues, or differences in how individuals from the lines select microhabitats across time. We did not find evidence of this last hypothesis, as the proportion of adult aphids on the adaxial surface changed consistently across both lines. Although the final

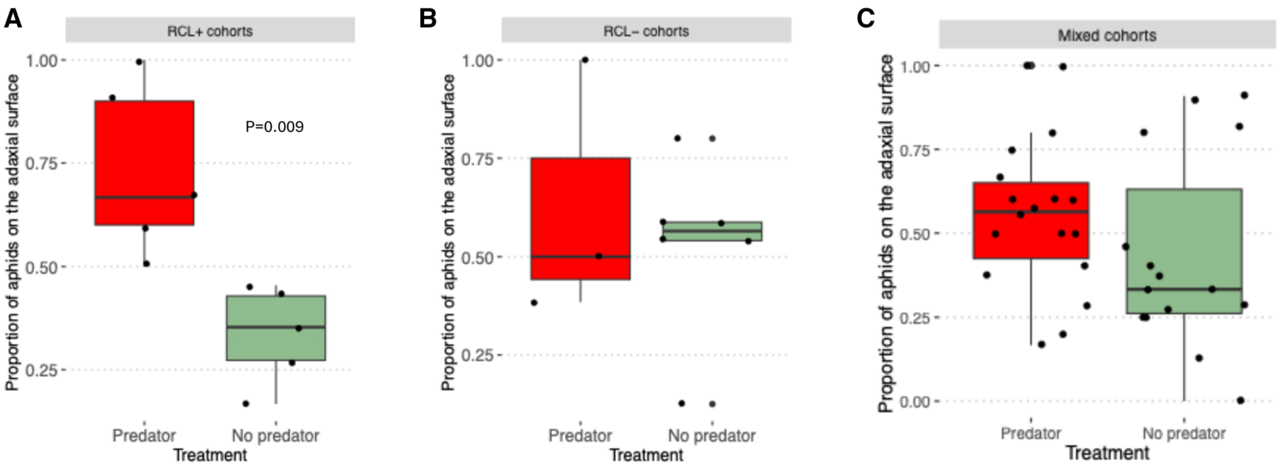

Fig. 6. Proportion of aphids found on the adaxial surface in (A) RCL+ cohorts, (B) RCL− cohorts, and (C) mixed cohorts with and without a predator present. Bold horizontal lines show medians, the lower and upper limits of the boxplot hinges correspond to the 25th and 75th percentiles. The upper and lower whiskers extend to highest and lowest values, respectively. Values exceeding 1.5 times the interquartile range are not included in the whiskers' extent. Dots show values for single data points. n.s. stands for nonsignificant (Student's *t*-test).

Table 2. Percentage of ladybird attacks to each aphid line and each surface in the first 30 min, and their binomial confidence intervals (CIs)

Attack frequency (total number = 35)	RCL+	95% CI values	RCL−	95% CI values
Abaxial surface	17.14%	0.081 to 0.327	22.86%	0.121 to 0.39
Adaxial surface	25.71%	0.142 to 0.421	34.29%	0.208 to 0.509

selection of microhabitat does not differ between lines, the absolute value of the slopes from across days of the experiment is positively correlated to that replicate's variance in line RCL− and less clearly for line RCL+ (Fig. 3). This suggests that particularly for the RCL− line, an aphids' position might tend to become more biased toward one or the other surface across time. The relatively more static behavior of RCL+ aphids could be deleterious if environmental challenges due to rainfall, high temperatures, or high UV intensity affect relative fitness on particular leaf surfaces. However, we recognize the option that a higher movement rate might reveal aphid presence to predators, incurring an indirect fitness benefit and/or trade-off of the transinfection with *Rickettsiella*.

Aphid movement from one surface to the other could also be driven by differences in nutrient availability (Nalam et al. 2021), leading to differences in fecundity or nymph performance. Endosymbionts are often closely associated with host nutrition (Douglas 1998, Akman Gündüz and Douglas 2009). Wu et al. (2022) have suggested that aphid fecundity might vary in function of gravity and leaf surface, but we did not observe differences in nymph production in *M. persicae* across surfaces regardless of infection status (Fig. 4).

Aphid responsiveness, movement between leaves and leaf surfaces, and the difference in color between our aphid lines could alter interactions with predators. In fact, Polin et al. (2015) showed how visual cues are important for ladybirds when predating pea aphids. They focused on the effect of native (as opposed to transinfected) *Rickettsiella* on aphid body color, whereby the infection changed aphids to green, including those that were originally red. Ladybirds attack red clones significantly more often than their green counterparts (Polin et al. 2015) which implies that *Rickettsiella* may provide a certain level of protection to its host against predation. In our choice experiment, there was only a weak tendency for green clones to be attacked less on both surfaces (Table 2), consistent to what might be expected based on Polin et al. (2015). However, these assays proved difficult due to variation in ladybird activity in the first 30 min. Variables such as changes in air pressure and temperature may have impacts on predator behavior (Logan et al. 2006), and we also acknowledge the importance of aphid alarm pheromones and their avoidance tactics (Ninkovic et al. 2013). It may be possible to test these in future assays.

To increase biological realism in our assays of habitat selection, we tended to use whole-plant experiments. This meant that we could not make detailed observations of specific behaviors and meant that any observations on the distribution of aphids on leaves confounded predation rate with habitat choice, although there were clear impacts of predation on aphid numbers. The differences observed in Fig. 6 between panels A and B could reflect selective predation by ladybirds for aphids on the abaxial surface or aphid movement. The scouting behavior of predators with a preference for the adaxial surface (Table 2) seems to counter the notion of higher predation on the abaxial surface, although perhaps RCL+ aphids are less evident on the adaxial surface. Aphids not infected with *Rickettsiella* might also move to the adaxial surface to escape predators later point. These conjectures require further experimental testing. It is also worth noting that some of the patterns we have detected are based on rather small sample sizes due to the nature of the assay and it would be worthwhile undertaking other assays where larger sample sizes can be considered, especially if these allow specific behaviors to be explored.

Our results do not suggest the occurrence of niche separation between the 2 aphid lines, as the infection frequency on the 2 surfaces is similar. However, the distribution of the 2 lines in the mixed cohort (Fig. 6) is in the opposite direction of the pattern observed in isolated cohorts of RCL+. Perhaps visual cues are not the only ones used by ladybirds, as suggested by Polin et al. (2015). In the absence of *Rickettsiella*, olfactory cues might become more important for the predator and such cues in turn could also be influenced by the endosymbiont. In the case of our mixed cohort experiment, different aphid scents and alarm pheromones, concentrated in the space of the arena, might have altered prey seeking by the ladybird.

Clearly aphid numbers can be reduced substantially on whole plants through ladybird presence (Fig. 5B and C). *Rickettsiella* has already shown to be effective in reducing aphid fecundity (Gu et al. 2023), and therefore has potential as biocontrol agent (Slavenko et al. 2024). The endosymbiont might therefore act synergistically with ladybird predators in reducing aphid populations. It is important that future studies explore this pattern further under field conditions. Nevertheless, the current work provides further support for the absence of any negative effects of this endosymbiont on the effectiveness of a lady bird predator, which is also consistent with findings from other predation experiments that do not focus on whole plants (Bitsch et al. 2025).

Acknowledgements

We thank Alex Gill and Sonia Sharma for aphid line collection; Dr Xinyue Gu for the transinfection of the RCL+ line; Dr Qiong Yang, Ashley Callahan and Monica Stelmach for advice on molecular procedures; Assoc. Prof. Paul Umina for academic advice.

Author Contributions

Eloïse Ansermin (Conceptualization [equal], Formal analysis [lead], Investigation [lead], Methodology [lead], Visualization [lead], Writing—original draft [lead], Writing—review & editing [equal]), Ary Hoffmann (Conceptualization [equal], Formal analysis [supporting], Funding acquisition [lead], Methodology [supporting], Supervision [lead], Writing—review & editing [lead]), and Perran Ross (Conceptualization [equal], Formal analysis [supporting], Funding acquisition [equal], Methodology [supporting], Supervision [lead], Writing—review & editing [lead])

Supplementary Material

Supplementary material is available at *Annals of the Entomological Society of America* online.

Funding

This work was undertaken as part of the Australian Grains Pest Innovation Program (AGPIP), supported through funding provided by the Grains Research and Development Corporation (UOM1905-002RTX) and the Australian Grains and Horticulture Pest Innovation Program (AGHPIP), supported through funding provided by the Grains Research and Development Corporation (UOM1906-002RTX, UOM2404-006RT), and by Hort Innovation Australia (ST23002) with additional support from the University of Melbourne and Cesar Australia. This work was made possible by the Melbourne Research Scholarship (University of Melbourne).

Conflicts of Interest

None declared.

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