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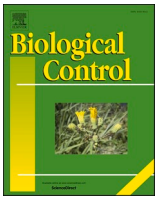

Facultative endosymbionts of aphids on strawberry crops affect aphid-parasitoid interactions

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HIGHLIGHTS

- Facultative endosymbionts of aphids are common in strawberry crop systems.
- Facultative endosymbionts can protect aphids against biocontrol parasitoids.
- Some parasitoid species are more affected by endosymbiont protection than others.

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ABSTRACT

Aphids are major agricultural pests and laboratory studies have shown that heritable bacterial endosymbionts can protect aphids against biocontrol parasitoids, but results from agricultural crop systems are lacking. Here we assess (1) which aphid species are present in Dutch strawberry crops, (2) which facultative endosymbionts they carry, and (3) how such facultative endosymbionts affect parasitism success of the biocontrol parasitoid wasps *Aphidius ervi* and *Praon volucre* on the most prevalent aphid species *Acyrthosiphon malvae*. By curing endosymbiont infections, we were able to determine the effects of endosymbionts and aphid genotypes on the parasitism success of both parasitoid species.

We show that aphid species composition on strawberry crops is dynamic across the season and that facultative endosymbiont (co-)infections are common. Infection with facultative endosymbionts protected *A. malvae* against *A. ervi*, but not against *P. volucre*. Finally, we also found variation in levels of endogenous resistance among *A. malvae* lines against *A. ervi*. Our study is the first to show that a common aphid pest on agricultural farms can be protected against biocontrol parasitoids by both facultative endosymbionts and endogenous resistance.

1. Introduction

Heritable bacterial endosymbionts are widespread among insects. Because they are predominantly vertically transmitted, they can add to the functional genetic repertoire of a species. Endosymbionts depend on their hosts for survival and reproduction, resulting in strong positive selection on endosymbiont-based traits that are advantageous to their host (Bennett & Moran, 2015; Estrela et al., 2016). One of the best studied examples of this are the many species of facultative bacterial endosymbionts of aphids (Guo et al., 2017; Shigenobu & Yorimoto, 2022), of which some species have been shown to affect the host-parasitoid dynamics of aphids and their parasitoid wasps by decreasing parasitism success (Heyworth & Ferrari, 2015; McLean et al., 2020; Oliver et al., 2003; von Burg et al., 2008).

Aphids are major agricultural pests, as they harm plants by phloem feeding, produce honeydew and transmit many economically important plant viruses (Blackman & Eastop, 2000). Commercially reared parasitoid wasps are often deployed in horticulture to biologically control aphid pests, as an environmentally safe alternative to insecticides. Although the importance of biological control (biocontrol) is

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increasingly recognized, aphid biocontrol is not always successful (Boivin et al., 2012; Glastuinbouw Nederland, 2022; Messelink et al., 2014; Sanchez et al., 2007). One factor that could explain aphid resistance against biocontrol parasitoids is genetically encoded defences, also known as endogenous resistance (Martinez et al., 2018; Martinez et al., 2014a; Sandrock et al., 2010). Alternatively, over the last years, concern has emerged that heritable facultative endosymbionts of aphids could negatively affect the success of aphid biocontrol in agricultural crop systems, especially in protected crops (Vorburger, 2018). However, 1) the prevalence of facultative endosymbionts in agricultural aphid pests remains poorly studied, and 2) insight into the effects of these endosymbionts on aphid-parasitoid dynamics in agricultural systems is lacking.

Endosymbiont prevalence is difficult to predict and seems to depend on many variables including the species of aphid (Henry et al., 2015; Zytynska & Weisser, 2016), season (Liu et al., 2019; Smith et al., 2015), temperature (Doremus et al., 2018; Smith et al., 2021), geography (Zytynska & Weisser, 2016), and host-plant genus (Henry et al., 2015; Xu et al., 2020). Consequently, the likelihood of aphids in a crop system harbouring (protective) endosymbionts will differ between systems. For example, facultative endosymbionts were never detected in aphids from Dutch sweet pepper greenhouses (Beekman et al., 2022) and were rare in aphids from pepper crops in Colombia (Gallo-Franco et al., 2019), but were found to be common in aphids from strawberry crops in France (Postic et al., 2020a).

The effects of endosymbionts on aphid-parasitoid interactions are also difficult to predict. The best studied protective endosymbiont is the gammaproteobacterium *Hamiltonella defensa* (Moran et al., 2005), which can protect its hosts against parasitoids when it is itself infected with a bacteriophage called *Acyrthosiphon pisum* secondary endosymbiont (APSE) (Weldon et al., 2013). Furthermore, specific strains of several other endosymbiont species have occasionally been shown to protect their aphid hosts from parasitoid wasps (Heyworth & Ferrari, 2015; McLean et al., 2020; von Burg et al., 2008). Protective phenotypes of endosymbionts are highly variable and depend on the species and genotype/strain of all players involved, as well as environmental factors such as temperature (Doremus et al., 2018). Since parasitoid wasps are commonly released as biocontrol agents against aphids, the specificity of protection can affect the success rates of certain biocontrol parasitoid species, while not affecting others. It is also possible that when multiple parasitoid species are released, the effects of protective endosymbionts mediate the competition between parasitoid species parasitizing the same host. Thus far, clear effects of endosymbionts on aphid-parasitoid dynamics have mostly been shown in laboratory cage studies where protective endosymbionts often lead to parasitoid extinction (Käch et al., 2018; Oliver et al., 2008; Sanders et al., 2016). In field studies, these effects are often much more nuanced, and can be mitigated by alternative natural enemies (Hrček et al., 2016) and the fitness-costs associated with carrying endosymbionts (Rothacher et al., 2016).

In conclusion, the presence and prevalence of facultative endosymbionts in aphids can differ strongly between crops and geographical locations, and potential protective effects against biocontrol parasitoids are difficult to predict. Therefore, to gain more insight into the potential effects of facultative endosymbionts on the success of biocontrol, we need 1) more knowledge on the prevalence of facultative endosymbionts in specific crop systems, and 2) to understand the effects of endosymbiont infections on common, non-model, aphid pest species. We investigated the prevalence of facultative endosymbionts in aphid species in Dutch strawberry as it is known to host many different aphid species (Jansen & Warnier, 2005; Klinkenberg, 1974; Lahiri et al., 2022; Postic et al., 2020b) that carried many facultative endosymbionts in a previous study conducted in France (Postic et al., 2020a). Furthermore, strawberry pests are predominantly managed using pesticides and have one of the highest levels of pesticide residues of all fruits and vegetables (Mantingh, 2022; Parker, 2015). Knowledge on how to improve biocontrol in strawberry will aid the shift towards more sustainable farming.

Our survey revealed that Dutch strawberry crop systems contain large numbers of aphid species, many of which harbour different species of facultative endosymbionts. We predicted that some species of endosymbionts can affect aphid-parasitoid dynamics, which in turn potentially affects the success of aphid biocontrol in agricultural systems. We studied the relationships between aphid endosymbionts and biocontrol parasitoids by investigating the effects of the most prevalent facultative endosymbionts, *H. defensa* and *Regiella insecticola*, on the parasitism success of the commonly used biocontrol parasitoids *Aphidius ervi* Haliday and *Praon volucre* (Haliday) on the common strawberry aphid pest *Acyrthosiphon malvae* (Mosley). We compared levels of parasitism success between endosymbiont-infected and endosymbiont-cured aphid lines with the same genetic background which allowed us to disentangle endosymbiont-based from aphid endogenous resistance.

2. Materials & methods

2.1. Aphid collection

Aphids were collected from 16 different strawberry-growing locations (henceforth called 'farms') in the Netherlands at two timepoints during the 2020 growing season (electronic supplementary material, Fig. S1 and Table S1). They were sampled for the first time between the 15th of April and the 3rd of June (from here on called 'spring'), and for a second time between the 9th and the 25th of September (from here on called 'summer'). We sampled different cropping systems including heated glasshouses, unheated polytunnels, and partly protected and unprotected raised trays. Nine farms grew the strawberries entirely indoors in glasshouses, four farms grew them entirely outdoors, and three farms had a combination of outdoor and indoor grown strawberries.

Aphids were sampled by choosing five sampling sites per farm, as far apart from each other as possible. In many cases, this resulted in four sites for the corners of each farm and one in the middle. However, there were exceptions since some farms did not grow the strawberries in square fields or greenhouses. In these cases, we still chose five sampling sites as far apart as possible. Each sampling site was thoroughly searched for aphids of as many different species as possible. We aimed to collect multiple aphids per colony per sampling site in 96-well plates containing 70% ethanol which were subsequently stored at –20 °C. Additional aphids from the same colony were only used as back-ups, not as separate samples, to avoid pseudoreplication.

2.2. Identification of aphids and facultative endosymbionts

Aphids were morphologically identified to the species level using the identification keys of Blackman and Eastop (2000). Next, DNA was extracted from individual aphids using a Chelex and proteinase K-based protocol as described by Beekman et al. (2022). When morphological identification was inconclusive, aphids were identified by DNA barcoding targeting the mitochondrial cytochrome *c* oxidase subunit I (COI) region with primer pair LepF/LepR (ATTCAACCAATCATAAAGATATTGG / TAAACTTCTGGATGTCCAAAAAATCA) (Hajibabaei et al., 2006). PCR reactions were carried out by adding 1 μl DNA to 9 μl GoTaq®-based PCR Master mix (Promega, Southampton, UK), prepared according to the manufacturer's instructions with a final primer concentration of 0.4 μM. The PCR programme was set to an initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 15 s, 48 °C for 30 s, and 72 °C for 40 s; followed by 72 °C for 5 min. PCR success was confirmed using gel electrophoresis with a 1% agarose gel stained with ethidium bromide and a GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Bleiswijk, the Netherlands). Sequences were obtained by sending the PCR products to Eurofins Genomics (Ebersberg, Germany) for Sanger sequencing. The resulting sequences were aligned against the NCBI nucleotide database using BLAST (Altschul et al., 1990) to identify or confirm the aphid species.

The presence of the facultative endosymbionts *H. defensa, R. insecticola, Fukatsuia symbiotica* (previously known as X-type/PAXS)*, Serratia symbiotica, Arsenophonus* sp., *Rickettsiella* sp., *Rickettsia* sp., and *Spiroplasma* sp. were determined for each individual aphid with diagnostic PCR as described by Beekman et al. (2022).

2.3. Screening for endosymbiont-based resistance

2.3.1. Experimental organisms and endosymbiont removal

Living geranium aphids, *A. malvae*, were collected from Dutch strawberry farms in 2019, 2020 and 2021 (electronic supplementary material, Table S2). Each aphid line was established from a single parthenogenic female and kept separate on a 91-mm-diameter sweet pepper (*Capsicum annuum* L.) leaf disc, placed abaxial side up on a layer of 1% agar, in sterile polypropylene culture vessels (Lab Associates, Oudenbosch, the Netherlands) closed with nylon-screened Donut Lids (BDC0001- 1; Bugdorm, MegaView Science, Taichung, Taiwan). The rearing vessels were stored upside down at 18 °C and a 16 h light/8h dark cycle in an incubator (Sanyo model MLR-352H, PHC Europe, Etten-Leur, The Netherlands). We opted for rearing the aphids on sweet pepper instead of strawberry leaves since rearing the aphids on strawberry was unsuccessful in the laboratory, both on strawberry leaves embedded in 1% agar, and on whole strawberry plants. In addition, sweet pepper leaf-disc rearing was already optimized in our laboratory. The presence of facultative endosymbionts in the living aphid lines was determined with diagnostic PCR, following the methods of Beekman et al. (2022). Each aphid line carried either *R. insecticola* or *H. defensa*, or was co-infected with both these facultative endosymbionts (electronic supplementary material, Table S2).

Facultative endosymbionts were selectively removed through microinjections with antibiotics, using a protocol modified from Sochard et al. (2020). Adult *A. malvae* aphids were CO_2-anesthetized, and injected dorsally, with either 2.3, 4.6 or 9.2 nL of an antibiotics mixture (ampicillin, cefotaxime, and gentamicin, dissolved in sterile ultrapure water, at 3.7 mg/mL each) using a Nanoject II microinjector (Drummond Scientific Company, USA). The different volumes were used to find the lowest effective dose so to minimize any untargeted detrimental effects on aphid health. Injected aphids were transferred to sweet pepper leaf discs embedded in 1% agar in 12-well plates (665180; Greiner Bio-One GmbH, Frickenhausen, Germany) with five adults per well. Offspring produced in the first three to four days after injection were removed. Next, the surviving injected adults were individually allowed to produce at least 20–30 nymphs, after which the presence of *H. defensa* and *R. insecticola* was checked with diagnostic PCR for 10 offspring per line, using *Buchnera aphidicola* (Munson et al., 1991), the obligatory endosymbiont of aphids, as a positive control. For lines A1*Hd*+ and A2*Hd*+ (the 'A' or 'R' indicates the genotype of the aphid line, and the '*Hd*+', '*Ri*+' or '*HdRi*+' indicates the presence of *H. defensa*, *R. insecticola*, or both in the aphid lines), 2.3 nL antibiotics mixture was sufficient to remove *H. defensa*. For lines R1*Ri*+ and R2*HdRi*+, 4.6 nL was needed to remove *R. insecticola* or the coinfection with *R. insecticola* and *H. defensa,* respectively. Line R3*HdRi*+ was not successfully cured of its endosymbionts and was thus excluded from the experiments. Henceforth, aphid lines cured from facultative endosymbionts are called 'cured' while aphid lines still infected with facultative endosymbionts are called 'endosymbiont-infected'.

2.3.2. Confirming infection status and determining endosymbiont titres

To confirm the infection status of all experimental lines during the experiments we used diagnostic PCR to show presence/absence of endosymbionts, and quantitative PCR (qPCR) to measure endosymbiont titres. The infection status was confirmed at three time points: before and after antibiotics treatment, as well as during the parasitism assays. We used diagnostic PCR, as described by Beekman et al. (2022), to check for *B. aphidicola*, *H. defensa,* and *R. insecticola* in 10 aphids at each time point from each aphid line. During the parasitism assay, a random sample of 10 aphids was taken from the three control treatments combined. Because in lines R1*Ri*- and R2*HdRi*-, *R. insecticola* was detected in 1/10 samples, an additional five aphids were tested for these lines.

Endosymbiont titres of nine-to-10-day old aphids were measured with qPCR, using primers from Qian et al. (2018) to target the 16S genes of *R. insecticola* and *H. defensa*, and the aphid ß-actin as a host reference gene. Endosymbiont titres were measured in 10 individuals right before microinjections, to know the baseline endosymbiont titres, as well as in five individuals of the F2 generation, which was assumed endosymbiont-free. For each line, five aphids were again tested with qPCR during the parasitism essays to confirm infection status. Aphids were snap-frozen in liquid nitrogen and stored at –80 °C until DNA extractions. DNA was extracted as described above under 'Identification of aphids and facultative endosymbionts'. Reactions and cycling conditions were according to the PCR kit protocol, with each reaction containing 5 µl 2x SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience, Boxtel, the Netherlands), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 0.5 µl DNA and 3.7 µl sterile ultrapure water. Samples were run in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Lunteren, the Netherlands). Cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 20 s. The relative abundance of the endosymbiont 16S DNA to the aphid ß-actin was calculated as described in statistics section 2.4.4.

2.3.3. Parasitism assays

We assessed the resistance of the endosymbiont-infected and cured *A. malvae* lines against the commonly used biocontrol agents *Aphidius ervi* and *Praon volucre*, two species of braconid parasitoids from the *Aphidiinae* subfamily. Prior to the parasitism experiment, the aphids were reared on bell pepper leaf discs because rearing them on strawberry was unsuccessful. Eventually, this problem was resolved by watering the strawberry plants with an aqueous fertilizer solution (22–4-3 NPK). Therefore, one month before the start of the parasitism assays, all aphid lines were transferred to insect cages (60 × 60 × 90 cm, mesh size 250 µm) containing five potted strawberry plants (*Fragaria* × *ananassa*, cv. Elsanta) to re-acclimate the aphids to strawberry and to boost population sizes. They were kept in a greenhouse at 18–23 °C at 29–74% humidity and a 12 h light/12 h dark cycle.

Parasitoids were kindly provided by Koppert Biological Systems (Berkel en Rodenrijs, the Netherlands). *Aphidius ervi* were delivered as mated wasps. *Praon volucre* was obtained by separating mummies from the Aphiscout mix (Koppert Biological Systems) by using the fact that *P. volucre* larvae spin their cocoon under, instead of inside, the aphid mummy. Until being used in the parasitism assays at five days old, both sexes of *P. volucre* were kept together (and thus the females were assumed mated) in an incubator (Sanyo model MLR-352H-PE, PHC Europe, Etten-Leur, The Netherlands) at 21 °C, 75% relative humidity and a 16 h light/8h dark cycle and were fed with honey. For both parasitoid species, immediately before use, individual female wasps were offered one aphid nymph randomly chosen from one of the cured aphid lines, to gain oviposition experience. Only parasitoid wasps that attempted to parasitize the aphid within three minutes were used in the parasitism assays.

The parasitism assays were performed on five-to-eight-week-old, potted strawberry plants placed in cylindrical insect cages (260 mm diameter × 650 mm height; Ento Nets POT-ZIP-10X-W, Atherstone, UK). Cotton wool was used to close any holes surrounding the zipper and cord-lock to prevent aphids and parasitoids from escaping. Each aphid line × parasitoid combination was tested five times in separate temporal blocks. For the cured aphid lines, the time between initial antibiotics treatment and the use in the parasitism experiment was 55–99 days. In each replicate, 30 second-to-third instar nymphs were placed on a plant and left to acclimatize for 24 h (experimental design in supplementary material, Fig. S2). The next day, a single female wasp was released in each cage and given 24 h to parasitize the aphids after which she was removed. Cages in which the parasitoid could not be retrieved, or was

found dead, were considered unsuccessful and these treatments were repeated in a new temporal block. Fourteen days after removal of the parasitoid, the number of mummies, representing the number of successful parasitism events, on each plant were counted. This timeframe was chosen to ensure that all successfully parasitized aphids would have developed into a mummy.

To determine whether there was a difference in survival probability of each aphid line in the absence of parasitoids, control treatments were included in which no parasitoid wasp was introduced. Survival probability of the nymphs in the control treatments was determined eight days after the initial placement of the nymphs on the plants, to prevent the offspring of the aphids to have grown to a size that would cause difficulties in counting. For each aphid line, the control treatment was replicated three times. As some of the aphids had reached adulthood after eight days and had started reproducing, we counted only the larger (adult) individuals to determine the survival probability.

2.4. Statistical analyses

The statistical analyses were performed in R v. 4.2.0 (R Core Team, 2020) using RStudio v. 1.4.1717 (RStudio Team, 2020). Data visualization was done using the *ggplot2* package v. 3.3.5 (Wickham, 2016) and the *plotly* package v. 4.9.4 (Sievert, 2020).

2.4.1. Endosymbiont-based resistance

The distribution of the datasets was tested using the *vcd* package v. 1.4–8 (Meyer et al., 2022). Differences in overall parasitism rates between endosymbiont-infected and cured aphids were analysed using generalized linear mixed models (GLMMs) from the *lme4* package v. 1.1–27.1 (Bates et al., 2015). We used a Poisson distribution with a logit-link function. The fit of the models was tested using the *DHARMa* package v. 0.4.5 (Hartig, 2021), which assesses the normality of the residuals. The *glmmTMB* package v. 1.1.4 (Brooks et al., 2017) was used in the case of overdispersion. The *p*-values were determined using the 'Anova' function of the *car* package (Fox & Weisberg, 2019), which uses a Wald chi-squared test. The effects of facultative endosymbiont presence status on the total number of mummies were tested for each parasitoid species separately, with aphid line and replicate (timepoint) as random factors.

Differences between the parasitism rates of endosymbiont-infected and cured aphid lines were also tested for each aphid clonal line separately. The obtained *p*-values were corrected for multiple hypothesis testing by using the FDR method (Benjamini & Hochberg, 1995).

2.4.2. Survival of the aphid lines

Differences between the survival probability of the endosymbiont-infected and cured aphid lines were also tested using GLMMs, both for the lines separately and for all endosymbiont-infected lines taken together against all cured lines taken together. The same methods as described above were used, but with a normal distribution instead of a Poisson distribution. We tested the dataset for normality with the *fitdistrplus* package v. 1.1–8 (Delignette-Muller & Dutang, 2015). The dataset did not significantly deviate from a normal distribution.

2.4.3. Endogenous-based resistance

We looked for a correlation between aphid genotype and parasitism success using GLMMs with the methods described above. A Poisson distribution was used, and replicate was added as a random factor.

2.4.4. qPCR titres of facultative endosymbionts

The qPCR titres were determined using the *tidyqpcr* package v. 1.0 (Haynes & Wallace, 2021) by calculating the relative abundance of the facultative endosymbionts, in terms of 16S rRNA gene abundance, compared to the host reference gene aphid β-actin abundance. Next, we averaged the relative abundance over the two technical replicates.

3. Results

3.1. Aphid community composition

We sampled aphids from 16 Dutch strawberry farms at two timepoints, spring and summer, in 2020 and detected a total of 22 different species. We sampled a total of 165 and 103 aphid colonies, for spring and summer, respectively. Twenty-one samples from the spring, and two from the summer collection were excluded due to parasitisation of all the aphids sampled from that colony, leaving 144 and 101 colonies for the spring and summer timepoints, respectively, totalling 245 sampled aphid colonies. The aphid species we encountered were *A. malvae*, *Aphis craccivora* Koch, *Aphis fabae* Scopoli, *Aphis gossypii* Glover, *Aphis nasturtii* Kaltenbach, *Aphis ruborum* (Börner)/*idaei* van der Groot, *Aulacorthum solani* (Kaltenbach), *Chaetosiphon fragaefolii/thomasi* (Cockerell), *Ericaphis fimbriata* (Richards), *Ericaphis scammelli* (Richards), *Macrosiphum euphorbiae* Thomas, *Macrosiphum rosae* (Linnaeus), *Metapolophium dirhodum* (Walker), *Myzus ascalonicus* Doncaster, *Myzus ornatus* Laing, *Myzus persicae* (Sulzer), *Rhopalosiphum nymphaeae* (Linnaeus), *Rhopalosiphum padi* (Linnaeus), *Rhodobium porosum* (Sanderson), *Sitobion fragariae* (Walker), *Wahlgreniella nervata* (Gillette), and an *Anoecia* species that did not have a match in the NCBI database (Fig. 1a; electronic supplementary material, Table S3).

Which aphid species was the most common differed between the two timepoints. During the spring timepoint, we found *A. malvae* at 10, *M. euphorbiae* at seven, and *A. solani* at six out of the 16 locations. The aphid species we found most during the summer timepoint were *A. nasturtii* at seven, *A. gossypii* at six, and *A. malvae* at six out of the 16 locations (Fig. 1a). We found six aphid species that were located at only one farm in only one of the five sampling locations within a farm at low density, while the more common aphid species such as *A. malvae* were usually found in more than one of the sampling locations within a farm. At two farms, no aphids were found at all during the summer timepoint.

We found large variation in the number of aphid colonies and species per farm. The number of aphid colonies we sampled from a farm ranged from three to 20 during the spring timepoint, and from zero to 22 during the summer timepoint. We found an average of 3.3 and 2.6 aphid species per farm during spring and summer, respectively. At some farms we found up to 10 different aphid species, but at other farms we encountered only one aphid species ranging from being in one location to being spread throughout all five sampling locations. Our experiment was not set up to test for any differences in aphid community composition between different types of strawberry farms, such as open systems versus tunnels versus greenhouses.

3.2. Diversity and prevalence of facultative endosymbionts

We screened all aphids for facultative endosymbiont presence using species-specific primers and most carried facultative endosymbionts. We detected a total of 48 unique aphid-endosymbiont combinations, including the aphids that did not carry a secondary endosymbiont.

In the 245 tested samples, 26.9% of all aphids carried *R. insecticola,* and 26.9% of all aphids carried *H. defensa*. *Arsenophonus* sp. was present in 38.4% of the samples from the *Aphis* genus ($n = 52$) but in none of the other aphid genera, resulting in an overall infection frequency of 8.1%. Both *F. symbiotica* and *S. symbiotica* were detected in a small number of aphids (3.6 and 2.9% respectively). None of the aphids from the *Myzus* genus ($N = 19$) carried any of the endosymbionts in our screen and we did not find the three endosymbionts *Rickettsiella* sp., *Rickettsia* sp., and *Spiroplasma* sp. Our results show that many aphid species can carry multiple species of endosymbionts, also often as co-infections.

The most encountered aphid, *A. malvae*, occurred 48 times in our dataset. Of these, 12.5% did not carry facultative endosymbionts, 29.2% carried *H. defensa*, 6.3% carried *R. insecticola*, and 52.1% carried both. The second most common aphid, *M. euphorbiae,* occurred 37 times. Of these, 67.6% did not carry facultative endosymbionts, 10.8% carried

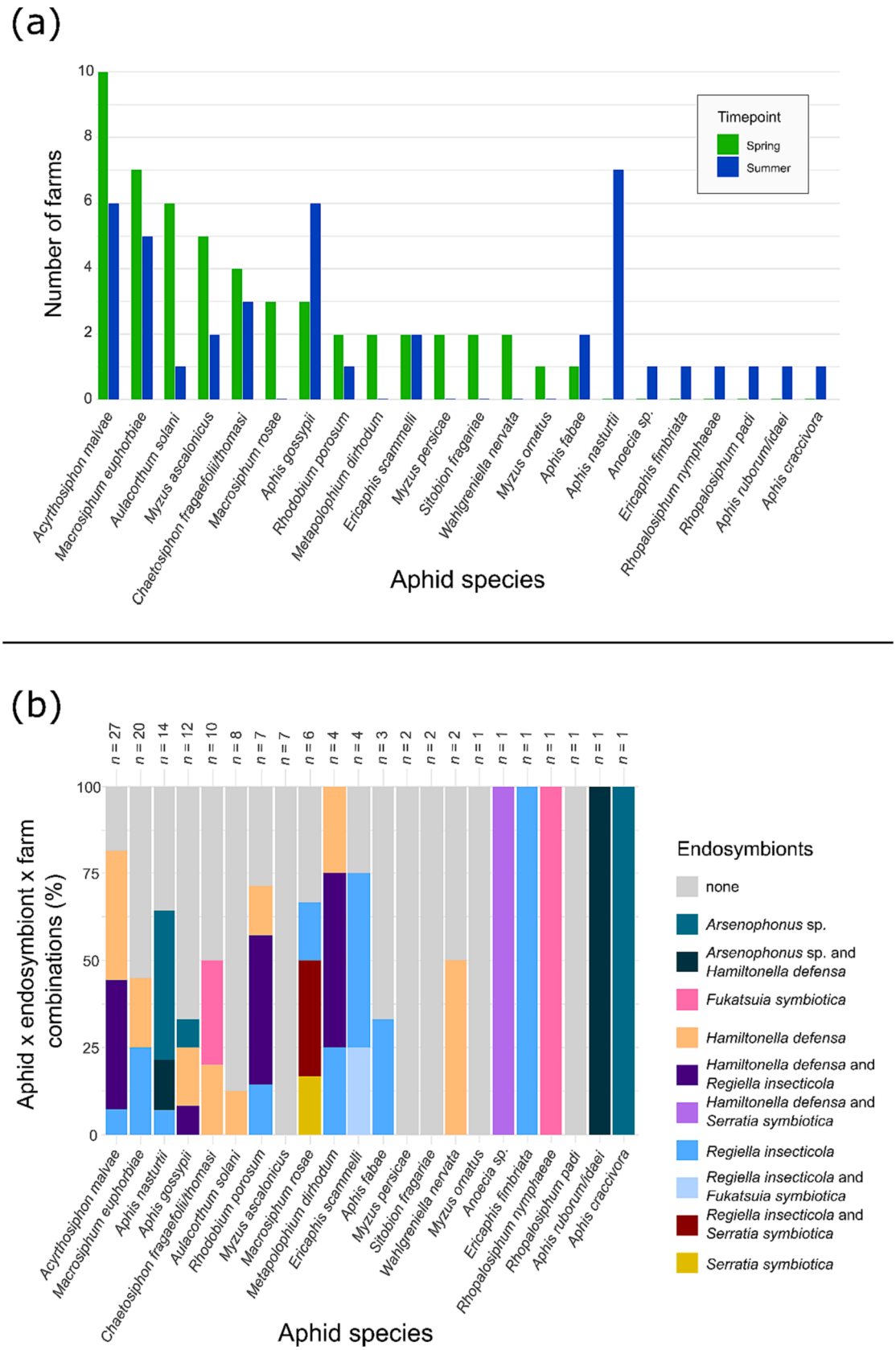

Fig. 1. Aphid species and facultative endosymbionts on strawberry crops in the Netherlands, sampled in 2020. (*a*) Overview of the aphid species found in strawberry farms divided over two sampling timepoints, spring (sampled between April and June), and summer (sampled in September). The aphids are ordered according to prevalence during the spring timepoint. (*b*) Overview of facultative endosymbiont presence in the same aphids as in (a) Each unique aphid-endosymbiont combination is represented once per farm per timepoint in this graph. '*n*' represents the number of individual aphids tested for this species, and they are ordered starting with the aphid species with the highest number of tested individuals on the left, to the aphid species with the lowest number of tested individuals to the right.

H. defensa, and 21.6% carried *R. insecticola*, and no double infections were found (electronic supplementary material, Table S3).

Figure 1b shows an overview of endosymbionts found in each aphid species. Each unique aphid-endosymbiont combination is represented once per farm per timepoint in this figure, regardless of in how many of the five sampling locations of the farm it was found.

3.3. Endosymbiont-based resistance to parasitoid wasps

3.3.1. Endosymbiont status and titres

To confirm the infection status of the *A. malvae* aphid lines we used both diagnostic PCR as well as qPCR at three timepoints during the experimental process: before endosymbiont curing, after antibiotics treatment and during the parasitism assays. Immediately after the antibiotics treatment, all 10 tested F1 individuals of each aphid line had the expected endosymbiont status, when checking with diagnostic PCR.

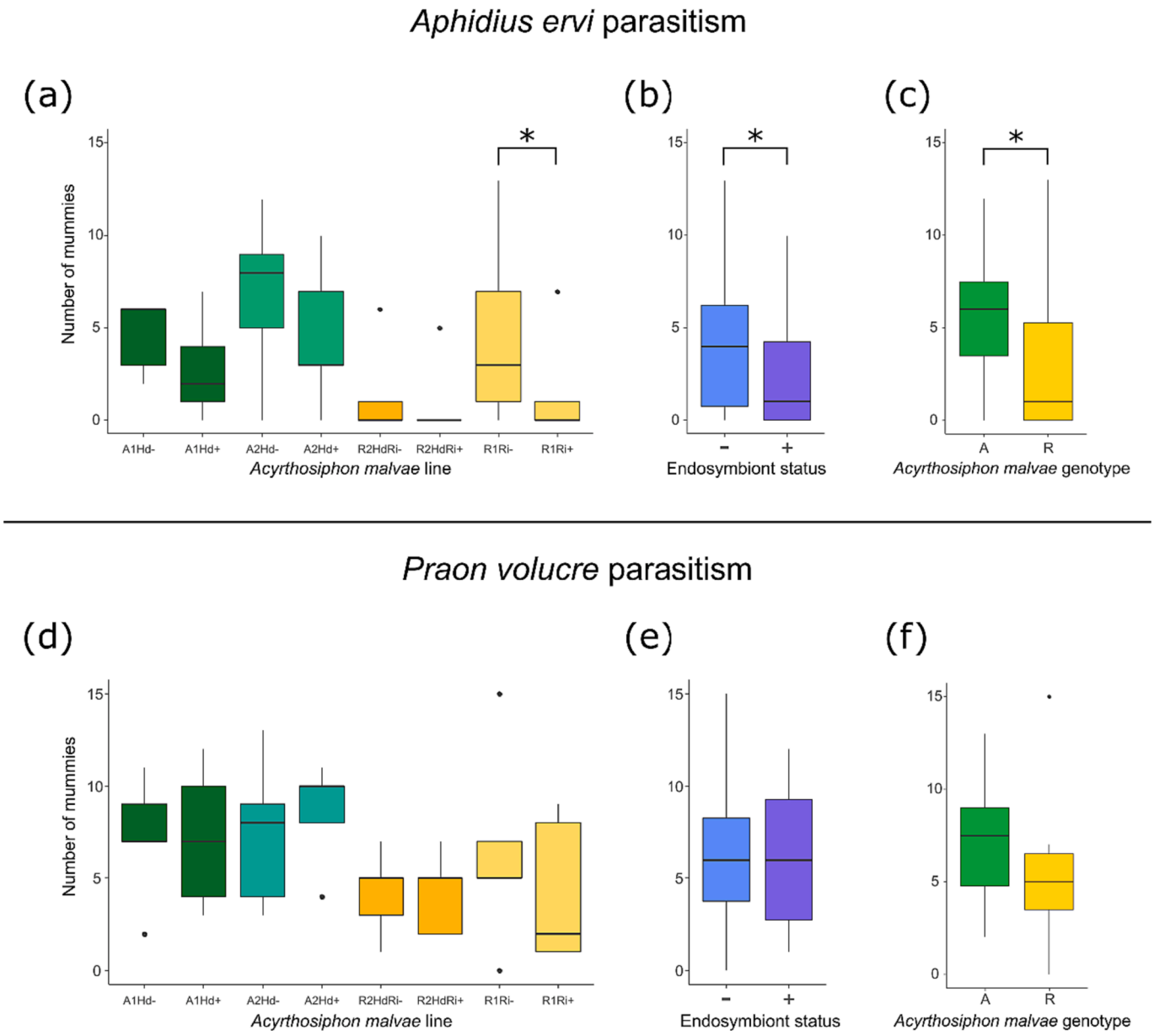

Fig. 2. Number of mummies formed during whole-plant laboratory parasitism assays of the parasitoid wasp species *Aphidius ervi* and *Praon volucre* on four lines of the aphid *Acyrthosiphon malvae*. Each aphid line is represented by a line carrying facultative endosymbionts (+) and a line that has been cured by antibiotics (-). The replicates are presented separately per parasitoid wasp ((*a*), *(b),* and (*c*) for *Aphidius ervi*, (*d*), (*e*), and (*f*) for *Praon volucre*). Panels (*a*) and (d) show the results for all aphid lines separately, while panels (*b*) and *(e)* show the same data grouped together according to endosymbiont status (- for the cured lines and + for the endosymbiont-carrying lines). The number of mummies formed by *A. ervi* on the cured aphids was significantly higher for line R1*Ri*+ compared to R1*Ri*- (GLMM: $\chi^2(1, N = 10) = 10.28$, $p = .005$), but not for lines A1*Hd*+ compared to A1*Hd*- (GLMM: $\chi^2(1, N = 10) = 2.14$, $p = .191$), A2*Hd*+ compared to A2*Hd*- (GLMM: $\chi^2(1, N = 10) = 3.25$, $p = .143$), and R2*HdRi*+ compared to R2*HdRi*- (GLMM: $\chi^2(1, N = 10) = 0.34$, $p = .562$) (*a*). When grouped, the number of mummies formed on the cured aphids was significantly higher (GLMM: $\chi^2(1, N = 40) = 11.44$, $p < .001$) (*b*). The number of mummies formed by *P. volucre* did not differ between cured and original lines, neither for any line separately (*d*) nor for all samples taken together (GLMM: $\chi^2(1, N = 40) = 0.09$, $p = .769$) (*e*). The number of mummies formed by *A. ervi* on the cured aphids of genotype A was significantly higher than the number of mummies formed on the cured aphids of genotype R (GLMM: $\chi^2(1, N = 20) = 5.60$, $p = .018$) (*c*). This difference was not significant for *P. volucre* (GLMM: $\chi^2(1, N = 20) = 1.37$, $p = .241$) *(f)*. Significant differences are shown with an asterisk ($p < 0.05$).

During the parasitism assay we checked the endosymbiont status of 10 aphids from the control treatment with diagnostic PCR. For both the R1*Ri*- and R2*HdRi*- lines, one out of 10 samples was positive for *R. insecticola*. An additional five samples of each line were tested and these tested negative, resulting in a total of 1/15 aphids still being infected with *R. insecticola* in these lines. All other aphid lines had the expected endosymbiont status according to the diagnostic PCR results.

Because diagnostic PCR only gives presence/absence-, but not density information, we additionally quantified endosymbiont titres using qPCR with endosymbiont specific primers and compared these to titres of the uncured *A. malvae* lines. In the cured lines, most samples had either undetectable or low titres of facultative endosymbionts in the F2 generation, except for one case in the group just after antibiotic treatment, where one out of five samples of line A2*Hd*- had a similar amount of *H. defensa* DNA as line A2*Hd*+. However, in the F1 generation after antibiotics treatment, as well as during the parasitism assay, *H. defensa* was not detected in any of the 10 samples tested with diagnostic PCR, indicating that a majority of the cured aphids did not carry *H. defensa*. For the graph and data showing the relative abundance of the endosymbiont DNA compared to the aphid host gene β-actin see electronic supplementary material Fig. S3 and Table S4.

3.3.2. Parasitism assays

To test the effects of the facultative endosymbiont presence in *A. malvae* on the parasitism success of biocontrol parasitoids, we studied the parasitisation rates of *A. ervi* and *P. volucre* on four aphid lines with and without endosymbionts. First, we tested whether there was a difference in survival in the absence of parasitoids. We found that the survival probability of all aphid lines was between 65.5% and 82.2% (electronic supplementary material, Table S5). No significant differences between the lines (GLMM: $\chi^2(3, N = 24) = 1.07$, $p = .785$) or between the endosymbiont-infected and cured lines (GLMM: $\chi^2(1, N = 24) = 0.13$, $p = .717$) were observed.

For *A. ervi*, we found significantly fewer mummies in the endosymbiont-infected aphid line R1*Ri*+ compared to the cured line R1*Ri*- (1.6 vs 4.8 mummies, respectively, GLMM: $\chi^2(1, N = 10) = 10.28$, $p = .005$). A non-significant result in the same direction was found when comparing line A1*Hd*+ to A1*Hd*- (2.8 vs 4.6 mummies, respectively, GLMM: $\chi^2(1, N = 10) = 2.14$, $p = .191$) and when comparing line A2*Hd*+ to A2*Hd*- (4.6 vs 6.8 mummies, respectively, GLMM: $\chi^2(1, N = 10) = 3.25$, $p = .143$). Line R2*HdRi*+ and R2*HdRi*- both had a low number of mummies and no significant difference (1 vs 1.4 mummies, GLMM: $\chi^2(1, N = 10) = 0.34$, $p = .562$) (Fig. 2a). When grouping all lines together, significantly fewer mummies were found for endosymbiont-infected aphid lines than for cured lines (2.5 vs 4.4 mummies, or 8.3% vs 14.7%, respectively, GLMM: $\chi^2(1, N = 40) = 11.44$, $p < .001$) (Fig. 2b). For *P. volucre*, we observed no difference in the number of mummies between the endosymbiont-infected and cured lines, neither when looking at any specific line (Fig. 2d) nor when taking all the lines together (6.05 mummies or 20.2% for the endosymbiont-positive lines and 6.3 mummies or 21% for the cured lines, GLMM: $\chi^2(1, N = 40) = 0.09$, $p = .769$) (Fig. 2e). For the data on the parasitism experiment on the endosymbiont-infected and cured lines, see electronic supplementary material Table S6.

3.4. Endogenous resistance

To study whether endogenous resistance plays a role in the success rate of parasitoids, we compared the number of mummies between the cured *A. malvae* lines A1*Hd*-/A2*Hd*- and R1*Ri*-/R2*HdRi*-. We know from microsatellite genotyping (unpublished data) that the combination "A1 and A2" and "RJ and RR" lines have the same multilocus genotype, therefore we assumed lines A1 and A2 and lines RR and RJ to be the same genotype, genotype A and genotype R, respectively. With *A. ervi*, the number of mummies on the genotype A lines was 5.7, which is 84% higher compared to the genotype R lines with 3.1 mummies (GLMM: $\chi^2(1, N = 20) = 5.60$, $p = .018$) (Fig. 2c). A non-significant result in the same direction was found for *P. volucre*, where the number of mummies on the genotype A lines was 7.3, which is 38% higher compared to the genotype R lines with 5.3 mummies (GLMM: $\chi^2(1, N = 20) = 1.37$, $p = .241$) (Fig. 2f).

4. Discussion

4.1. High diversity of aphid species with facultative endosymbionts in strawberry crops

Our survey revealed that strawberry crops are hosts to many different aphid species that regularly carry facultative endosymbionts. We encountered a total of 22 aphid species, and 48 unique aphid-endosymbiont combinations. It is likely that more aphid-endosymbiont combinations exist, especially in the aphid species that we sampled less often. Two of the detected aphid species, *Anoecia* sp. and *R. padi*, have not been reported on strawberry (Blackman & Eastop, 2019). As these aphids were sampled only once and outdoors, they may have accidentally arrived onto the plants. Another consideration point is the possibility that some facultative endosymbionts were missed during our analyses since nucleotide variation in primer binding sites can occur between more divergent strains of bacteria, leading to false negative PCR results due to reduced primer biding efficiency. Additionally, since we only used species-specific primers, other less common and/or undescribed endosymbiont species could have been missed.

The most common endosymbionts were *R. insecticola* and *H. defensa* which were both present in 26.9% of the samples. Thus, our survey revealed that biocontrol agents encounter a broad arsenal of different aphid-endosymbionts combinations in strawberry crops, as was also found in protected strawberry crops studied in France (Postic et al., 2020b, 2020a).

4.2. Facultative endosymbionts protect against A. ervi but not P. volucre

To study if the most abundant endosymbionts (*R. insecticola* and *H. defensa*) can make aphids unsuitable hosts for generalist biocontrol parasitoids, we focused on the aphid species we sampled most often, *Acyrthosiphon malvae*, which frequently carried these endosymbionts. We found that carrying facultative endosymbionts protected *A. malvae* against *A. ervi,* but not against *P. volucre*. These results are in line with previous laboratory studies where *H. defensa*-infected pea aphids (*Acyrthosiphon pisum* (Harris)) were protected against *A. ervi* but not against *Praon pequodorum* Viereck (Martinez et al., 2016). Martinez et al. assign this difference in sensitivity between parasitoid species to the fact that *H. defensa* APSE3 kills parasitoid eggs shortly after hatching (Martinez et al., 2014b), and *P. pequodorum* eggs have a thicker chorion and hatch much later than *A. ervi* eggs, thus conferring an advantage to the larvae of *P. pequodorum*. Protective effects of *H. defensa* have commonly been reported, especially in *A. pisum* against *A. ervi* (Martinez et al., 2014b; McLean & Godfray, 2015; Oliver et al., 2003) as well as in *A. fabae* against *Lysiphlebus fabarum* (Cayetano & Vorburger, 2015; Dennis et al., 2017). However, there are also aphid species in which no protective traits are reported for naturally occurring *H. defensa* strains, for example in the case of *M. euphorbiae,* where no protection of *H. defensa* was found against the two parasitoid species *A. ervi* (Clarke et al., 2017; Postic et al., 2020a) and *Aphidius rhopalosiphi* (Wu et al., 2022), even though this aphid species is often infected with *H. defensa* (H. V. Clarke et al., 2017, 2018; Henry et al., 2015; Postic et al., 2020a). While any protective effects of *H. defensa* were not significant in our experiments, our study is the first to show that *R. insecticola* can protect aphids against *A. ervi*. This symbiont is best known for protecting aphids against entomopathogenic fungi (Łukasik et al., 2013; Scarborough et al., 2005), although a protective strain against *Aphidius colemani* was found once in *M. persicae* in Australia (Vorburger et al., 2010). Our study thus adds to the evidence that *R. insecticola* can protect multiple aphid

species and against multiple species of *Aphidius* parasitoids.

4.3. Varying levels of endogenous resistance against A. ervi but not P. volucre

As we studied the protective effects of endosymbionts in the same aphid genetic background, endosymbiont-based resistance could be disentangled from endogenous resistance effects. Indeed, we found clear differences in levels of endogenous resistance of different *A. malvae* multilocus genotypes against *A. ervi* parasitisation. Similar observations of varying levels of endogenous resistance have been made for *A. pisum* (Doremus et al., 2018; Martinez et al., 2016; Martinez et al., 2014a; McLean & Parker, 2020), *M. persicae* (von Burg et al., 2008) and *M. euphorbiae* (Clarke et al., 2017), although never before for *A. malvae*. Interestingly, we did not find a difference in endogenous resistance against *P. volucre*, showing that not only endosymbiont-based, but also endogenous resistance can be specific to parasitoid species, as is also described for *A. pisum* (Martinez et al., 2016). The ability for both endogenous and endosymbiont-based resistance could be energetically costly, but we show that also in the R-line of *A. malvae* with already high endogenous resistance, endosymbiont presence results in increased resistance against *A. ervi.* In summary, we found that *A. malvae* has a greater variability of resistance against *A. ervi* than against *P. volucre*, both for endosymbiont-based and endogenous resistance. Potentially this specificity to protect against *A. ervi* but not *P. volucre* relates to how common *A. malvae* is attacked by these species, as previous research has shown that specific strains of *H. defensa* carried by aphid species often only protect against the most commonly attacking parasitoid species (Wu et al., 2022).

4.4. Incomplete curing of endosymbiont infections

It is important to note that not all cured aphid lines were completely cured of facultative endosymbionts. Whether this was caused by a contamination of the line, or because the curing was not entirely successful and some of the offspring of the cured individual we started the line with were still carrying an undetectable amount of endosymbionts is unclear. One of the five tested aphids of the F2 generation of line A2*Hd*- still carried *H. defensa* according to the qPCR results. However, no infected aphids were detected in this line in later generations, neither with qPCR nor with diagnostic PCR. Additionally, in the aphids taken from the control treatments of the parasitism experiment, 1/15 from both line R1*Ri*- and R2*HdRi*- unexpectedly tested positive for *R. insecticola*. However, the presence of endosymbiont-infected aphids in the cured populations will only have resulted in lower parasitism rates than expected for these 'cured' lines, and thus the protective effects, which we already found, would only have been even stronger when all aphids were properly cured.

4.5. Low overall parasitism rates

The overall parasitism success in our study was low for both *A. ervi* (8.3% for the endosymbiont-carrying and 14.7% for the cured lines), and *P. volucre* (20.2% for the endosymbiont-carrying and 21% for the cured lines). The small number of mummies found in our study could be caused by our experimental design. As we performed our parasitism experiments in cages, the parasitoids had to actively search for the aphids which could hide in between the leaves of the strawberry plant, in contrast to Petri dish-experiments where aphids cannot hide and almost no host-searching is required. It is also possible that since our parasitoids had 24 h to parasitize 30 aphids, superparasitism could have occurred which in some cases can cause aphids to die without turning into a mummy (Hertäg & Vorburger, 2018), but in other cases it has been shown to help parasitoids overcome endosymbiont protection (Oliver et al., 2012). We believe that cage experiments, compared to Petri dish experiments, more closely represent the circumstances under which biocontrol parasitoids need to operate in crop systems.

4.6. Varying levels of selective pressure for parasitoid resistance between different crop systems

The hypothesis that protective endosymbionts could affect the success of biocontrol is especially relevant in systems where there is high selective pressure of parasitoid wasps on endosymbiont-protected aphids. It could be expected that this selection pressure will be higher in organically managed, closed systems, where aphid control solely relies on released natural enemies, compared to open systems, and systems where insecticides are also used. In our study, we collected aphids from both indoor and outdoor grown crops, and all the farms used insecticides. Consequently, there was no exceptionally high selection pressure of parasitoid wasps on symbiont-protected aphids, and we expect that the aphid and symbiont populations we sampled probably closely represent those of natural systems. It is known that in natural systems, endosymbiont infections occur at intermediate frequencies (Henry et al., 2015; Vorburger & Rouchet, 2016; Zytynska & Weisser, 2016), which, in the case for protective endosymbionts, has been hypothesized to arise from balancing selection at the aphid level, with protection in the presence of parasitoids and fitness costs in their absence (Oliver et al., 2014). Indeed, we regularly found variation in endosymbiont infection status, both in terms of presence and species, within an aphid species at a farm. Although our study system was unsuitable to study if organic pest management strategies select for parasitoid-resistant aphid populations, we do show that protective endosymbionts and varying levels of endogenous resistance, upon which selection could act, are indeed present in aphids on strawberry crops. Future studies could compare the prevalence of facultative endosymbionts in aphids, and proportions of aphids with of varying levels of endogenous resistance, in conventional versus organic systems to evaluate if there is indeed strong selection for these protective phenotypes in biologically managed agricultural systems.

In conclusion, the growing awareness of the effects of pesticides on global health and biodiversity, pesticides being phased out, and the increasing problems with insecticide-resistant pest populations, increases the importance of biocontrol for sustainable food production. The role of heritable bacterial endosymbionts on pest-biocontrol agent interactions has long been overlooked. Here, we showed that protective endosymbionts are present in strawberry crop systems and that these specifically affect the interactions between a common aphid pest and their commonly used biocontrol parasitoids. Our results thus show that knowledge on the prevalence of facultative endosymbionts in common pest species and the effects of these endosymbionts on the biocontrol success of common biocontrol species could be incorporated into deciding which biocontrol species to deploy. A challenging but crucial next step in this research field would be to translate these findings from the two-species interactions studied in the laboratory to the much more complex interactions in the field by studying the effects of endosymbiont protection on biocontrol success on the farms themselves.

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CRediT authorship contribution statement

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Methodology, Writing – review & editing. **Marcel Dicke:** Conceptualization, Supervision, Writing – review & editing. **Bas J. Zwaan:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Eveline C. Verhulst:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Bart A. Pannebakker:** Conceptualization, Funding acquisition, Project administration, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105383.

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