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Title:
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Date:
2025-01-01

Citation:
Ross, P. A., Tyrilos, M. C., Durugkar, N., Gill, A., de Jonge, N., Yang, Q., Gu, X., Hoffmann, A. A. & Kristensen, T. N. (2025). Deleterious effects of the endosymbiont Rickettsiella viridis in Myzus persicae are environmentally dependent. Journal of Pest Science, 98 (1), pp.375-388. https://doi.org/10.1007/s10340-024-01786-x.

Persistent Link:
https://hdl.handle.net/11343/352451

Journal of Pest Science
https://doi.org/10.1007/s10340-024-01786-x

ORIGINAL PAPER

Deleterious effects of the endosymbiont *Rickettsiella viridis* in *Myzus persicae* are environmentally dependent

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Received: 18 November 2023 / Revised: 28 March 2024 / Accepted: 16 April 2024

Abstract

Endosymbionts living within insect cells can modify host fitness and could provide new tools for pest control. The endosymbiont *Rickettsiella viridis* has been transferred experimentally into the green peach aphid, *Myzus persicae,* a globally important agricultural pest. This *Rickettsiella* spreads via vertical and horizontal transmission and induces host fitness costs which could potentially suppress pest populations. Endosymbiont prevalence can fluctuate in natural populations, and it is important to identify factors that contribute to their spread or loss. Here, we explore the effects of *Rickettsiella* infection when aphids are reared on eight different host plants or exposed to thermal, starvation and desiccation stresses. *Rickettsiella* infection reduced *M. persicae* fecundity and longevity across all host plants, but the magnitude of costs varied among host plants and generations. *Rickettsiella* was horizontally transmitted and spread in caged populations at initial ratios of 1:2 *Rickettsiella* (+): *Rickettsiella* (−) on all host plants, but with limited long-term persistence under cycling 20–30 °C. We also identified temperature-dependent costs of *Rickettsiella* infection on heat knockdown time, chill coma recovery, and starvation tolerance. Finally, we present evidence that *Rickettsiella* infection reduces host activity levels under heat stress. Our results suggest that *Rickettsiella* infections induce a variety of deleterious effects but with complex environment-dependent interactions. This work helps understand ecological conditions that enhance or limit the spread of these endosymbionts in aphid populations.

Keywords Aphid · Endosymbiont · *Rickettsiella* · Environmental stress · Host plants

Introduction

Endosymbionts reside within the cells of insects where they can dramatically alter host biology. Almost all aphid species harbor *Buchnera aphidicola*, an obligate endosymbiont that provides essential amino acids to its host (Moran 2021). Facultative endosymbionts are not required by their host and are also widespread in aphids, with several genera being shared across multiple species (Henry et al. 2015; Zytynska and Weisser 2016; Guo et al. 2017). Facultative endosymbionts are found at variable frequencies in populations, with their prevalence shaped by factors including temperature, interactions with parasitoids and pathogens and host genotype diversity (Smith et al. 2021; Carpenter et al. 2021; Zepeda-Paulo and Lavandero 2021; Zytynska et al. 2023). This highlights the fact that benefits and costs of endosymbionts on hosts depend strongly on the environment (Russell and Moran 1586; Liu et al. 2019; Higashi et al. 2020).

Facultative endosymbionts have useful properties that are already starting to be used to control insect pests and arbovirus vectors. Some endosymbiont strains protect hosts against virus infection (Teixeira et al. 2008; Higashi et al. 2023), with *Wolbachia*-infected mosquitoes successfully released to reduce arbovirus transmission by mosquitoes (Nazni et al. 2019; Utarini et al. 2021). Reproductive incompatibilities and deleterious effects induced by endosymbionts can also be used to suppress insect populations, with programs now being implemented against arbovirus vector mosquitoes (Crawford et al. 2020; Consortium PW-S 2021). Other

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Communicated by Antonio Biondi.

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options for using endosymbionts in agricultural pest and disease control are also being explored (Gong et al. 2020; Gu et al. 2023).

Myzus persicae is an important agricultural pest globally and a potential target for endosymbiont release programs (Gu et al. 2023). A transinfection of *Rickettsiella viridis* in *M. persciae* enacts severe fitness costs and can spread through caged aphid populations by horizontal transmission through a bok choy host plant as well as vertical transmission from mother to offspring (Gu et al. 2023). The infection could potentially suppress wild aphid populations through deleterious effects, but any candidate release strain must be thoroughly evaluated prior to release given that interactions among aphids, their endosymbionts and the environment can be complex (Smith et al. 2021; Zepeda-Paulo and Lavandero 2021).

Myzus persicae has a broad host plant range with over 40 plant families (Blackman and Eastop 2000). It reproduces sexually only on its primary host, peach (*Prunus persica*), and via parthenogenesis on secondary host plants (Emden et al. 1969). Reproductive output varies among host plants, but some aphid genotypes show evidence of local adaptation to certain hosts with improved performance (Weber 1985; Fenton et al. 2009). Facultative or secondary endosymbionts can also influence performance on different host plants (Chen et al. 2000; Zélé et al. 2018) and can facilitate host plant adaptation. For instance, infection with *Regiella insecticola* greatly increases the performance of pea aphids on white clover (Tsuchida et al. 1989), while field studies have found associations among host plant species and endosymbiont prevalence (Leonardo and Muiru 2003; Brady and White 2013; Tian et al. 2019). Transfers of endosymbionts to novel aphid hosts can enhance performance on some plants (Wagner et al. 2015) or even allow aphid species to reproduce on previously unsuitable hosts (Tsuchida et al. 2011).

Apart from host plant, temperature is another key factor influencing the host fitness effects of endosymbionts and their persistence in natural populations (Tougeron and Iltis 2002). Endosymbionts can influence host stress tolerance, with examples including costs of *Wolbachia* to cold tolerance in *Drosophila* (Kriesner et al. 2016) and the protection of pea aphids against heat shock by *Serratia* (Montllor et al. 2002). We also previously found that the persistence of *Rickettsiella* in populations of *M. persicae* was transient at a mild temperature of 25 °C compared to 19 °C where *Rickettsiella* persisted at a high frequency, and that *Rickettsiella* reduce heat tolerance of their host (Gu et al. 2023). Mechanisms underlying costs and benefits associated with endosymbionts under heat stress are expected to vary among endosymbiont species and may involve cell lysis, responses to gene regulation in both the endosymbiont and the host (which may interact), and stress responses triggered by the endosymbionts and their interactions with *Buchnera* (Heyworth et al. 2020). For example, fecundity in *Acyrthosiphon pisum* following heat exposure is reduced by a *Buchnera* mutation that suppresses the expression of a small heat shock protein gene (Zhang et al. 2019). Likewise, a single nucleotide deletion in *Buchnera* affects a heat-shock transcriptional promoter encoding a small heat-shock protein and that this mutation governs thermal tolerance of the *A. pisum* host (Dunbar et al. 2007). Endosymbionts and hosts therefore show interacting effects of gene regulation which can contribute to host fitness in different environments. These results illustrate the environmental dependency of host effects of endosymbionts and raise the question of whether the effects of novel infections in a new host represented by *Rickettsiella* in *M. persicae* will depend on host plant and other environmental conditions.

Here, we investigate potential environmental factors impacting spread of *Rickettsiella* by rearing lines of *M. persicae* infected or uninfected by *Rickettsiella* (*Rickettsiella* (+) and *Rickettsiella* (−-), respectively) on different host plants and at different rearing temperatures, as well as by testing the response of these lines to thermal, starvation and desiccation stresses. We also assessed horizontal transmission of *Rickettsiella* to an uninfected genotype and tracked the persistence of the infection in mixed populations on different host plant species under warm greenhouse conditions. Our results help inform the potential use of *Rickettsiella* in aphid pest control and provide general insights into interactions among aphids, endosymbionts and environmental conditions, thus providing important knowhow for the use of endosymbionts in release programs and factors that needs to be considered.

Methods

Aphid lines and maintenance

We used two *M. persicae* lines denoted *Rickettsiella* (+) and *Rickettsiella* (−), which were both derived from a single isofemale line provided by the Grains Innovation Park (Horsham, Victoria, Australia). Because *Rickettsiella* does not occur naturally in *M. persicae* (Yang et al. 2023), the *Rickettsiella* (+) aphid line was stably infected through microinjection of hemolymph from an *A. pisum* donor as described previously (Gu et al. 2023), while the *Rickettsiella* (−) aphid line was naturally uninfected. In the horizontal transmission and stress tolerance experiments (see below), we also included an uninfected line collected in Aalborg, Denmark, DK (GPS coordinates: 56.99786, 9.86549) on the exotic garden plant *Schefflera arboricola* in 2022 denoted *Rickettsiella* (-) DK. Aphid genotype and *Rickettsiella* infection status were tested in each line with aphid genotypes identified using microsatellite markers (Sloane et al. 2001;

Umina et al. 2014) and *Rickettsiella* detected by qPCR (see below) before experiments commenced. The DK line was confirmed as representing a different genotype to the Australian lines.

Aphid lines were maintained at 11–12 °C with a 16:8 light:dark cycle in controlled climate chambers (KBWF 240, Binder GmbH, Tuttlingen, Germany) on 35 mm bok choy (*Brassica rapa* subsp. *chinensis*) leaf disks on a layer of 1% agar in Petri dishes (60 × 15 mm, FALCON Easy Grip Tissue Culture Dish, Polystyrene Non-pyrogenic) according to methods described previously (Gu et al. 2023). Two to four weeks prior to experiments, aphids were transferred to 1–2-month-old whole plants in insect rearing cages (W 24.5 × D 24.5 × H 63.0 cm, 160 μm aperture mesh) and reared at 19–20 °C in controlled climate chambers (MC1750, Snijders Labs, Tilburg, The Netherlands) to expand the population. Adults from whole plants were then transferred to 35 mm diameter bok choy leaf disks in Petri dishes (20 adults per 60 × 15 mm dish) and allowed to reproduce at 19 °C to obtain < 24 h old nymphs for experiments. For experiments involving older aphids, nymphs were transferred to 35 mm bok choy leaf disks in petri dishes and reared at a controlled density of 10 aphids per dish until the desired age.

Host plant effects

Plant varieties and maintenance

We performed a series of experiments testing the effects of species of host plant on *M. persicae* life history traits, endosymbiont densities and *Rickettsiella* horizontal transmission and spread through caged populations. Eight plant species were grown for experiments under greenhouse conditions at Aalborg University, Denmark (Table 1). As *M. persicae* is highly polyphagous, plants were selected to cover a range of plant families with known host colonization. We chose plants that are commonly grown in farms or gardens in Denmark and where seeds were readily available. Seeds were sown in Plantesæk soil (with sphagnum and nutrients, pH 5.6–6.4) in plastic pots (90 × 90 × 100 mm PÖPpelmann Gmbh & Co. KG, Lohne, Germany) and placed in BugDorm-4E4590 (93.0 × 47.5 × 47.5 cm, 160 μm aperture) cages (Megaview Science C., Taichung, Taiwan) in a greenhouse (13.3 × 3.6 m). Temperature and humidity were recorded with data loggers (Thermochron 1-Wire, Dallas Semiconductors, Sunnyvale, CA, USA and Lascar EL-USB-2-LCD, Sweden). Temperature was partially controlled to a target of 25 °C using heat pumps (Century Carrie Residential, Hong Kong), while humidity was uncontrolled but remained above 50% (Figure S1). Multiple seeds were sown per pot and then thinned to a single plant. Plants were 1–3 months old when used in experiments, with plants of a similar age used for all treatments of a given experiment. No pesticides (including seed treatments) were used throughout the study.

Life history measurements

Nymphs (< 24 h old) from the *Rickettsiella* (+) and *Rickettsiella* (−) lines were transferred individually to 20 mm diameter leaf disks from the eight host plants (Table 1) in 1% agar Petri dishes (35 × 10 mm) and maintained at 19 °C with an 16:8 light:dark cycle in controlled climate chambers (KBWF 240). We established 25–30 replicates for each combination of host plant and line. Due to high frequencies of early mortality and escapees on some plants, we set up additional replicates to replace individuals that died within the first two days of the experiment. Aphids were transferred to new Petri dishes with fresh leaf disks every 6–7 d. Individuals were scored daily for their development time (time to first nymph), fecundity and age at death according to methods described previously (Gu et al. 2023). On Day 18 for aphids reared on sunflower and Day 13 for all other host plants, adult apterous aphids were photographed with a Zeiss Axiocam 105 color camera under a dissecting microscope. Images were analyzed with ImageJ (Schneider et al. 2012) to obtain body length measurements according to methods described previously (Gu et al. 2023).

Table 1 Host plants used in the study

Species	Common name	Cultivar	Family	Supplier
Brassica rapa	Bok choy	Chinensis	Brassicaceae	HORNUM (Fausol, Bjæverskov, Denmark)
Brassica napus	Canola	–	Brassicaceae	Friske Spirer (Denmark)
Trifolium resupinatum	Persian clover	*majus* Boss	Fabaceae	HORNUM (Fausol, Bjæverskov, Denmark)
Beta vulgaris	Beetroot	Conditiva	Amaranthaceae	HORNUM (Fausol, Bjæverskov, Denmark)
Vicia faba	Broad bean	–	Fabaceae	HORNUM (Fausol, Bjæverskov, Denmark)
Capsicum annuum	Capsicum	California Wonder	Solanaceae	HORNUM (Fausol, Bjæverskov, Denmark)
Solanum tuberosum	Potato	Anouk	Solanaceace	Palm Kartofler ApS (Blommenslyst, Denmark)
Helianthus annuus	Sunflower	Teddybear	Asteraceae	HORNUM (Fausol, Bjæverskov, Denmark)

One week after most individuals within a host plant treatment had started producing nymphs asexually, we collected nymphs that were less than 24 h old and reared aphids individually on the same host plant that they were derived from for a second generation. We then repeated the measurements described above with up to 30 replicates. Aphids reared on beetroot and sunflower were not measured in the second generation due to high early mortality causing a loss of replicates. In both generations, aphids that died before reaching adulthood were excluded from the analysis.

Body color and endosymbiont density

Nymphs (< 24 h old) from the *Rickettsiella* (+) and *Rickettsiella* (−) lines were transferred to 30 mm leaf disks from each host plant (Table 1) in agar Petri dishes in groups of 10 and reared to adulthood. On Day 14, up to 20 adult aphids from each line and host plant were removed from their Petri dishes using a paint brush, placed on a white piece of filter paper and photographed with a Zeiss Axiocam 105 color camera under a dissecting microscope. Images were processed and analyzed with ImageJ (Schneider et al. 2012) and the RGB measure plugin (https://imagej.nih.gov/ij/plugins/rgb-measure.html) according to methods described previously (Gu et al. 2023) to obtain an average body color separated into three components (hue, saturation and lightness). Aphids were stored in 100% ethanol and 16 adults from each line and host plant were assessed for their *Buchnera* and *Rickettsiella* density (see below). Their offspring were reared on the same host plant for a second generation in groups of 10 and we measured endosymbiont densities again from 16 adults when 14 d old. Aphids reared on sunflower were excluded from both body color and endosymbiont density measurements due to low survival. Body color was only measured after the first generation of rearing.

Horizontal transmission

We assessed horizontal transmission of *Rickettsiella* from the *Rickettsiella* (+) line to the *Rickettsiella* (−) DK line. The experiment involved placing one *Rickettsiella* (+) and one *Rickettsiella* (−) DK nymph (2 d old) together on a single 20 mm leaf disk in an agar Petri dish according to methods described previously (Gu et al. 2023). We set up 25 replicates for each host plant except for sunflower (due to high mortality in previous experiments). Aphids were left for one week, then both aphids were stored in 100% ethanol and screened for the presence and relative density of *Rickettsiella* (see below). Replicates where one or both aphids died were discarded.

Rickettsiella population dynamics

We tested the ability of *Rickettsiella* to spread through caged *M. persicae* populations containing mature (> 1 month old) host plants in pots (70 × 70 × 160 mm). We set up 5 replicate cages (30 × 30 × 62 cm, mesh 160 µm aperture) for each of the 8 host plants, each containing a single potted plant. We then placed 30 *Rickettsiella* (+) and 60 *Rickettsiella* (−) aphids of mixed age (1:2 ratio of *Rickettsiella* (+) to *Rickettsiella* (−)) in closed petri dishes. Petri dishes were placed at the base of each plant and opened to allow the aphids to colonize the plant. Population cages were maintained in a greenhouse at Aalborg University, Denmark, with the experiment commencing on the 8th of August 2022. Temperature was partially controlled and typically cycled daily between approximately 20 and 30 °C (Figure S1). The position of each cage was randomized every week to reduce potential effects of position on temperature and lighting that could influence plant and aphid population growth and *Rickettsiella* spread. Every three weeks, aphids were removed from plants and 50 aphids per plant were transferred to a clean plant and cage according to methods described previously (Gu et al. 2023). We stored the remaining aphids in 100% ethanol and 15 individuals from each plant were screened for the presence and relative density of *Rickettsiella* at weeks 3 and 9 (see below). Populations reared on sunflower were excluded from the analysis due to low aphid population sizes.

Stress tolerance

We performed a series of experiments comparing the tolerance of the *Rickettsiella* (+), *Rickettsiella* (−) and *Rickettsiella* (−) DK lines reared on bok choy at different temperatures to heat, cold, starvation and desiccation stresses. Groups of ten nymphs (< 24 h old) from each line were transferred to 30 mm bok choy leaf disks in agar Petri dishes (60 mm × 15 mm) and reared at constant temperatures of 20 °C, 23 °C, and 28 °C with a 16:8 light:dark cycle in controlled climate rooms. Seven d old individuals were then used in the stress tolerance experiments described below. In each experiment, 30 individuals were tested per line for each rearing temperature.

Heat tolerance

We measured heat tolerance using a knockdown assay according to methods described previously (Gu et al. 2023). Aphids were placed individually in 5 mL glass vials and sealed with a cap. Vials were attached to a metal rack and submerged in a glass tank filled with demineralized water. The water was maintained at a constant 41 ± 0.1 °C with a PROLINE RP 1845 thermostat

(LAUDA, Germany). Heat knockdown time was recorded by measuring the time it took for individuals to cease movement. This was confirmed by using a metal stick to poke the cap of the vials to provoke movement. Aphids were removed from the tank after all had been scored, then examined under a microscope after 1 h to confirm their death. This experiment was conducted in nine total batches with 30 individuals measured per batch, with all treatments represented in each batch.

In a second experiment, we used a Drosophila Activity Monitor system (DAM2, TriKinetics, US) to measure the locomotor activity of aphids under heat stress. Aphids were placed individually in transparent polycarbonate tubes that were then sealed at both ends. Tubes were arranged in the DAM2 system with the beam sensor aligning with the center of each tube. DAM2 systems were transferred to a climate chamber (KBWF 240) set at 40 °C for 4 h. Aphid locomotor activity was estimated by recording the number of times each aphid passed through the sensor every 30 s. To estimate heat tolerance with this method, we recorded the last time point where activity was detected for each individual. The assay was carried out in three batches, with each batch including three DAM2 systems and 30 individuals in each system. Aphids with no movement after the first minute were excluded from the analysis.

Cold tolerance

We assessed the cold tolerance of aphids using a chill-coma recovery assay. Aphids were placed individually in 5 mL glass vials with their caps sealed then put into a cloth bag. The bag was submerged in a glass tank filled with coolant (POLAR) that prevented the liquid from freezing. Metal sinkers were used to prevent the vials from floating on the surface of the water. We first conducted pilot experiments at a range of temperatures (− 9, − 8, − 7, − 5 and 0 °C) and durations (1, 2 or 3 h) to find a treatment that would induce a chill coma in all aphid lines while minimizing mortality. For the main experiment, aphids were exposed to -8 °C for 3 h as *Rickettiella* (−) DK aphids did not go into a coma under less severe treatments. After the exposure period, vials were removed and observed with a magnifying glass and ring light. Chill coma recovery time was measured by recording the time it took for aphids to regain the ability to stand on their legs and move their legs or antennae. Aphids were observed for 2 h at room temperature and aphids that did not move within this period were considered dead. Aphids that recovered were placed individually on 20 mm bok choy leaf disks in agar Petri dishes (35 × 10 mm). Petri dishes were moved to a climate chamber at 20 °C and scored daily for their fecundity and mortality (see above) for 7 d. This experiment was conducted in three batches.

Starvation resistance

We conducted a starvation assay to measure the ability of aphids to survive without food. Aphids were placed individually in Petri dishes (35 × 10 mm) with 2% agar and no host plant and maintained at 20 °C. Aphids were scored for mortality every 8 h under a dissecting microscope until all of them were dead (with no movement detected after prodding with a paintbrush). Aphids that escaped during the experiment were excluded from the analysis.

Desiccation resistance

We assessed desiccation resistance by exposing aphids to low humidity conditions without access to water or food. Aphids were placed individually in empty 5 mL glass vials with the top covered by gauze and tied with a rubber band. Vials were placed in a desiccator with silica gel at 20 °C to maintain a relative humidity of 16% as measured by Thermochron data loggers. Aphids were scored for mortality every 4 h without opening the desiccator by looking at the aphids using a magnifying glass ring light until all aphids had died.

Endosymbiont screening

DNA was extracted from individual aphids using 150 µL 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) according to Lee et al. (Lee et al. 2012). We then determined the presence or absence of *Rickettsiella* and densities of *Buchnera* and *Rickettsiella* in individual aphids from experiments. We were using a qPCR assay adapted from Gu et al. (Gu et al. 2023) for a Stratagene (Agilent) M3005xP, 96 well qPCR machine, where an aphid actin marker was used as the reference. Briefly, qPCR analysis was performed using Brilliant III Ultra-Fast SYBR Green qPCR Master Mastermix (Agilent Technologies), in 20 µL reactions with 30 nM ROX, 400 nM of each primer and 1 µL template DNA. All analyses were performed in duplicates on a Stratagene (Agilent) M3005xP, with a 96-well thermal block. qPCR conditions were as follows: PCR activation at 95 °C for 2 min, followed by 40 cycles of denaturation (95 °C for 10 s) and combined annealing and extension (60 °C for 20 s). A dissociation curve was subsequently generated for each assay. The difference between the average of 2 consistent Ct values from actin and the endosymbiont marker was used to calculate the density of the endosymbiont, using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All analyses were performed in SPSS Statistics 26 for Windows. In the host plant experiments, we used general linear models (GLMs) to test the effects of host plant, *Rickettsiella* infection and interactions among these factors for each trait measured. Because we measured life history traits in both alate and apterous individuals separately, we also included an additional analysis where we considered wing status as a factor. We used Sign tests to compare changes in *Rickettsiella* frequencies across time points in the population cage experiments. In the stress tolerance experiments, we used GLMs to test the effects of rearing temperature, aphid line and interactions between aphid line and rearing temperatures for all assays. Data for heat knockdown and starvation were log transformed. This transformation improved normality of residuals even though not all data were normally distributed. We performed Tukey's post-hoc tests with a Bonferroni correction for multiple comparisons to compare differences between aphid lines within a given temperature.

Results

Deleterious effects of *Rickettsiella* persist across host plants

We measured the development time, fecundity, longevity and body length of *Rickettsiella* (+) and (−) *M. persicae* reared on a variety of host plants across two generations (Fig. 1). In the first generation, we found significant effects of host plant on development time (GLM: $F_{7,402} = 107.887$, $P < 0.001$), lifetime fecundity ($F_{7,378} = 30.096$, $P < 0.001$), longevity ($F_{7,376} = 11.784$, $P < 0.001$) and body length ($F_{7,326} = 111.156$, $P < 0.001$). Aphids reared on sunflower were smaller, slower to develop and produced fewer offspring than aphids reared on the other plants but had the highest longevity (Fig. 1). *Rickettsiella* infection had deleterious effects on both fecundity ($F_{1,378} = 90.316$, $P < 0.001$) and longevity ($F_{1,376} = 97.083$, $P < 0.001$) across all host plants. *Rickettsiella* infection did not influence early

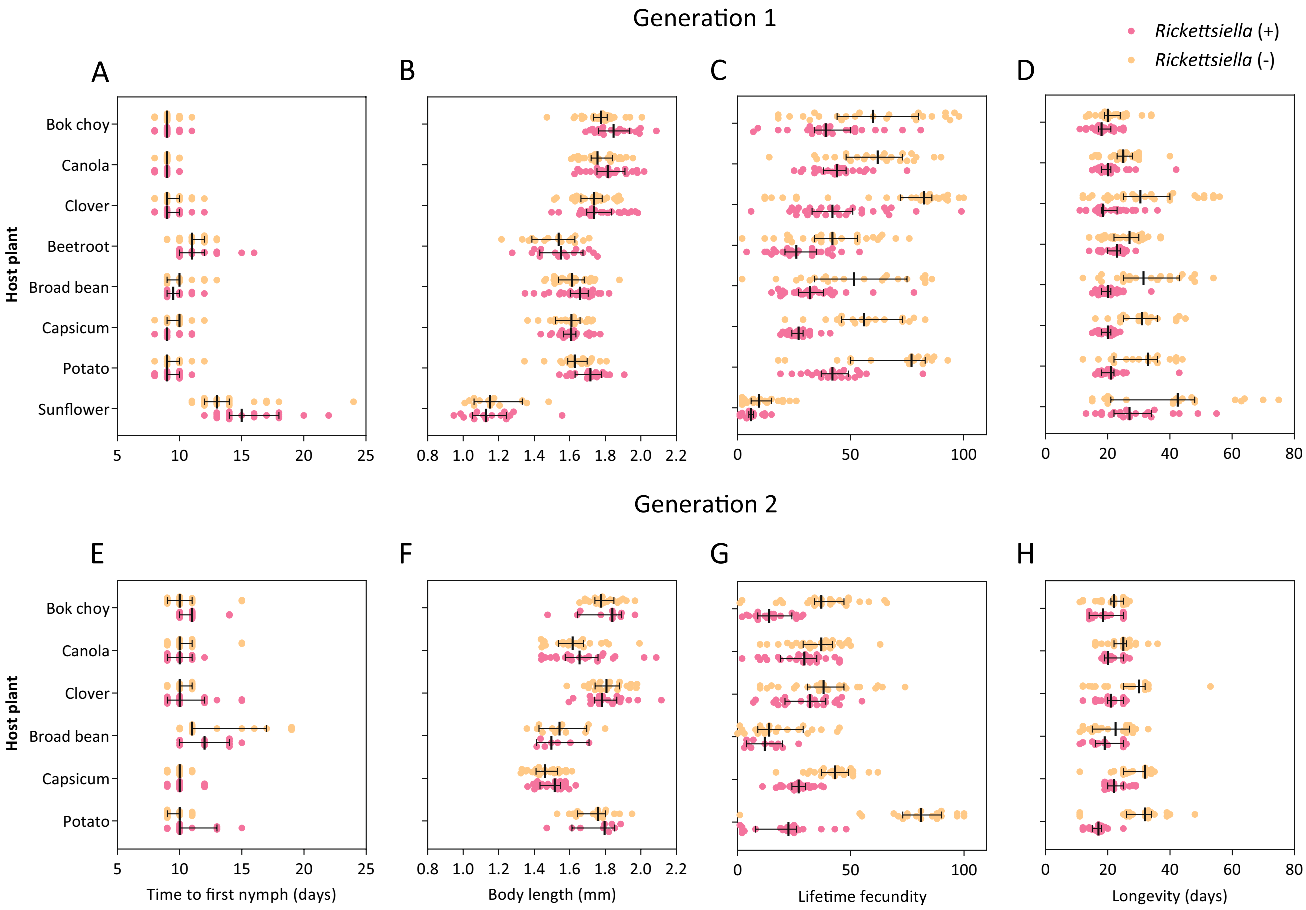

Fig. 1 Life history traits of *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* reared on different host plants. Development time (**A**, **E**), body length (**B**, **F**), fecundity (**C**, **G**) and longevity (**D**, **H**) were measured across two consecutive generations of rearing (generation 1: **A**-**D**, generation 2: **E**–**H**). Dots represent data for individual aphids while vertical lines and error bars are medians and 95% confidence intervals

reproduction, but the fecundity of *Rickettsiella* (+) aphids sharply decreased as aphids aged compared to *Rickettsiella* (−) aphids (Figure S2). While there was no significant effect of infection type on development time ($F_{1,402}=0.252$, $P=0.616$) there was an effect on body length ($F_{1,326}=8.787$, $P=0.003$) where *Rickettsiella* (+) aphids were slightly larger. We also found significant interactions between infection type and host plant for development time ($F_{7,402}=2.457$, $P=0.018$) and longevity ($F_{7,376}=3.134$, $P=0.003$), where costs of *Rickettsiella* infection to longevity were more severe on some plants (e.g., potato) compared to others (Fig. 1).

In the second generation, strong effects of host plant persisted for all traits (GLM: development time: $F_{5,249}=14.239$, $P<0.001$, lifetime fecundity: $F_{5,257}=25.576$, $P<0.001$, longevity: $F_{5,271}=6.210$, $P<0.001$, body length: $F_{5,199}=45.663$, $P<0.001$). While there was no significant effect of *Rickettsiella* infection on development time ($F_{1,249}=0.279$, $P=0.598$) or body length ($F_{1,199}=0.202$, $P=0.654$), *Rickettsiella* infection had deleterious effects on both fecundity ($F_{1,257}=138.860$, $P<0.001$) and longevity ($F_{1,271}=58.968$, $P<0.001$) across all host plants (Fig. 1), consistent with the first generation. There were also significant interactions between infection type and host plant for both fecundity ($F_{5,257}=21.237$, $P<0.001$) and longevity ($F_{5,271}=6.087$, $P<0.001$) where the magnitude of costs of *Rickettsiella* infection varied among the host plants. We note potential cross-generational effects of rearing, with the *Rickettsiella* (+) line performing relatively poorly during its second generation of rearing on potato, though we were unable to compare generations directly.

In our experiments, we observed that 14.3% (generation 1) and 4.4% (generation 2) of individuals developed into alate (winged) adults, with percentages ranging from 0 to 25% across treatments (Table S1). In an additional analysis on the first generation that also considered wing status, we found significant effects of wing status on development time (GLM: $F_{1,388}=52.111$, $P<0.001$) and fecundity ($F_{1,362}=6.644$, $P=0.010$), where alate aphids were slower to begin producing offspring with lower fecundity compared to apterous aphids (Figure S3). We found no significant effect of wing status on longevity ($F_{1,362}=1.460$, $P=0.228$) and no significant interactions with any other factor for each trait (all $P>0.052$), suggesting that the fitness costs of *Rickettsiella* infection are not influenced by wing status.

Host plant and *Rickettsiella* infection influence body color

We tested whether the commonly observed effects of *Rickettsiella* infection on aphid body color persisted when aphids were reared on different host plants (Fig. 2). Both host plant (Hue: $F_{6,254}=10.333$, $P<0.001$, Saturation: $F_{6,254}=2.311$, $P=0.034$, Lightness: $F_{6,254}=16.567$, $P<0.001$) and *Rickettsiella* infection (Hue: $F_{1,254}=1085.403$, $P<0.001$, Saturation: $F_{1,254}=280.505$, $P<0.001$, Lightness: $F_{1,254}=1049.768$, $P<0.001$) influenced body color across all three components (hue, saturation and lightness). The effects of *Rickettsiella* infection were particularly strong and consistent, with *Rickettsiella* (+) aphids being a greener hue, less saturated and darker in color compared to *Rickettsiella* (-) aphids across all host plants (Fig. 2). We also found significant interactions between host plant and *Rickettsiella* infection for two of the color components (Hue: $F_{6,254}=1.479$, $P=0.186$, Saturation: $F_{6,254}=4.381$, $P<0.001$, Lightness: $F_{6,254}=5.571$, $P<0.001$). For example, the differences between *Rickettsiella* (+) and (−) aphids were more distinct on beetroot compared to other plants, though these interactions were much less pronounced than the overall effects of *Rickettsiella* infection and host plant.

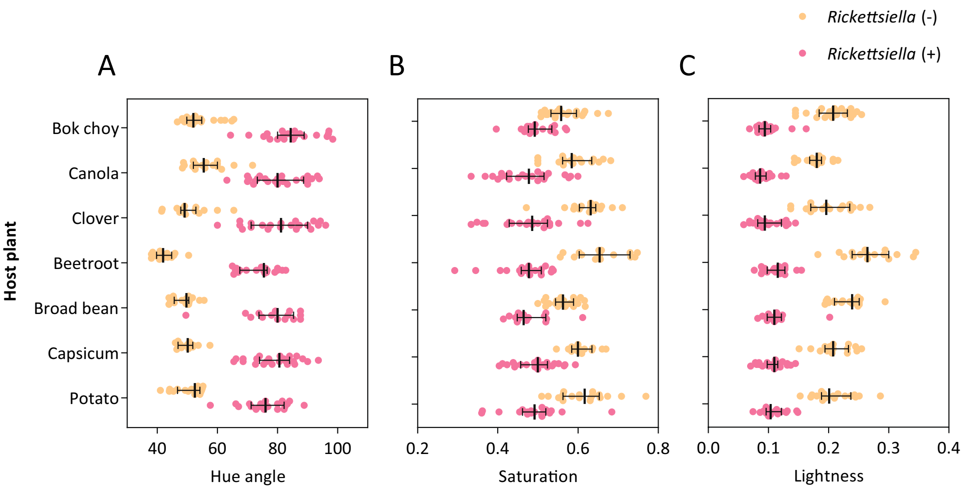

Fig. 2 Body color of *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* adults after being reared on different host plants. Body color was split into three components: **A** hue, **B** saturation and **C** lightness. Dots represent data for individual aphids, while vertical lines and error bars are medians and 95% confidence intervals

Variable endosymbiont densities across host plants

We then measured the density of both *Buchnera* and *Rickettsiella* endosymbionts in aphids reared on each host plant across two generations (Fig. 3). We found significant effects of host plant on both *Buchnera* (GLM: Generation 1: $F_{6,200} = 2.339$, $P < 0.001$, Generation 2: $F_{6,206} = 4.175$, $P = 0.001$) and *Rickettsiella* (Generation 1: $F_{6,99} = 2.225$, $P = 0.047$, Generation 2: $F_{6,104} = 4.605$, $P = 0.001$) densities in both generations (Fig. 3). We also note that the *Rickettsiella* (+) line tended to have higher *Buchnera* densities than the *Rickettsiella* (−) line, with significant effects of *Rickettsiella* infection status in generation 1 ($F_{1,200} = 7.772$, $P = 0.006$) but not in generation 2 ($F_{1,206} = 1.293$, $P = 0.257$). We also found significant interactions between host plant and *Rickettsiella* infection status (Generation 1: $F_{6,200} = 2.339$, $P = 0.033$, Generation 2: $F_{6,206} = 4.952$, $P < 0.001$), where the overall pattern of higher densities in the *Rickettsiella* (+) line was reversed for bok choy in generation 1 and for clover in generation 2 (Fig. 3). We also compared the density of both endosymbionts within individual aphids and found a significant positive correlation between them (Spearman's rank-order correlation: $\rho = 0.514$, $P < 0.001$, $n = 215$, Figure S4). Overall, there is no evidence for loss of *Rickettsiella* infection or reduced densities of *Buchnera* on any host plant, with all lines testing positive unambiguously for their respective endosymbionts.

Horizontal transmission of *Rickettsiella* between aphids across host plants

We also investigated the potential for *Rickettsiella* to spread to an uninfected *M. persicae* strain occupying the same host plant. We placed pairs of *Rickettsiella* (+) and *Rickettsiella* (−) DK aphids on individual leaf disks and left them for one week before screening both individuals for the presence of *Rickettsiella*. We found evidence of horizontal transmission of *Rickettsiella*, with ≥ 60% of *Rickettsiella* (−) DK aphids testing positive for *Rickettsiella* on each host plant (Table 2), though densities were typically much lower than stably infected *Rickettsiella* (+) aphids (mean Ct value: 21.58).

Transient spread of *Rickettsiella* in *M. persicae* across host plants in a warm greenhouse environment

We then measured the frequency of *Rickettsiella* in caged *M. persicae* populations on different host plants under

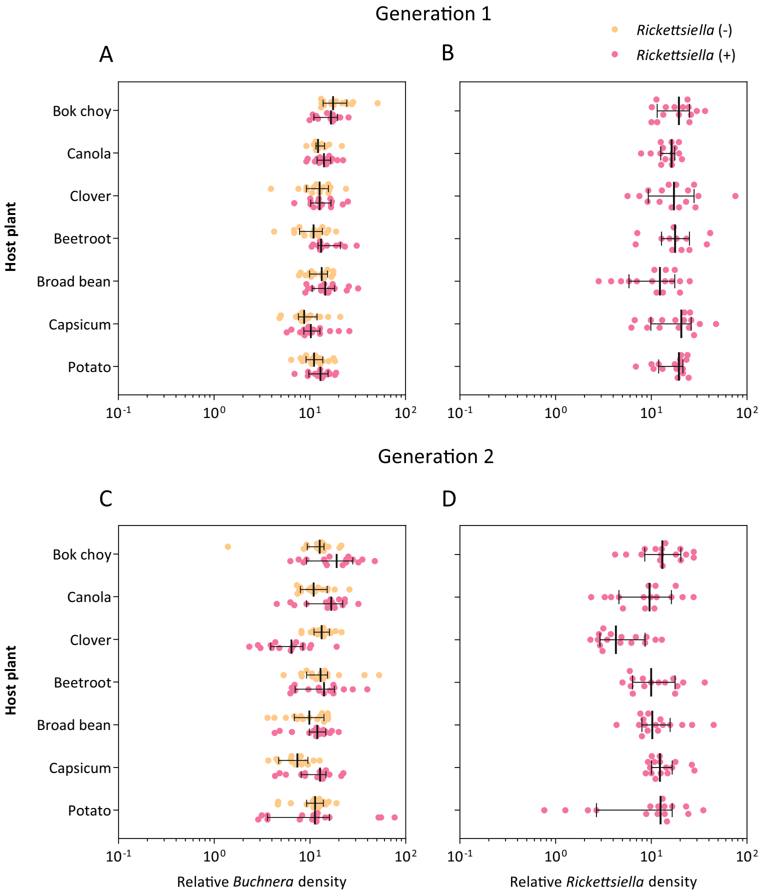

Fig. 3 Relative densities of *Buchnera* (**A**, **C**) and *Rickettsiella* (**B**, **D**) in *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* adults reared on different host plants. Endosymbiont densities were measured after aphids were reared on host plants for one (**A**, **B**) or two (**C**, **D**) generations. Endosymbiont densities were calculated relative to the actin marker and transformed by $2^{-\Delta\Delta Ct}$. Dots represent data for individual aphids while vertical lines and error bars are medians and 95% confidence intervals

Table 2 Frequency of horizontal transmission of *Rickettsiella*, tested using pairs of *Rickettsiella* (+) and *Rickettsiella* (−) *M. persicae* on leaf disks from a range of host plants

Host plant	Number positive (n tested)	Positive %	Mean Ct value (lower, upper 95% confidence interval)
Bok choy	9(15)	60.00	31.38 (30.00, 34.64)
Canola	13(19)	68.42	32.22 (30.73, 34.05)
Clover	18(23)	78.26	33.80 (32.28, 35.18)
Beetroot	5(7)	71.43	30.66 (27.12, 35.92)
Broad bean	14(18)	77.78	33.96 (32.16, 35.24)
Capsicum	15(17)	88.24	31.28 (29.76, 33.31)
Potato	11(17)	64.71	32.42 (29.05, 35.78)

greenhouse conditions (average temperature of 23.06 °C) at an initial ratio of 1:2 *Rickettsiella* (+) to *Rickettsiella* (−). The proportion of aphids testing positive for *Rickettsiella* increased in all replicate cages by Week 3 (Sign test: $Z=5.916$, $P<0.001$, $n=35$) then decreased by Week 9 (Sign test: $Z=5.745$, $P<0.001$), regardless of host plant (Fig. 4). At Week 9, only 5.6% (28/500) of individuals across all cages had *Rickettsiella* Cp values below 30, suggesting that most detections represent aphids that horizontally acquired *Rickettsiella* rather than through stable vertical transmission. When using this threshold, *Rickettsiella* frequencies exceeded the initial 1:2 ratio in only two replicate cages of beetroot plants, suggesting uncommon long-term persistence.

Rickettsiella infection decreases heat and cold tolerance

We investigated the impact of *Rickettsiella* infection on aphid stress tolerance at different rearing temperatures. In these experiments, we also included the naturally uninfected line collected from Denmark, *Rickettsiella* (−) DK). We first conducted a heat knockdown assay in a water bath at a constant 41 °C. We found significant effects of both aphid line (GLM: $F_{2,261}=145.519$, $P<0.001$) and rearing temperature ($F_{2,261}=114.433$, $P<0.001$) on heat knockdown time (Fig. 5A). Heat knockdown time increased at higher rearing temperatures, while *Rickettsiella* (+) aphids had faster knockdown times compared to *Rickettsiella* (−); thus, there was reduced heat tolerance in the *Rickettsiella* infected line. The *Rickettsiella* (−) DK line had faster knockdown times compared to the other two lines, suggesting an effect of aphid genotype. There was also a significant interaction between aphid line and rearing temperature ($F_{4,261}=10.684$, $P<0.001$), where the extent of increase in heat knockdown time at higher rearing temperatures differed among the lines and effects of *Rickettsiella* infection were weaker at higher temperatures (Fig. 5A).

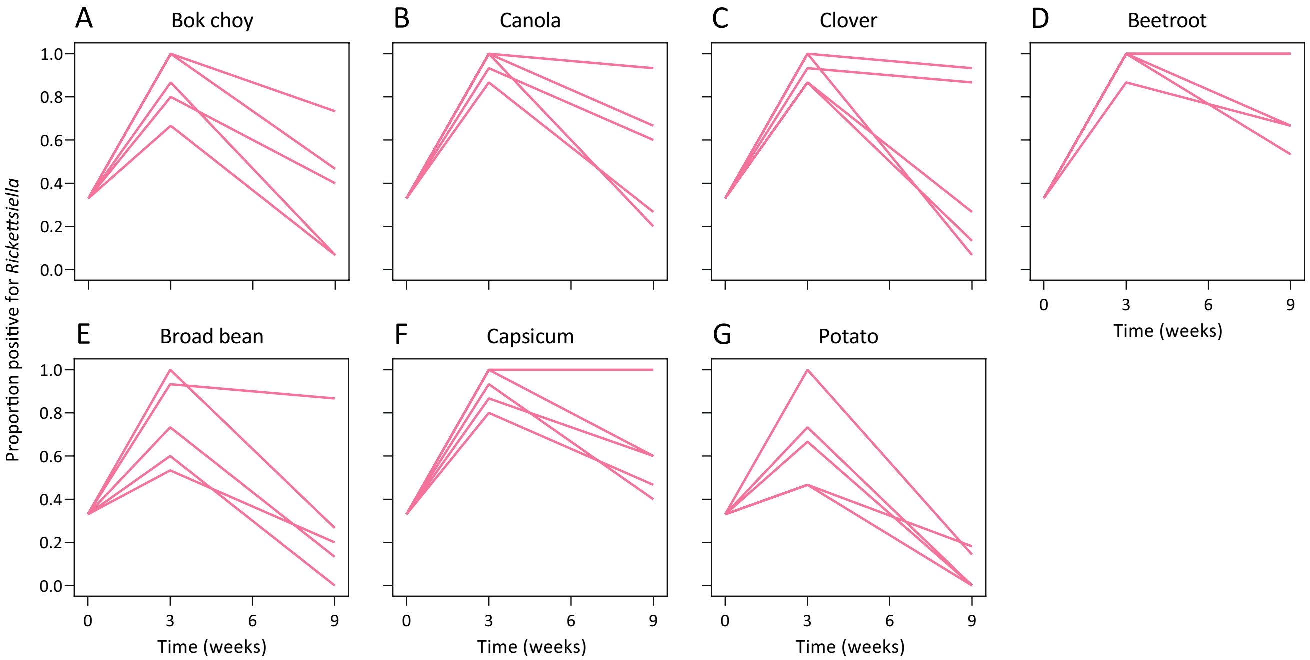

Fig. 4 *Rickettsiella* dynamics in *M. persicae* populations on different host plants (**A**-**G**) at an initial ratio of 1:2 *Rickettsiella* (+) to *Rickettsiella* (−). Experiments were run with 5 replicate cages per host plant in a greenhouse environment (Figure S1). Lines show the proportion of aphids testing positive for *Rickettsiella* with qPCR assays from a sample of 15 aphids per cage, per time point (weeks 3 and 9). *Rickettsiella* positivity was defined as having a Cp value < 40 and a Tm value within range of the positive controls

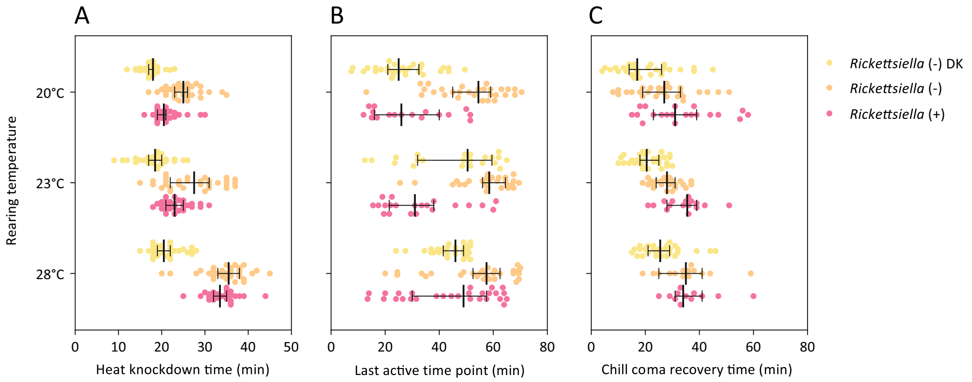

Fig. 5 Heat (**A**-**B**) and cold (**C**) tolerance of *Rickettsiella* (−) DK, *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* when reared at different temperatures. **A** Heat knockdown time was measured by exposing aphids to a constant 41 °C in a water bath. **B** Heat knockdown time was estimated using a DAM system in an incubator set to constant 40 °C by measuring the last active time point. Individuals with no activity beyond the first minute of the experiment were excluded. **C** Chill coma recovery time of aphids exposed to −8 °C for 3 h in a water bath. Dots represent data for individual aphids while vertical lines and error bars are medians and 95% confidence intervals

We then used DAM systems placed at a constant 40 °C to further test the effects of rearing temperature and aphid line on heat tolerance. We estimated heat tolerance by identifying the last active point for each aphid after which it was assumed to be knocked down. Here we also found significant effects of both aphid line (GLM: $F_{2,222} = 44.647$, $P < 0.001$) and rearing temperature ($F_{2,222} = 16.050$, $P < 0.001$) on heat knockdown time as well as an interaction effect ($F_{4,222} = 4.352$, $P = 0.002$, Fig. 5B). Patterns were broadly consistent with the water bath assay except that the *Rickettsiella* (+) line performed relatively more poorly compared to the two uninfected lines. This may in part be due to lower overall activity of *Rickettsiella* (+) aphids, where we found significant effects of aphid line ($F_{2,261} = 15.466$, $P < 0.001$) on mean activity at 40 °C (Figure S5).

To investigate the effects of rearing temperature and aphid line on cold tolerance, we measured the chill coma recovery of aphids following exposure to -8 °C for 3 h in a water bath. *Rickettsiella* (−) DK aphids had a higher post-exposure survival rate than the other lines but there was no clear effect of *Rickettsiella* infection (Table S2). We found significant effects of aphid line (GLM: $F_{2,190} = 29.939$, $P < 0.001$) and rearing temperature ($F_{2,190} = 6.643$, $P = 0.002$) on chill coma recovery time (Fig. 5C), but no significant interaction between the two factors ($F_{4,190} = 0.310$, $P = 0.871$). Chill coma recovery time tended to decrease with lower rearing temperatures and was fastest for the *Rickettsiella* (−) DK line. *Rickettsiella* (−) aphids also tended to have a faster recovery time than *Rickettsiella* (+) aphids, though there were only significant differences at 23 °C in pair-wise comparisons at each temperature (Tukey's post hoc test). We then measured the fecundity of aphids that recovered from the chill coma and found significant effects of aphid line ($F_{2,190} = 15.672$, $P < 0.001$) but not rearing temperature ($F_{2,190} = 1.830$, $P = 0.163$) on total fecundity 7 d after recovery, with no two-way interaction ($F_{4,190} = 0.148$, $P = 0.964$). Consistent with the patterns of recovery time, *Rickettsiella* (−) DK aphids had the greatest fecundity while *Rickettsiella* (+) aphids had a relatively low fecundity with a median of 0 regardless of rearing temperature (Figure S6).

Temperature-dependent effects of *Rickettsiella* infection on starvation and desiccation resistance

Finally, we tested the ability of aphids to tolerate starvation and desiccation. We found significant effects of aphid line ($F_{2,258} = 5.721$, $P = 0.004$) and rearing temperature ($F_{2,258} = 101.636$, $P < 0.001$) on starvation survival time, with shorter survival times at higher rearing temperatures (Fig. 6A). There was also a significant interaction between aphid line and rearing temperature ($F_{4,258} = 6.384$, $P < 0.001$), where *Rickettsiella* (+) aphids had lower survival when reared at 20 and 23 °C but higher survival than the *Rickettsiella* (−) line when reared at 28 °C. In the desiccation experiment, we also found significant effects of line ($F_{2,259} = 3.970$, $P = 0.020$), but not temperature ($F_{2,259} = 0.968$, $P = 0.381$) or the interaction between the two factors ($F_{4,259} = 1.077$, $P = 0.368$) on survival time (Fig. 6B). In pair-wise comparisons at each temperature (Tukey's post hoc test), the *Rickettsiella* (+) line had a significantly higher survival time than the *Rickettsiella* (−) line when reared at 28 °C but not the other temperatures, and the effects were relatively small (differences of a few hours).

Discussion

Here we demonstrate pervasive deleterious effects of *Rickettsiella* infection in *M. persicae* that are modulated by the environment. *Rickettsiella* infection reduced aphid fecundity

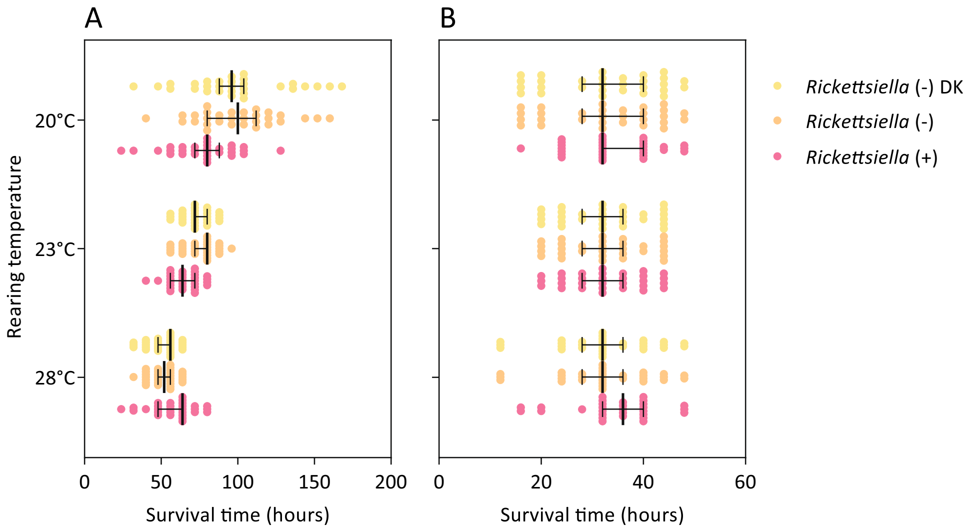

Fig. 6 Starvation (**A**) and desiccation (**B**) resistance of *Rickettsiella* (-) DK, *Rickettsiella* (−) and *Rickettsiella* (+) *M. persicae* when reared at different temperatures. **A** Starvation resistance was measured by providing aphids with access to 2% agar only. **B** Desiccation resistance was measured by placing aphids in chambers at a relative humidity of 16% and with no access to food/water. Dots represent data for individual aphids while vertical lines and error bars are medians and 95% confidence intervals

and longevity across all host plants tested, but the magnitude of costs varied among host plants and across generations. Similarly, effects of *Rickettsiella* infection on stress tolerance were also dependent on the aphid rearing temperature and for some traits infection increased rather than decreased host performance at warm rearing temperatures. Our horizontal transmission and population cage experiments and endosymbiont density data suggest that host plant type is not a strong driver of *Rickettsiella* spread despite clear differences in aphid performance and costs of *Rickettsiella* infection. While our results suggest that temperature has a strong influence on the phenotypic effects of *Rickettsiella*, these effects will likely be influenced by complex interactions with additional environmental factors (Smith et al. 2021; Zepeda-Paulo and Lavandero 2021; Zytynska et al. 2023).

We found clear differences in life history traits among aphids reared on different host plants, consistent with previous studies (Lowe 1973; Nikolakakis et al. 2003), but fitness costs of *Rickettsiella* were observed regardless of host plant. This is consistent with our data showing high densities of *Rickettsiella* and persistent body color changes when aphids are reared on different plant species. The deleterious effects of *Rickettsiella* appear to accumulate with age (Figure S2), which may contribute to higher fitness costs of the endosymbiont on plants where lifespans are longer. The age-dependent fitness costs of *Rickettsiella* are reminiscent of *w*MelPop, a variant of the *Wolbachia* endosymbiont in *Drosophila melanogaster* that causes extreme fitness costs that accumulate over time (Min and Benzer 1997; Yeap et al. 2011). This may be related to over-replication within host tissues given that *Rickettsiella* densities tend to increase during development (Soleimannejad et al. 2023), or may be associated with *Buchnera* depletion with age (Nishikori et al. 2009). We also considered effects of *Rickettsiella* on wing polymorphism given that other studies have suggested effects of other facultative endosymbionts including *Regiella* and *Serratia* on wing polymorphism (Leonardo and Mondor 2006; Kang et al. 2022), but found no consistent trends (Table S1).

In our horizontal transmission experiments, we detected *Rickettsiella* in aphids that were initially uninfected across all tested host plants at a moderate to high frequency. Our finding that horizontal transmission occurs across a range of host plant types suggests a limited role of plant defenses or immunity in limiting transmission between aphids. We acknowledge that our design did not allow us to demonstrate whether horizontal transmission was strictly via the host plant, and it is possible that some transmission could occur via honeydew or physical contact (Chiel et al. 2009; Pons et al. 2019). While the frequency of horizontal transmission here was relatively high, we did not measure the frequency of vertical transmission in aphids that tested positive for *Rickettsiella*. Previous work indicates that vertical transmission following horizontal transmission occurs but rates are low (Gu et al. 2023).

In our population cage experiments, *Rickettsiella* initially spread but then declined in almost all cages regardless of host plant. Our results suggest that *Rickettsiella* infections are unlikely to persist under warm conditions, but there are challenges in interpreting these data. Due to widespread horizontal transmission, binary infection frequencies based on sensitive qPCR assays can be uninformative because some individuals that test positive may not be stably infected and transmit *Rickettsiella* to the next generation. Furthermore, while horizontally acquired infections typically have lower *Rickettsiella* densities, this is not necessarily always the case. We suspect that the initial peak at Week 3 is driven by horizontal transmission that is largely transient and insufficient to overcome the fitness costs of infection, eventually leading to a decline in frequency. This pattern is consistent with our previous laboratory experiment at 25 °C (Gu et al. 2023), slightly warmer than the average temperature in our greenhouse experiments but with a lower variance. While fitness costs of *Rickettsiella* were weaker at higher rearing temperatures, there may be other fitness costs or reduced horizontal transmission efficiency at warmer temperatures, particularly when vertical transmission is stable at warmer temperatures (Gu et al. 2023). The population dynamics may be further complicated by potential fitness costs of horizontally acquired *Rickettsiella*. While we did not measure fitness costs directly following horizontal transmission here, this is an important consideration for future work.

We found that the clone derived from Australia outperformed the clone collected in Denmark in heat tolerance assays but with lower performance in the chill coma assay. Previous studies comparing *M. persicae* clones for thermal tolerance have not identified any clear evidence for climate-related adaptation in this species (Alford et al. 2012a; Vorburger 2004), likely due to dispersal of aphid genotypes between sampling locations and limited divergence between them. A previous study on *M. persicae* (Alford et al. 2012b) found little evidence for latitudinal variation in climate-related responses among clones from different parts of Europe, perhaps with the exception of activity levels under hot temperatures. We did find that *Rickettsiella* (+) aphids had lower activity levels compared to *Rickettsiella* (−) aphids at 40 °C which may influence their performance at high temperatures under field conditions and perhaps their susceptibility to predation.

The widespread deleterious effects described in this study support the potential of *Rickettsiella* to suppress wild aphid populations in a range of contexts. Our experiments also help to address some safety concerns of releasing a novel aphid endosymbiont strain. One risk is that aphids carrying *Rickettsiella* will perform better than wild-type aphids and cause additional damage. Our results suggest that this is unlikely, though additional work is required to explore other variables, particularly different aphid genotypes, given that endosymbiont effects can vary across genotypes (e.g., (Heyworth and Ferrari 2015)). This study along with Gu et al. (Gu et al. 2023) suggests that *Rickettsiella* might have more potential to suppress *M. persicae* at higher latitudes, altitudes and cooler seasons. Several outstanding questions remain. For instance, how long does *Rickettsiella* persist in host plants and is the infection likely to spread to other aphid species occupying the same host plant, especially given that *M. persicae* has a much broader host range than the native host from which this *Rickettsiella* was sourced (Emden and Harrington 2017)?

Author contributions

PAR, MCT, AAH and TNK designed the experiments, PAR, MCT, ND, AG performed the experimental work. ND, NdJ and QY performed the molecular work. PAR, MCT, AG and XG contributed resources. PAR and MCT wrote the first draft of the manuscript. All authors revised the manuscript.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-024-01786-x.

Acknowledgements We thank Christian Dupont Danielsen, Andreas Mølgaard Andersen, Sara Rosenørn de Lasson Sørensen, Holly Kang, Monica Stelmach and Ella Yeatman for technical assistance. This work was supported by research grants from VILLUM FONDEN (40841 and 58645) as well as funding provided to the Australian Grains Pest Innovation Program (AGPIP) by the Grains Research and Development Corporation (UOM1905-002RTX) and The University of Melbourne.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions. This work was supported by research grants from VILLUM FONDEN (40841 and 58645) as well as funding provided to the Australian Grains Pest Innovation Program (AGPIP) by the Grains Research and Development Corporation (UOM1905-002RTX) and The University of Melbourne.

Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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