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Title: **Attacking the clones: understanding aphid pest resistance to biological control.**

Understanding potato aphid resistance to parasitoids.

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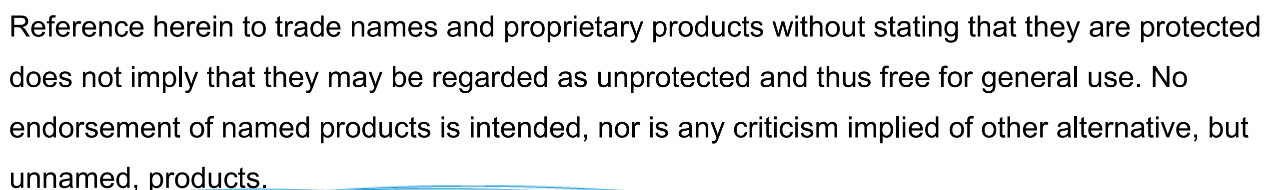

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1. Industry Summary

The potato aphid (*Macrosiphum euphorbiae*) is an economically important pest of strawberry crops in the UK. The presence of this aphid and the honeydew it produces can affect plant photosynthesis by promoting fungal growth (sooty moulds), affecting fruit formation and its marketability. Integrated pest management in strawberry crops is focused on the use of biological controls for different pests and the minimal use of synthetic chemical insecticides. In the case of aphids, individual or a mix of six aphid parasitoid species are currently used in both protected and outdoor crop environments. However, there has been an increase in the frequency of reports of early-season control problems of potato aphids in strawberry crops. Parasitoid resistance in aphids has been reported for different species including the potato aphid in potato crops in the UK, however, aphid resistance to parasitoids has not been confirmed to be a problem in strawberry crops. Aphid resistance to parasitoids can be genetically encoded or a trait conferred by facultative bacteria living inside the insects. The main objective of this project is to understand the role of potato aphid clonal diversity on its interactions with parasitoids used in biological control programmes in strawberry crops. The specific objectives of this project were:

1) Determine the clonal diversity of the potato aphid attacking strawberry crops in the UK.

2) Understand the role of aphid genetics and secondary endosymbiont composition on shaping the potato aphid-*Aphidius ervi* interaction.

3) Determine the genetic diversity of the main parasitoid species (*Aphidius ervi*) used against potato aphid, as supplied by different biological control companies, to understand whether genetic diversity can be used to overcome aphid resistance to this parasitoid species.

4) To test under commercial crop conditions whether genetic variation in the potato aphid and the endosymbionts they carry confers resistance to released parasitoids, by assessing how the aphid-endosymbiont-parasitoid community dynamic fluctuates through the season.

In terms of results, first, parasitism assays were completed using the 14 aphid clonal lines. Data from these parasitism bioassays showed significant differences in parasitoid susceptibility between the 14-potato aphid clonal lines, with two lines from Staffordshire where a parasitism proportion <20% to the parasitoid *A. ervi* provided by a commercial supplier was recorded. Subsequently, a more detailed assay with two clonal lines per genotype was undertaken to understand the role of aphid genotype in determining the role of potato aphid clonal variation in shaping its interaction with the parasitoid *A. ervi.* The results showed no effect of genotype on parasitism proportions, nor for parasitoid acceptance for oviposition. However, an effect of genotype on aphid defensive behaviours such as cornicle secretions was described and differences in parasitoid cleaning time were also recorded. A second assay with two clonal lines per endosymbiont infection status (i.e

Hamiltonella defensa, Serratia symbiotica, Regiella insecticola, no infection) showed an effect of endosymbiont infection status on parasitism proportion, acceptance and oviposition. An effect of infection status was recorded for aphid defensive behaviours, certain parasitoid searching behaviours, parasitoid acceptance and parasitism success. With potato aphid clonal lines infected with Hamiltonella defensa, Serratia symbiotica recording lower overall parasitism efficiency *by A. ervi.* These results show that the interaction between the potato aphid and *A. ervi* is predominantly shaped by endosymbiont infection, however the interaction is complex.

In terms of the parasitoid *Aphidius ervi* itself, it has been established that there was a low genetic diversity in the wasps supplied at the beginning of the season by commercial companies in the UK. There was also, low genetic differentiation between the different commercial suppliers. This can influence the effectiveness of parasitoid populations against aphid populations with variation in traits such as parasitoid susceptibility. The parasitism assessment of the *A. ervi* populations against different clonal lines of potato aphid, showed that variation in parasitism proportion was recorded depending on the source of *A. ervi.* However, a stronger variation was noted when the parasitoid interacted with previously described "resistant" clonal lines, with less genetically diverse parasitoid populations doing worse than more genetically diverse ones.

Finally, the results from the 2023-2025 field survey have shown that there is a high intraspecific diversity of the potato aphid attacking strawberry crops. It is important to notice that most of the genotypes showed low abundances and poor representation throughout the year. However, 9 genotypes were deemed of importance as it was present throughout the survey period with high relative abundances. In terms of secondary endosymbionts, a mix of uninfected aphids and single and multiple endosymbiont infections were found throughout the season with a dominance of *Regiella insecticola* at the end of all seasons. However, only evidence of a weak link between endosymbiont infection with parasitism pressures was found, and a moderate relationship between potato aphid genotypes and parasitoid community β diversities was also described.

Key findings and conclusions:

- There is genetic diversity of *Macrosiphum euphorbiae* infesting strawberry crops grown in the UK.
- Different clonal lines of *Macrosiphum euphorbiae* are infected by single, double or triple infections of secondary endosymbionts including: *Hamiltonella defensa, Serratia symbiotica, Regiella insecticola, Rickettsia* sp.
- Different clonal lines of *Macrosiphum euphorbiae* collected from strawberries in the UK have shown variability on their susceptibility to the parasitoid *Aphidius ervi* provided by a commercial supplier.

- The genetic background of *Macrosiphum euphorbiae* influences certain aphid defensive behaviours and has an effect in some parasitoid host searching behaviours but it is not a factor in the variability of *Aphidius ervi* (provided by a commercial supplier) acceptance or success.
- Infection of *Macrosiphum euphorbiae* with certain secondary endosymbionts influences some aphid defensive behaviours, parasitoid host searching behaviours*, A. ervi* acceptance and success (provided by a commercial supplier).
- There is little genetic differentiation between *Aphidius ervi* wasps supplied by different commercial suppliers.
- *A. ervi* source has an effect on its parasitism success on different clonal lines of *M. euphorbiae.*
- There is high genotypic diversity and endosymbiont infection statuses among aphids attacking strawberry crops.
- Under field conditions, potato aphid endosymbiont infection has a weak link with parasitism pressures.
- Under field conditions, potato aphid genotype can affect parasitoid community turnover in the season.

This project will benefit strawberry growers, any other agricultural sectors where biological control agents are used for pest control (especially aphids) and biological control companies. This is because the main findings can be translated to any context where pests are supressed by natural enemies (especially parasitoids), as what is described here is not an isolated problem associated with strawberry crops. In addition, the information provided in this document could be the base for a step change in mass-rearing programs and quality control assurance in the commercial production of biological controls. The key takeaways for from this project are:

- Potato aphid populations are highly intraspecifically diverse in terms of genotypes and endosymbiont associations in strawberry crops.
- Intraspecific variation in potato aphid populations is linked to variations in parasitism success by *Aphidius ervi*, although the effect on other parasitoid species has been seldomly explored.
- Endosymbiont infection of potato aphid can modulate aphid defensive behaviour and parasitoid searching behaviour, with implications for parasitoid acceptance.
- Endosymbiont infection of potato aphid can be associated with lower levels of physiological susceptibility to the parasitoid *A. ervi*.
- The genetic diversity of populations of the parasitoid *A. ervi* is negatively affected by commercial mass-rearing practices.
- *A. ervi* populations attacking potato aphid in strawberry crops are more closely related to natural populations instead of commercially supplied individuals.
- *A. ervi* source and its genetic diversity can affect parasitism success of different potato aphid populations. Especially when resistant pest’s populations are present.
- The evaluation of other commercially supplied natural enemies’ success and genetic diversity is advocated.

- Field sampling is crucial to understanding the role of potato aphid intraspecific variation on parasitism success under real life conditions.
- The integration of pest intraspecific variation into the design and implementation of IPM programs is highly recommended.
- Local variation of pest populations should be explored for tailored and more efficient IPM programs.
- Field findings highlight the need for monitoring aphid population structure and symbiont prevalence when planning parasitoid releases.
- Increased understanding of the ecological complexity influencing biological control outcomes contributes to reduced pesticide reliance and more resilient control systems for strawberry crops and other agricultural sectors.

The following report is an abridged version of the PhD thesis.

2. Introduction

Global strawberry production was estimated at 9.1 million tonnes in 2021 in 389 000 hectares, representing a 3% rise on yield and an expansion of 1.55% in crop area over the preceding year (Kouloumprouka Zacharaki *et al.*, 2024). These changes reflect both the crop's growing economic importance and increasing consumer demand (Simpson, 2018). Increases in global production has, in part, been attributed to the ability to grow this crop in diverse climatic conditions (Samtani *et al.*, 2019). On the other hand, the growing demand can be linked to public awareness of the antioxidant and anti-inflammatory properties of the fruit (Husaini and Neri, 2016).

The UK strawberry production was over an area of 4,527 Ha, yielding 21.8 tonnes/Ha in 2024, with a value of approximately £389 million (Department for Environment Food & Rural Affairs, 2025). Strawberries account for a large proportion of the UK's fruit production value following apples and it is the main soft fruit crop in terms of cultivated area (Department for Environment Food & Rural Affairs, 2025). Consumers in the United Kingdom value strawberries for their quality and flavour, therefore crop production inputs (i.e., pesticides) that can affect the quality of the fruit have to be balanced with yield optimization (Sampson *et al.*, 2011; Simpson, 2018). In the most recent governmental report on pesticide usage in soft fruit crops it was noted that 13,550 ha of strawberries were treated with insecticides in 2022, but the area treated with biological control was even higher at 45 142 ha (double the record from 2018) (Ridley *et al.*, 2020). Currently, the most important pests of strawberry crops in the UK are western flower thrips (*Frankliniella occidentalis* Pergande, 1895), the European tarnished plant bug (*Lygus rugulipennis* Poppius, 1911), common green capsid (*Lygocoris pabulinus* (L., 1761)), other species of thrips, such as *Thrips fuscipennis* Haliday, 1836, whiteflies, weevils, moth caterpillars (Noctuidae and Tortricidae), spotted wing drosophila, *Drosophilia suzukii* (Matsumura), and different species of aphid (Cross,

Fitzgerald and Down, 2005; Husaini and Neri, 2016; Fountain, 2020; Mitchell and Karley, 2020). Almost all these pests are polyphagous with only few adapted to feed only on strawberry plants. Nevertheless, one of the most economically important pests of strawberry crops is aphids, including the potato aphid, *Macrosiphum euphorbiae* (Thomas), which can be found in large numbers in strawberry crops (Cross, Fitzgerald and Down, 2005; Mitchell and Karley, 2020). Other species, such as the strawberry aphid, *Chaetosiphon fragaefolii* (Cockerell), the shallot aphid *Myzus ascalonicus* Doncaster, 1946 and the melon-cotton aphid, *Aphis gossypii* Glover, can also impact greatly fruit production. The presence of large populations of any aphid species can cause leaf and fruit distortion, which reduces the quality and value of the harvested crop (Cross, Fitzgerald and Down, 2005).

The potato aphid is a medium to large species native to North America but with a cosmopolitan distribution, with characteristically long antennae, legs, siphunculi and cauda (Blackman and Eastop, 2006; Vilcinskas, 2016). The colour of this species is usually green with red eyes; however, some populations can be red (Figure 1.7). Nymphs complete their development in 9-11 days at 20ºC going through four nymphal instars, which are often paler in colour than the adults but are characterised by having a darker central stripe on the dorsal surface (Blackman and Eastop, 2006). This species is typically heteroecious and holocyclic in their original region by laying eggs on their primary hosts, plants from the *Rosa* genus, as a physiological response to continuous short days (day length below 9-10 hours a day) (Trionnaire *et al.*, 2008). Populations in Europe are, however, mainly anholocyclic and overwinter as adults on secondary host plants, that are usually herbaceous weed species (Blackman and Eastop, 2006; Raboudi *et al.*, 2012).

Figure 1.7. Potato aphid morphs. Green morphotype on the left, red morphotype on the right. (Adapted from Utah Vegetable Production & Pest Management Guide, accessed on the 22 September 2022).

The potato aphid is a polyphagous species that feeds on up to 200 plants from over twenty families including economically important species, in the family *Solanaceae* (such as potatoes), *Fabaceae* (such as beans) and soft fruits (van Emden and Harrington, 2017). Infestations by this species are common in both field and protected crops and can cause direct and indirect feeding damage (Saguez, Giordanengo and Vincent, 2013). Direct feeding damage on strawberry plants results in the leaves and fruits becoming deformed in shape. Indirect feeding damage, includes the production of honeydew, which can lead to the development of sooty moulds (Jansen, 2005). Sooty moulds are a group of fungi species, such as *Alternaria* spp. and *Cladosporium* spp., that produce dark growth and spores which are usually only surface contaminants but that can greatly affect plant performance by reducing photosynthesis (Chomnunti *et al.*, 2014). As aphid excreted honeydew contains high levels of sugars, it is the perfect nutrient source for the fungi. These fungi can therefore coat not only leaves but also fruits, restraining their formation and affecting the marketability of the product (Insausti *et al.*, 2015; Halder and Seni, 2020).

Potato aphid control is currently incorporated into IPM strategies in strawberry crops, which include diverse methods and the mixed use of insecticides and biological controls. Biological control plays a crucial role in strawberry IPM, mainly because the control of pests such as whiteflies, thrips and spider mites relies almost exclusively on generalist predators (the specialist mite *Phytoseiulus persimilis* Athias-Henriot being one exception) and parasitoids such as *Encarsia* sp.(Hoogerbrugge *et al.*, 2011), thus the use of chemical insecticides can disrupt the control of those pests. In addition, chemical control of the potato aphid (as with other aphid species) has become problematic due to the withdrawal of commonly used insecticides (Fountain, 2020) and the increasing risk of the development of insecticide resistance via different mechanisms, such as the overexpression of carboxylesterases which are enzymes that are involved in the metabolism and detoxification of insecticides, in some wild populations that can potentially infest protected crops (Insecticide Resistance Action Group, 2022).

Different generalist and specialist natural enemies can be used to control the potato aphid. This includes, as for other aphid species, predators, such as adult and larval ladybirds, lacewings and predatory midges. However, parasitoids of the Aphidiinae subfamily, such as *A. ervi* in Europe and *A. nigripes* in North America, are considered the main biological controls (Cross *et al.*, 2001). In strawberry crops, a mix of six hymenopteran parasitoid species (*Aphidius colemani* Viereck, *A. ervi, Aphidius matricariae* Haliday, *Ephedrus cerasicola* Stary, *Aphelinus abdominalis* (Dalman) and *Praon volucre* (Haliday)) have been widely used to control aphid pests with good results reported (De Menten, 2011). However, the most important species of parasitoid introduced as biological controls for potato aphid in protected strawberry crops are *A. ervi* and *P. volucre* (Sampson *et al.*, 2011). Despite this, current information suggests that some populations of *M.*

euphorbiae in the UK have moderate to high levels of resistance to the parasitoid *A. ervi* (Clarke, 2013; Whitehead, 2019), something that has been proved to be common in other aphid species as well (Li *et al.*, 2002, McLean and Parker, 2020). The reason for low susceptibility levels in potato aphids to *A. ervi* seems to be a case of clonal resistance associated to aphid genotype (Clarke *et al.*, 2017). However, it is unknown if there is any resistance to other parasitoid species that are currently used for *M. euphorbiae* control or if populations attacking other crops are also developing resistance.

Aphidius ervi is a small (Figure 1.8), generalist and cosmopolitan aphid parasitoid of the family Braconidae, sub family Aphidiinae. This species is a solitary koinobiont that tends to parasitise larger aphid species such as the potato aphid, pea aphid (*Acyrtosiphon pisum* (Harris)) and the glasshouse potato aphid (*Aulacorthum solani* (Kaltenbach)) but has a range of 20 or more species of aphid hosts (Helyer, Cattlin and Brown, 2014). Therefore, *A. ervi* is widely used as a biological control in many agricultural systems in which its hosts can be found, including the horticultural sector (Pennacchio *et al.*, 1994). The development of this species lasts approximately 12-14 days at 20°C on potato aphid, however, the development time is highly dependent on temperature and other physiological factors. The efficacy of this parasitoid may be reduced below 10°C and above 30°C, but this species can overwinter in the pupal stage when the aphid is mummified (Kos *et al.*, 2009).

Figure 1.8. Female of *Aphidius ervi*. (Kos *et al.*, 2009)

The aim of this dissertation is to understand the importance of the potato aphid, *Macrosiphum euphorbiae* genotype and endosymbiont based intraspecific diversity on its interactions with the parasitoid, *Aphidius ervi* used as a biological control in strawberry crops. As such, this project incorporates evolutionary perspectives on ecological interactions to advance the field of biological control (Sentis *et al.*, 2022). Furthermore, this work builds on recent

developments in the understanding of the importance of intraspecific variation in the modulation of the interaction between natural enemies and their hosts (Gimmi and Vorburger, 2021).

3. Materials and methods

3.1. Insect cultures

The plants used for aphid culturing and all the experimental procedures were commercially supplied strawberry crowns (*Fragaria* x *ananassa* var. Elsanta) cold-stored at 2°C. The crowns were removed from the fridge when needed and grown in insecticide free and peat free compost (John Innes No.2 potting-on compost, Westland Garden Health, Dugannon, Northern Ireland, UK) in recyclable pots (13.8 cm diameter x 13.2 cm height, Aeroplas Ltd, Tipton, UK) at the Jean Jackson glasshouse at Harper Adams University.

3.1.1. Aphid clonal lines

Potato aphid colonies, *Macrosiphum euphorbiae*, were collected from strawberry fields (*Fragaria* x *ananassa*) from areas across the UK between November 2021 and July 2022, with the exception of HAU/01, which was already in culture at Harper Adams University and had been collected in 2018. An individual aphid from each colony was selected and transferred to a young strawberry plant within a small pop-up cage insect cage (30cm x 30 cm x 30cm, Edu-Sci Ltd, Tyne and Wear, UK) to start a culture of a clonal line. Each culture was labelled with the clonal line name, the location and date collected (Table 2.1). The culture was discarded if any aphid was found to be parasitized or having a fungal infection, so only cultures started from healthy individuals that produced offspring were maintained. Aphid cultures were kept in a controlled environment room (Fitotron® SGR, Weiss Technik, Loughborough, UK) at 20°C, 60% humidity and 16h light: 8h dark lighting conditions.

Once the cultures were successfully established, they were maintained under the same environmental conditions and watered at least once a week. The colonies were cultured on a 10-day basis by transferring aphids to fresh plant material by cutting a leaf infested with aphids from the old strawberry plant and transferring this onto the crown of a clean new strawberry plant. The old strawberry plant was immediately discarded. Care was taken to clean the surface where the

culturing was taking place between clonal lines to avoid cross-culture contamination. When experiments were taking place and larger numbers of aphids were needed, a second plant was added to the culture cage to build up the population numbers.

3.1.2. Standardised cohorts of potato aphids

For each experiment, age standardized cohorts of apterous aphids were generated. This was done by placing at least 6 strawberry plants within an insect proof mesh cage (47.5 cm × 47.5 cm × 47.5 cm, BugDorm-4S4545, MegaView Science Co. Ltd, Taichung, Taiwan) and transferring 20 apterous adult aphids onto each plant by using a size 000 paintbrush. After 24 hours, adult aphids were removed from the plants using a size 000 paintbrush to leave only first instar nymphs. These nymphs were left to develop to a suitable age for each experiment (usually third or fourth instar). The standardised cohorts were maintained under the same environmental conditions described above.

Table 2.1. Date and location of collection of the founding aphids for the clonal lines of *Macrosiphum euphorbiae* kept in culture and used for experiments.

***Macrosiphum euphorbiae* clonal line**	**Date collected**	**Collection location**	**Colour**
HAU/01	2018	NIAB, East Malling, Kent, England	Green
HAU/02	November 2021	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/03	November 2021	NIAB, East Malling, Kent, England	Green
HAU/04	November 2021	NIAB, East Malling, Kent, England	Red
HAU/05	April 2022	Harper Adams University, Shropshire, England	Green
HAU/06	July 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/07	August 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/08	August 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/09	August 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/10	August 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/11	August 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/12	August 2022	Littywood Farm, Bradley, Staffordshire, England	Green
HAU/13	July 2022	Hereford, Herefordshire, England	Green
HAU/14	July 2022	The James Hutton Institute, Invergowrie, Scotland	Green
HAU/15	September 2022	Littywood Farm, Bradley, Staffordshire, England	Red

3.1.3. Parasitoid cultures

An *Aphidius ervi* culture reared on pea aphid (*Acyrtosiphon pisum*), which had been reared on field beans (*Vicia faba*, Green Manure Seeds, Tetbury, Gloucestershire, UK), was already available at Harper Adams University. This culture was refreshed yearly by introducing ~ 500 mummies of the same species from the same commercial company, Biobest UK (Biobest UK Limited, Ashford, Kent, UK) as the original source of the culture to make a stock culture, this was to avoid inbreeding depression. The aphid host selected was the same as the wasps were reared on commercially, but other commercial culturing details were unknown.

The stock culture was maintained in a medium meshed insect rearing cage (47.5 cm × 47.5 cm × 47.5 cm, BugDorm-4S4545, MegaView Science Co. Ltd, Taichung, Taiwan) on the pea aphid clonal line LL01 supplied by the James Hutton Institute on field beans. This specific pea aphid line does not have secondary endosymbionts and has a high reproductive rate. Culturing of the stock culture of parasitoids was done by introducing a small pot with three field bean plants infested with pea aphids into the parasitoid cage every week and discarding everything but the leaves on the old field bean plant (to avoid throwing away mummies). Both the parasitoids and the pea aphids were maintained under the same environmental conditions described above. A ball of cotton wool soaked in 20% honey solution was also added to the cage and refreshed every three days to provide food for the wasps.

3.1.4. Parasitoids standardised cohorts

Standardised cohorts of *A. ervi* were stablished when needed for experiments by selecting 10 female *A. ervi* wasps presumed mated from the stock culture and introducing them into a small pop-up cage (30cm x 30 cm x 30cm, Edu-Sci Ltd, Tyne and Wear, UK) with one field bean plant infested with pea aphid clonal line LL01, a ball of cotton wool soaked in 20% honey solution was added to the cage to provide food. After 24 hours the females were removed and transferred back to the stock culture.

Eight days after the female parasitoids were removed, the cage was checked for mummies daily. Once mummies had formed, they were gently removed from the plant material or walls of the cage using soft forceps and a 000 paintbrush. Each mummy was placed in a 1.5 mL Eppendorf

tube (Eppendorf Tube 3810X, Eppendorf UK Limited, Stevenage, UK) with a small cotton ball covering the lid space for ventilation and labelled with the date of collection and batch number. After day 10, the individual mummies were checked daily for recently emerged adult parasitoids, which were transferred into new labelled Eppendorf tubes in pairs (one male and one female) in daily cohorts with a small ball of cotton wool soaked in 20% honey solution at the bottom. Adult female parasitoids were used in the experiments up to three days after emerging to limit any potential effects of age on fitness. Parasitoids were handled after being anesthetised using CO_2.

All the steps of the parasitoid cohort standardisation process were done under the same environmental conditions described above.

3.2. Molecular biology

3.2.1. Preparation of total DNA

Total DNA was extracted from whole potato aphids and whole *A. ervi* wasps using the destructive protocol (DY14) of the DNeasy® Blood and Tissue Kit (Qiagen, Crawley, UK) for DNA extractions from insects. DNA was extracted from frozen aphid samples previously stored at -20 °C. An individual aphid (adult if possible) from each sample was extracted and placed in a 1.5 mL Eppendorf tube with 180 µL of phosphate buffered saline (PBS) and a new disposable polypropylene micropestle (Starlab (UK) Ltd, Milton Keynes, UK) used to grind the tissue. Once the tissue was ground, 20 µL of proteinase K and 200 µL of Buffer AL were added to the sample, which was subsequently vortexed and incubated at 56°C for 10 minutes. After the incubation period, 200 µL of 100% ethanol were added, the sample vortexed again and the mixture pipetted into a DNeasy Mini Spin column within a 2 mL collection tube and centrifuged at 8,000 rpm for 1 min using a centrifuge (Sorvall legend centrifuge 17R, Thermo Scientific). The spin column was transferred into another collection tube, 500 µL of buffer AW1 added and centrifuged at 8,000 rpm for 1 min. After this the spin column was again transferred into a new collection tube, 500 µL of buffer AW2 added and centrifuged at 14,000 rpm for 3 min. Finally, the spin column was transferred into a clean 1.5 mL Eppendorf tube, 200 µL of elution buffer (buffer AE) was added directly into the membrane and incubated for 10 minutes at room temperature. Following the incubation, the columns were centrifuged at 8,000 rpm for one minute to elute the DNA. For each

sample the total DNA obtained was divided into one working aliquot of 20 µL and 3 backup aliquots of 60 µL to be stored separately at -20 °C.

A similar protocol was used when extracting DNA from empty mummy cases of parasitised aphids, the only changes made were the use of 40 µL of proteinase K, incubation at 56 °C overnight and the use of only 100 µL of elution buffer (buffer AE), plus incubation at room temperature for 20 minutes at the last step.

3.2.2. Estimation of DNA concentration and dilution

The total DNA concentration obtained from the aphids and parasitoids was initially estimated using the NanoDrop 2000/2000c Spectrophotometer (ThermoFisher Scientific, Loughborough, UK). This was complemented by using the Qubit 4 fluorometer (ThermoFisher Scientific, Loughborough, UK) following the Qubit dsDNA BR Assay Kit protocol for a more accurate quantification of DNA. All samples were diluted to 5 ng/µL when possible, for posterior PCR amplification.

3.2.3. Genotyping using microsatellites / short tandem repeats (STR)

Amplification of microsatellites by Polymerase Chain Reaction (PCR)

The aphid clonal lines kept in culture, parasitoid samples described in Chapter 6 and field samples described in Chapter 7 were genotyped based on the allele sizes of polymorphic microsatellite loci. In the case of aphid laboratory cultures, seven initial microsatellites were tested. However, only five microsatellite loci were used to confirm the integrity of all clonal lines over time and for field samples only the four more variable microsatellite loci were used. In the case of parasitoids, six microsatellite loci were used. All microsatellites had a 5’ fluorescent label for its posterior use in capillary electrophoresis. The primers for microsatellite amplification of aphid DNA were described by (Raboudi *et al.*, 2005), while the primers for parasitoids were described by (Zepeda-Paulo *et al.*, 2015) and used in 25 µL reactions.

All the PCRs were carried out using Cytiva PuReTaq Ready-To-Go™ PCR Beads (Cytiva - Global Life Sciences Solutions, Buckinghamshire, UK), comprising ~2.5 units of puReTaq DNA polymerase and reaction buffer, each dNTP at 200 µM in 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$. Each bead was mixed with 22 µL of UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific, Loughborough, UK), 1 µL of forward primer, 1 µL of reverse primer and 1.5 µL of DNA template. When the PCR programmes were finished, the amplicons were stored at -20 °C.

The presence of microsatellite products and the approximate scoring of the sizes was determined by separating the DNA fragments on a 2% agarose gel with GelRed® Nucleic Acid Gel Stain (Biotium Inc, California, US) using electrophoresis. The 2% agarose gel was made by mixing 2g of molecular biology grade agar (Agarose I, ThermoFisher Scientific, Loughborough, UK) on 100 mL of 10X Tris-borate-EDTA (TBE) buffer (ThermoFisher Scientific, Loughborough, UK) and heating the solution until homogenisation. After pouring the agarose solution into the chamber, combs were pleased to create wells. Once the gels were solidified, the combs were removed, and the gel transferred into a multiSUB Midi, Midi Horizontal Electrophoresis System (Thistle Scientific, Glasgow, UK). Each PCR product was mixed 5:1 with a 6X Gel Loading Dye (ThermoFisher Scientific, Loughborough, UK) and loaded into the wells of the gels accompanied by a DNA ladder (EasyLadderI, Meridian BioScience UK Ltd, London, UK). The gels were run at 120 V for 45 minutes using a nanoPAC-300P Power Supply (Thistle Scientific, Glasgow, UK) source and observed under UV light on a gelLITE, UV transilluminator (Techmate Ltd, Milton Keynes, UK).

Scoring the STR products using capillary electrophoresis

To determine the size of the microsatellite products capillary electrophoresis was used. This process was done by a third party, Eurofins Genomics, by using their standard service. PCR products were sent in a concentration of 80 ng/µL and a total volume of 20 µL. The capillary electrophoresis was performed in an ABI 3130 XL DNA analyser (Applied Biosystems/Life Technologies Ltd, Paisley, UK). Initial analysis was conducted using GeneMapper software v 6.0 (Applied Biosystems/Life Technologies Ltd, Paisley, UK).

3.2.4. Diagnostic PCR reactions for endosymbionts

The presence of the most frequently detected secondary endosymbionts in each aphid clonal line and field samples was determine by PCR reactions using already described primers specific to the 16S rRNA gene of each bacterium. The secondary endosymbionts that were tested for in clonal lines are: *Hamiltonella defensa, Serratia symbiotica, Regiella insecticola, Fuckatsuia symbiotica, Rickettsia* sp.*, Spiroplasma* sp.*, Ricketsiella* sp*.* Field samples were only tested for the first three facultative endosymbionts. All samples were subjected to amplification of the 16S rRNA gene to confirm the successful extraction of bacterial DNA from the aphid samples. In addition, amplification of the linked 16S-23S rRNA gene was undertaken to confirm the presence of any bacteria but *Buchnera aphidicola*. As these genes are only unlinked on the primary endosymbiont *Buchnera aphidicola* the amplification is not possible for this bacterium*,* so a positive result indicated the presence of other endobacteria.

The diagnostic PCRs for presence/absence of endosymbionts were carried out following the same protocol already described. The negative control was UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific, Loughborough, UK) and positive controls, when available, comprised DNA extracted from potato aphid cultures cultured on potatoes and acquired from the James Hutton Institute. These cultures were known to harbour either *Hamiltonella defensa* (clonal line RB15/10)*,* or *Regiella insecticola* (clonal line MW16/38). Positive controls were not available for the other facultative endosymbionts, but amplicon identity was confirmed by sequencing.

In addition to the amplification of the 16S genes for each bacterium, two housekeeping genes, *accD* and *murE*, were amplified for the samples positive for *H. defensa* and the presence and variation of APSE (*Acyrthosiphon pisum* secondary endosymbiont) bacteriophage was also determined by amplifying two regions (P3-P24 and P35-P41) using specific primers described by (Degnan and Moran, 2008). For any samples positive for APSE presence, the amplification of three previously associated toxin genes (*stdX*, *ctdB*, *Ydp*) was undertaken by using specific primers described by (Degnan and Moran, 2008) in order to characterise the infection. All the positive PCR amplicons from this process were sent for Sanger sequencing at Eurofins Genomics for confirmation and variation analysis.

PCRs for the APSE regions and toxin genes were performed using the LongAmp™ Hot Start *Taq* 2X Master Mix (New England Biolabs, Massachusetts, US), using 12.5 µL of the Master Mix, 1 µL of 10 µM Forward Primer, 1 µL of 10 µM Reverse Primer, 1.5 µL of template DNA at 5 ng/µL and 9 µL of UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific,

Loughborough, UK). The clonal line RB15/10 from The James Hutton Institute was used as positive control for APSE, however no positive control was available for toxin genes.

Products for diagnostic PCRs were visualized on a 2% agarose to confirm the presence or absence of each secondary endosymbiont. PCR products from the housekeeping genes and APSE regions/toxins were visualized on a 0.6% gel (0.6 g of Molecular Biology grade agarose in the mix), using the 1 Kb Plus DNA Ladder (ThermoFisher Scientific, Loughborough, UK) before sending for sequencing. The APSE amplicons were diluted to 100 ng/µL for visualisation.

3.2.5. Barcoding using the Cytochrome oxidase sub-unit I (COI) gene and sequencing

The determination of the variation of the COI gene in parasitoids and aphid mummies for Chapter 7 was undertaken by PCR reactions using the widely used pair of primers LCO1490-HCO2198 (Folmer *et al.*, 1994). PCR amplifications were carried out following the original paper without changing any conditions, using *A. pisum* as a positive control and UltraPure™ DNase/RNase-Free Distilled Water as negative control. The PCR products were amplified and visualised on a 2% agarose gel.

Sequencing was carried out by using the Ligation Sequencing Kit V14 and library preparation was performed following the protocol Ligation sequencing amplicons V14 (SQK-LSK114) for sequencing with MinION technology (Oxford Nanopore Technologies, Oxford, UK). The amplicon pool corresponding to either parasitoids or aphid mummies was sequenced in a MinION R10.4.1 flow cell and a Mk1C at a low-speed setting of 260 bps in order to obtain highly accurate reads with live basecalling enabled in MinKNOW stored as Fastq files. Further data analysis was done using the ONTbarcoder pipeline for barcode calling, demultiplexing and sequence consensus (Srivathsan *et al.*, 2024).

3.3. Laboratory experiments

3.3.1. Black-box type assays

Experimental arenas for the parasitism bioassays were prepared by introducing one fully expanded strawberry leaf into a small deli pot (4.5 cm diameter x 3 cm tall, 2 oz compostable PLA portion cup with lid, Greenware, Vegware Ltd. Edinburgh, UK) with the petiole inserted into 1 oz of water at the bottom of the pot. Thirty potato aphid nymphs of a given clonal line at third or fourth instar were transferred to an arena. Each small pot containing the strawberry leaf and aphid nymphs were placed inside a 720 ml insect pot with mesh snap lid (BugDorm, MegaView Science Co. Ltd, Taichung, Taiwan) (Figure 2.1). The aphids were left to settle on the leaf for at least one hour.

A single female *A. ervi* parasitoid from the age standardized culture, and presumed mated was then introduced into the experimental arena. The parasitoid was observed for 10 minutes to ensure it was actively foraging and attacking aphids, after which the parasitoid was left within the arena for one hour. However, if no activity was observed in the first 10 minutes, the female was replaced.

After parasitoid removal, each arena was left in a controlled environmental room maintained at 18 °C and 60 % RH with a 16:8 h photoperiod for at least 25 days or until all mummified aphids had eclosed. At day six a fresh leaf was introduced into the arena, close enough to the original leaf to allow the still-living aphids to move there if necessary. After day 8, the experimental arenas were checked for mummified aphids daily. Each mummified aphid was transferred at day 11, using soft forceps and a 000-paint brush, to a sealed Eppendorf tube containing a small ball of cotton wool for individual recording.

Figure 2.1. Experimental arena for the black-box type assay.

3.3.2. Experimental set-up for observing aphid-parasitoid interactions

Each experimental arena was set up by pouring, into a 90 mm Petri Dish (Sterilin Ltd/ThermoFisher Scientific, Loughborough, UK), 20 mL of 1% agarose (Agar, Bacteriological N.2, Neogen, Michigan, US) dissolved in sterile distilled water. As the agarose cooled, a single strawberry leaf, cut from a two-week plant, was gently pushed onto the surface of the agarose with the adaxial surface uppermost (Figure 2.2). Once the agarose had cooled, 20 nymphs aged 3-4 instar were carefully transferred to the leaf using a 000 paintbrush and left to settle for at least an hour before the start of the experiment.

A single female *A. ervi* parasitoid from the age standardized culture, presumed mated was then introduced into the experimental arena. The interaction between aphids and parasitoids was observed (notes were taken) and recorded from the moment the parasitoid was introduced. If the parasitoid did not show any sign of searching activity for the first 5 minutes, it was replaced, and the observation re-started. The interactions were recorded for 20 minutes using a Canon EOS 700D Digital SLR (Canon Europa N.V, The Netherlands) with a MACRO lens 0.25 m/0.8 ft (18-55 mm) and the recording saved in a hard drive. Insect behaviours were analysed using a pipeline in the software The Observer XT software (Noldus Information Technology, Wageningen, The Netherlands).

Once 20 minutes elapsed, the parasitoid was removed. After this, each arena was left in a controlled environment room and checked daily.

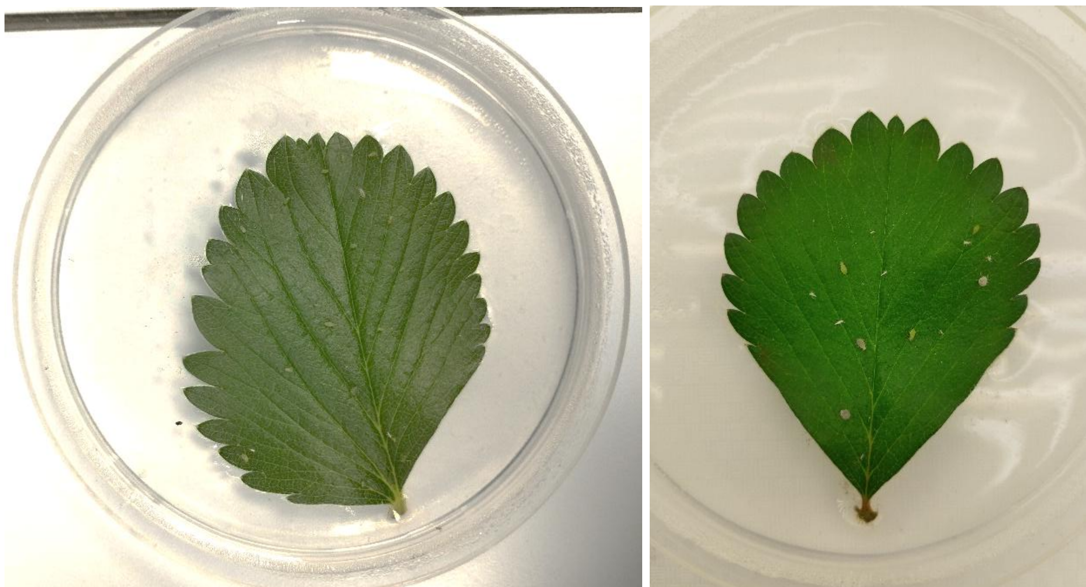


Figure 2.2. On the left the experimental arena for the recording type assay on day 0 and on the right an experimental arena on day 10 (different degrees of aphid mummification can be observed).

3.4. Parasitoids for genetic diversity assessment

Commercial *A. ervi* individuals were acquired from five different biological control suppliers selling the species in the United Kingdom as a crop protection product. The name of the commercial suppliers was anonymised with a number from one to five assigned to each company and kept the same for all experiments. The products were obtained in March 2024 and September 2024 to assess genetic differentiation and genetic diversity, and samples from only three commercial suppliers (1,3 and 5) were obtained in January 2025 for objective 4. Wild *A. ervi* parasitoids were obtained by taking strawberry plants infested with 200 potato aphids from clonal line HAU/01 to two locations considered not to be associated with commercial production of horticultural crops or use of commercial biological control agents (visual check on Google Maps for glasshouses and polytunnels in a 10 km radius from each site), with site number 1 being St Mary's allotment in the city of Leamington Spa in Warwickshire (coordinates: 52°16'48.0"N 1°30'36.0"W) and site number 2 being the Greenhills allotments in the town of Church Stretton in Shropshire (52°31'48.5"N 2°48'46.0"W). Wild parasitoids were collected in March 2024 and September 2024 by leaving aphid infested plants at the sites for 24 hours and then returning the plants with aphids to laboratory conditions (20°C, 60% RH, 16:8 L/D). In the laboratory, plants were contained in a small pop-up meshed cage (30x30x30 cm, Life Unearthed, London, UK) and after 10 days the plants were checked for the presence of mummified aphids, which were transferred individually to 1.5 mL Eppendorf tubes and adult parasitoids recovered if they emerged. Field-caught *A. ervi* parasitoids were collected in March-April 2024 and September 2024 from potato aphid mummies found in four polytunnels at a commercial strawberry production site Littywood Farm Ltd (52°45'59.6"N 2°09'47.9"W). The mummies collected from the field were placed individually in 1.5 mL Eppendorf tubes under laboratory conditions until adult parasitoids emerged. All parasitoids and their respective empty aphid mummies from which they had emerged were stored in 96% ethanol at -20 °C until molecular processing. Parasitoid identity as *A. ervi* was confirmed using morphological characteristics (Starý *et al.*, 2010).

3.5. Field sampling

Sampling site and process

Sampling was carried out at Littywood Farm in Bradley, Staffordshire, United Kingdom (ST18 9DW, Coordinates: 52°45'59.6" N 2°09'43.6"W), which is a soft fruit farm with strawberry, blackberry and cherry crops on their premises. Potato aphids were monitored and sampled over two full growing seasons and one spring (March 2023- April 2025) in four commercial polytunnels with a non-coloured cover of two different sizes (long: 130m x 9m and short: 82m x 9m) containing strawberry plants (*Fragaria* x *ananassa*). The long polytunnels (numbered 1 and 2) were separated from the short ones (numbered 3 and 4) by a ~45 meters strip of uncultivated land left to grow wild (Figure 7.1A). All polytunnels were surrounded by other polytunnels where strawberry crops are grown except polytunnel 4, which is next to a cherry plantation. Polytunnels 1 and 2 were planted with the variety Katrina over the two seasons and polytunnels 3 and 4 were planted with the variety Murano for the 2023 season and Katrina for the 2024 and 2025 season. All plants were grown in Biogrow Growbag Duo Strawberry coco growing media (100 cm x 20 cm x 10 cm, Royal Brinkman, East Yorkshire, UK).

Each polytunnel contained 6 rows of tabletops equally spaced except rows 3-4 in the middle as these were separated by a 2 m gap to allow tractor operations, which facilitated having 3 total sampling tracks (Figure 7.1B). Polytunnels 1 and 2 had 8 equally spaced sampling points along each sampling track while polytunnels 3 and 4 had 5 equally spaced sampling points along their sampling tracks. These sampling points were selected at the beginning of the 2023 season and re-visited each sampling date. For both polytunnel sizes, the sampling points were separated by 16 meters length wise. On each sampling date, the polytunnels were sampled in a systematic way by visiting all the sampling points and checking for potato aphid colonies on 2 strawberry plants at each side of the sampling track (i.e., on each tabletop). A total of 24 sampling points and 96 strawberry plants were checked for the large polytunnels, and a total of 15 sampling points and 60 strawberry plants for the short polytunnels per sampling date. The same plants were re visited on each sampling date and for each one the total number of potato aphids was counted and a representative sample of the aphids from each plant was collected and relocated to the laboratory. The representative sample consisted of approximately 60 % of a plant’s potato aphid population so as not to not disturb the aphid population composition by removing a colony or affecting the parasitoid community by removing all successful parasitoids from the area. All aphid mummies (i.e., dead aphids after parasitisation) observed in each sampling point were collected. Sampling dates were equally spaced throughout each season at every three calendar weeks, so the number of seasonal sampling dates was 12 for 2023 and 2024, and two for the spring of 2025.

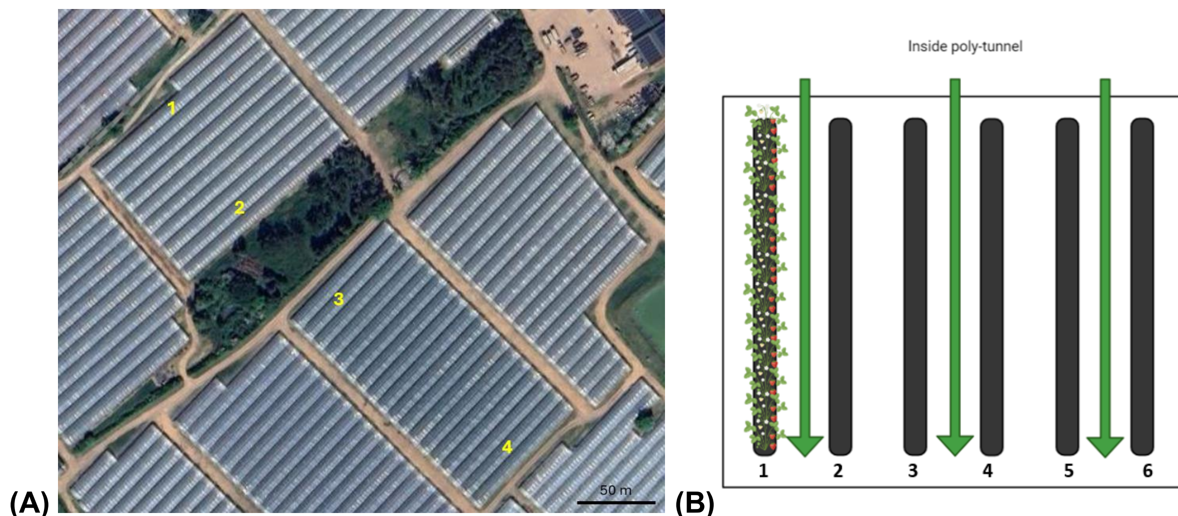


Figure 7.1. **(A)** Satellite picture of the sampling location, yellow numbers indicating the polytunnels used for this study. Image taken from Google Maps. **(B)** A scheme of the inside of the polytunnels, with the tabletops in back and the sampling track represented as green arrows. Polytunnel size varied with a length of 130 meters for polytunnels 1 and 2, and 85 meters for polytunnels 3 and 4.

Proxy for parasitism pressure and parasitoid identity

To estimate the parasitism pressure exerted on potato aphid populations, aphids relocated from the field to the laboratory were counted and maintained in 5 cm x 8 cm x 2 cm Blackman boxes (Blackman, 1974) on their original plant material and separated by sampling point for a period of 15 days or until they showed signs of mummification (i.e., swelling or colour changes). These samples were checked every two days and if mummies were detected then they were separated into individual 1.5 mL Eppendorf tubes (ThermoFisher, UK) until parasitoid emergence. Mummies collected directly from the field were brought into the laboratory and individually maintained in 1.5 mL Eppendorf tubes and monitored daily until parasitoid emergence. Overall, 2962 aphids from season 2023, 2650 aphids from 2024 and 55 aphids from the spring 2025 were monitored for parasitism. The proxy for parasitism pressure was calculated by dividing the number of parasitoids emerged by the total number of potato aphids that were brought into the laboratory (either alive or mummified) for each sampling point and date. In this way, the parasitism proxy could also be summarised by sampling track, polytunnel or in total per sampling date as needed. The parasitism proxy excluded hyperparasitoids, as hyperparasitism pressure was also estimated separately. Any living potato aphids left after the 20-day parasitism monitoring period were preserved in 80 % ethanol at -20 °C until further processing. Any emerged parasitoids were also preserved under the same conditions. Considering the raw results of parasitism pressure, three distinct parasitism periods were

arbitrarily stablished for the two complete seasons as “pre-maximum parasitism”, “maximum parasitism” (when parasitism proportion reaches >10%) and “post- maximum parasitism” for analysis.

Identification of parasitoids to species level for primary parasitoids and at least to genus for hyperparasitoids was done following available morphological keys (Starý *et al.*, 2010; Tomanović *et al.*, 2022).

Macrosiphum euphorbiae genotyping and secondary endosymbiont screening

Six sampling dates per sampling season for 2023 and 2024 were selected for molecular processing and analysis based on the variation of aphid abundance and parasitism pressure over the season, while only the samples collected from the last sampling date in spring 2025 were included as it was the only one where parasitism was recorded before stopping the sampling. A total of 13 sampling dates were processed for aphid holobiont information in this study. For each sampling date, aphids from four sample points per polytunnel were selected at random to give a total of 16 representative aphids per key sampling date (n = 208).

3.6. Statistical analysis

Statistical analyses were carried out using R version 4.4.2 (R Core Team, 2024). In all cases data distributions were checked using the Shapiro-Wilk test while homogeneity of variance was assessed by the Bartlett test. In case the data conformed with normal parameters, the analysis consisted of fitting an analysis of variance (ANOVA) model by using the function “aov” and calculating an analysis of variance table with the function “Anova” of the car package with replicate and block as random factors. Following a significant ANOVA result, pairwise comparisons using Tukey’s HSD post-hoc tests were used to establish which groups were different by using the function “TukeyHSD”. In case the data was found not to be normal, a GLM model with specific distribution depending on data structure was fitted, and an analysis of variance was done using the *car* package and posterior pairwise-comparisons of the means using the package *emmeans* and the function with the same name.

Specifics on the data analysis can be found in the full-length dissertation.

4. Results

4.1. Initial characterisation of *Macrosiphum euphorbiae* clonal lines

Of the 15 clonal lines of potato aphid available for this project, nine genotypes that have not been publicly described before were recorded. Of the nine genotypes, four consisted of multiple clonal lines, and two of aphid clones with different colours. Genotype 14 differed from genotype 16 by a small difference in the size of microsatellite Me9. This small difference was confirmed each time the clonal lines were checked for integrity. Four of the seven secondary endosymbionts that were tested for, were identified in the potato aphid clonal lines collected for this project. *Hamiltonella defensa* was present in four clonal lines, *S. symbiotica* in three*, R. insecticola* in ten and *Rickettsia* sp. in seven. Of the 15 clonal lines, only two did not harbour any of the bacteria tested. Five clonal lines had single infections, five had double infections, and three had triple infections when collected. Triple and double infections were not stable and were lost after a year in culture. All infections with *Rickettsia* sp. and most of the infections with *R. insecticola* were lost, but all infections with *H. defensa* and *S. symbiotica* remained present throughout the duration of the project. None of the clonal lines tested positive for *F. symbiotica*, *Spiroplasma* sp. and *Rickettsiella* sp. These samples tested positive for the 16S rRNA gene but were negative when amplifying the 16S-23S rRNA region, which indicates that no other bacteria but *Buchnera aphidicola* was present at a detectable level.

The proportion of mummified aphids differed significantly between all the clonal lines (χ_2= 821.25, *df*=14, p<0.001) (Figure 3.1). The sex ratio of the wasps that emerged differed significantly between all the clonal lines (χ_2= 138, *df*=14, p<0.001). In terms of wasp development times, males took 13.2 days to develop, and females took 21.3 days, and clonal line had a significant effect on development time for both sexes (χ_2= 85.2, *df*=14, p<0.001).

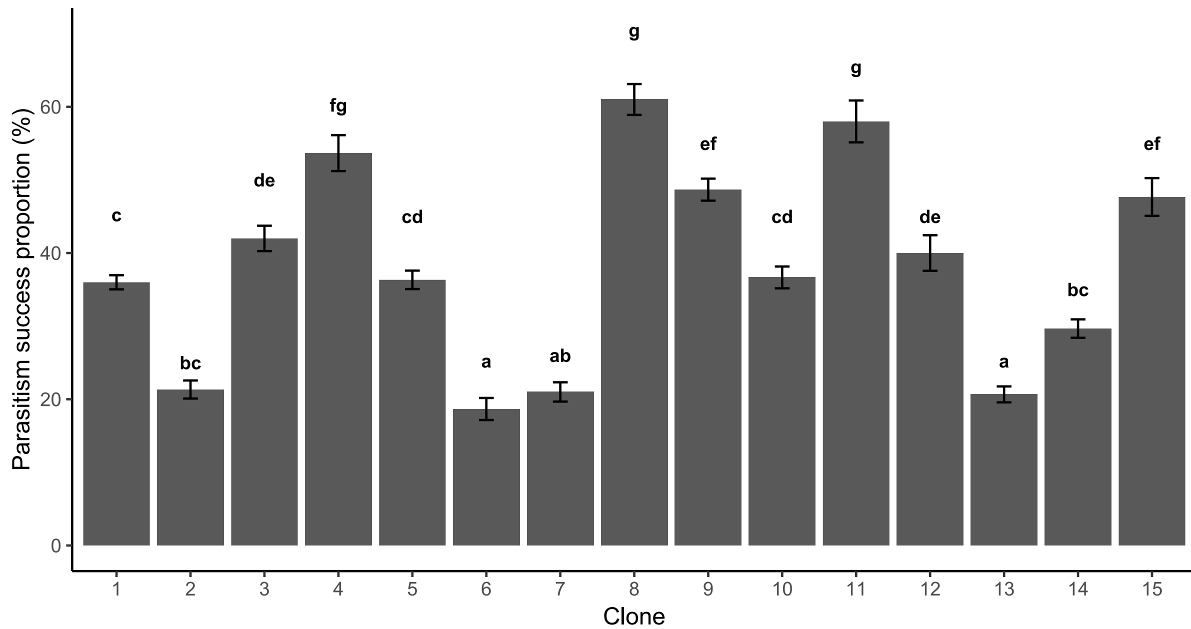


Figure 3.1. Proportion of mummified aphids from each *Macrosiphum euphorbiae* clonal line. Number of replicates was 10. Error bars show ± S.E. whilst bars labelled with different letters differ significantly at the 95% level of confidence (Pairwise comparisons).

4.2. The interplay of aphid genotype and confinement method on potato aphid fitness

Considering the overall fitness of the different potato aphid genotypes, no statistical differences between genotypes were found for the treatment with whole plants (F= 0.30, *df*= 3, P>0.05). However, genotype 11 with a mean of 13.83 points had the highest fitness score when using whole plants covered with a mesh bag followed by genotype 10. On the other hand, genotype fitness varied significantly when using clip cages (F= 2.86, *df*= 3, P<0.05), with genotypes 9 and 10 performing better with a mean of 11.86 and 11.63 points respectively (Figure 4.3).

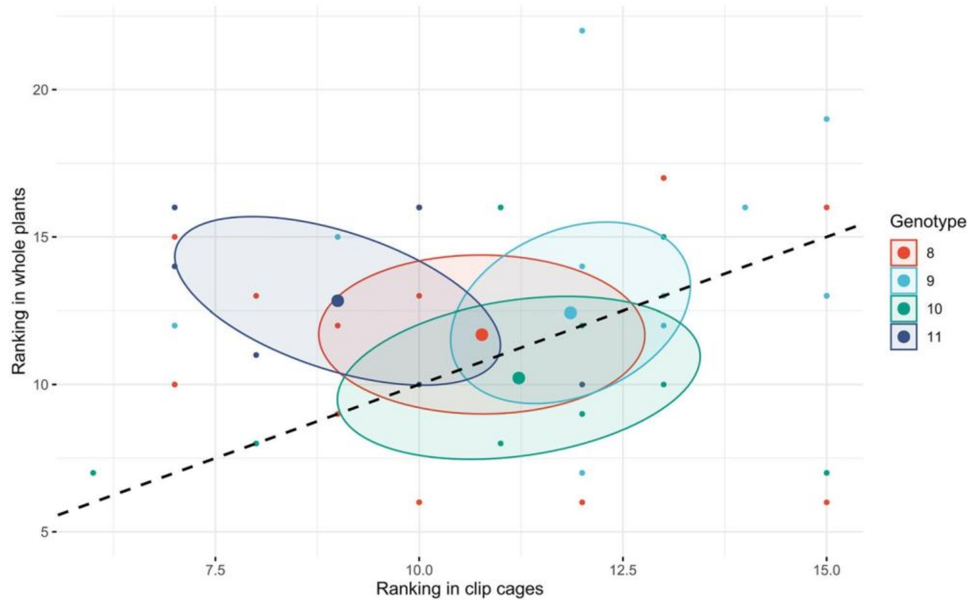


Figure 4.3 The overall fitness ranking of four *Macrosiphum euphorbiae* genotypes when contained on whole plants versus in clip cages. The means for each genotype is represented by a large dot of the same colour. The black dashed line is a 1: 1 line of equivalence.

More results associated with this topic have been published as:

Laura Marcela Martinez-Chavez, Joe M. Roberts, Alison J. Karley, Bethan Shaw, Tom W. Pope. (2024). "The clip cage conundrum: Assessing the interplay of confinement method and aphid genotype in fitness studies", Insect Science, 31(5), pp. 1591-1602.

4.3. The aphid-parasitoid interaction: behaviours and outcomes.

4.3.1. Genotype data set

The PERMANOVA test and the exploratory NMDS showed differences in the overall behavioural responses depending on genotype (F= 2.68, *df* = 2, p= 0.03), while the dispersion test showed no significant differences in dispersion (F=0.357, *df* = 2, p>0.05). The pairwise comparisons, showed that only genotype 9 differed significantly from genotype 14 considering all behaviours (F=3.67, *df* = 1, p=0.035), although this was not supported when corrected for multiple testing (p.adjusted = 0.096). When fitting the *outcomes* variables to the three-dimensional NMDS, neither acceptance proportion (r^2= 0.033, p>0.05) or efficiency (r^2= 0.018, p>0.05) had a significant correlation with the ordination. Parasitism proportion was weakly but significantly associated (r^2 =

0.114, p = 0.040) with NMDS1 (negative association) and NMDS2 (positive association). Thus, this suggested that aphid or parasitoid behavioural variation does not significantly explain any of the *outcomes* variation at the multivariate level.

Of all the *behaviours* variables that significantly contributed to the NMDS ordination, only parasitoid cleaning time and number of cornicle secretion events differed between potato aphid genotypes. Although aphid genotype did not have a direct effect on parasitoid acceptance, parasitism proportion or parasitism efficiency (Figure 5.3).

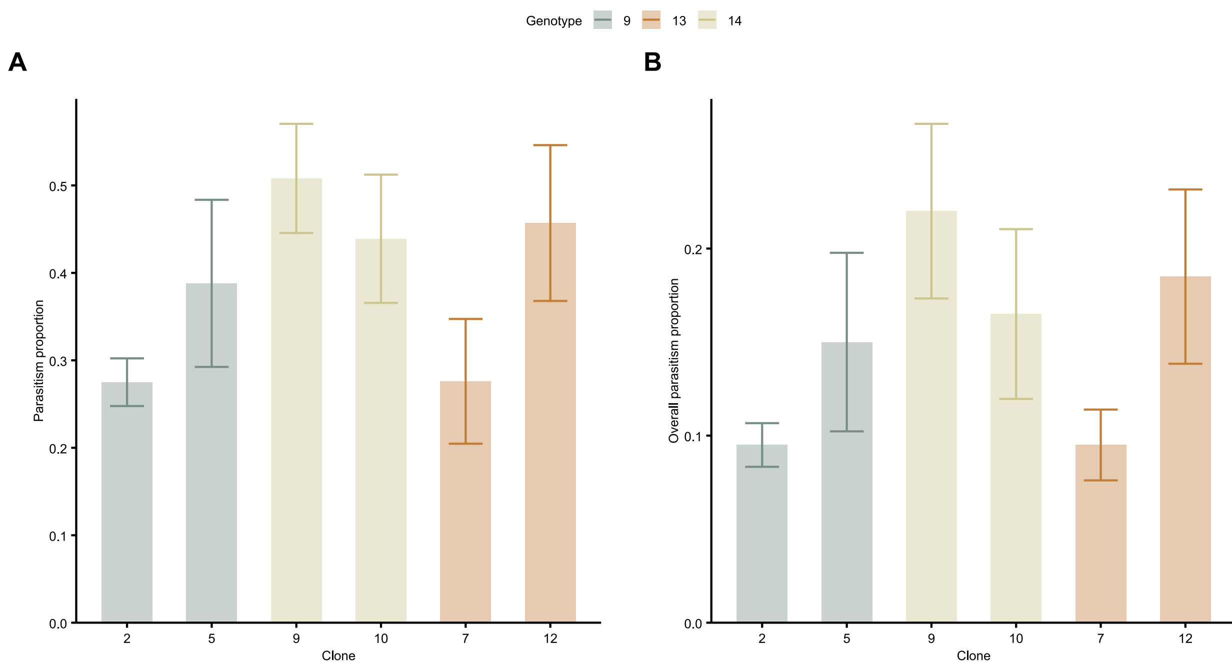


Figure 5.3. The mean (± S.E.) of **(A)** the parasitism proportion and **(B)** the overall parasitism proportion (efficiency) of female parasitoids *Aphidius ervi* when interacting with the different clonal lines of *Macrosiphum euphorbiae.* The clonal lines are coloured by genotype. For each clonal line n=10 replicates. The absence of uppercase or lowercase letters in the graph indicate no differences between aphid genotypes or clonal lines.

4.3.2. Endosymbiont data set

The PERMANOVA test and exploratory NMDS showed differences in the overall behavioural responses depending on endosymbiont infection status (F= 4.35, *df* = 3, p<0.001), while the dispersion test showed no significant differences in dispersion (F=1.164, *df* = 3, p>0.05). The pairwise comparisons, showed that behavioural responses related to aphids that are

uninfected differed from the ones related to aphids infected with *H. defensa* (F=6.311, *df*= 1, p= 0.006, p.adjusted = 0.036) and from aphids infected with *S. symbiotica* (F=7.196, *df*= 1, p= 0.003, p.adjusted = 0.018).

When fitting the *outcomes* variables to the three-dimensional NMDS, parasitism proportion did not have a significant correlation with the ordination (r^2= 0.050, p>0.05). However, acceptance proportion was strongly and significantly associated with the NMDS (r^2 = 0.459, p = 0.001) with the vector's direction NMDS1 = 0.58, NMDS2 = 0.81 while parasitism efficiency was significantly and positively associated with the NMDS (r^2 = 0.222, p = 0.001). This suggest that behavioural variation does significantly explain parasitoid acceptance and efficiency at the multivariate level.

Of all the *behaviours* variables that significantly contributed to the NMDS ordination, only number of parasitoid probes and number of parasitoid ovipositions differed when wasps interacted with potato aphids infected with different endosymbionts. In terms of host acceptance, the clonal line for which the highest acceptance proportion was recorded was HAU/01 with a mean of 48.8 ± 6.5 (S.E.) %, while the lowest was recorded for HAU/07 with a mean of 25.3 ± 4.7 (S.E.) % (Figure 5.6.A). Statistical analysis showed that endosymbiont infection affected parasitoid acceptance proportion (χ_2=17.42, *df*= 3, p<0.001) and clonal line added to the model (χ_2=160.77, *df*= 0, p<0.001). The post-hoc analysis showed that all infections were statistically different except the pairs *H. defensa* – *S. symbiotica* and *R. insecticola* – uninfected aphids. Post-hoc analysis did not support pairwise differences between clonal lines within endosymbiont infections. In the case of parasitism success, clonal line HAU/03 recorded the highest value with a mean of 57.1 ± 8.5 (S.E.) %, while clonal line HAU/02 recorded the lowest value with a mean of 26.3 ± 5.6 (S.E.) % (Figure 5.6.B). Statistical analysis showed that endosymbiont infections had a significant effect on parasitism proportion (F= 4.875, *df*= 3, p<0.001) but not clonal line within endosymbiont infection status (F=1.747, *df*= 4, p>0.05). Post-hoc analysis showed the same differences as those described for acceptance proportion. Finally, for parasitism efficiency clonal line HAU/14 recorded the highest value with a mean of 33.5 ± 5.7 (S.E.) %, while clonal line HAU/07 recorded the lowest value with a mean of 13.0 ± 3.1 (S.E.) % (Figure 5.6.C). The statistical analysis showed that endosymbiont infection status (F= 8.31, *df*= 3, p<0.001) affected the outcome but clonal line within endosymbiont infection status did not (F=0.318, *df*= 4, p>0.05). Post-hoc analysis showed the same differences as those described for acceptance proportion and parasitism success.

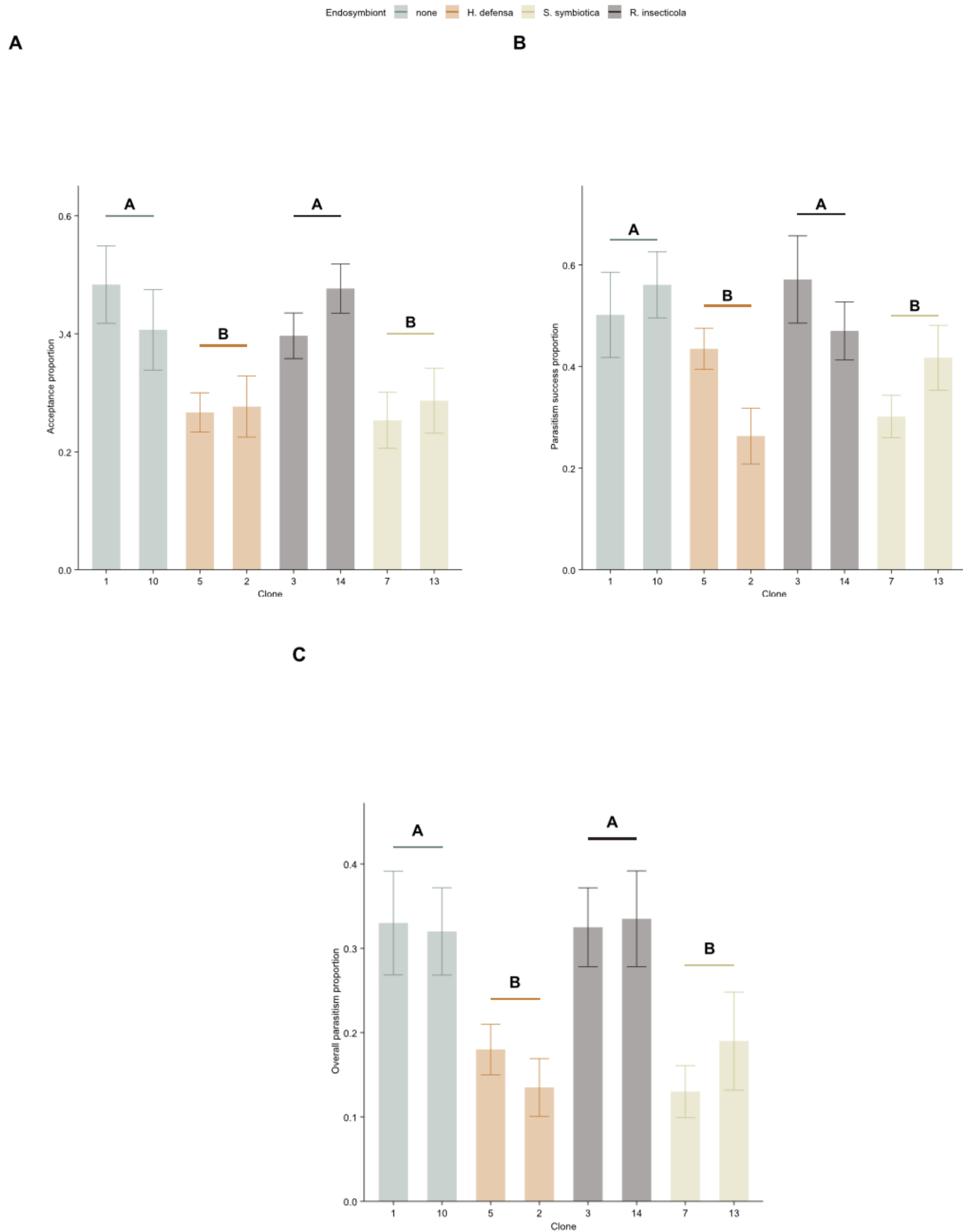


Figure 5.6. The mean (± S.E.) of **(A)** the acceptance proportion, **(B)** the parasitism proportion and **(C)** the overall parasitism proportion (efficiency) of female parasitoids *A. ervi* when interacting with the different clonal lines of *Macrosiphum euphorbiae.* The clonal lines are coloured by endosymbiont infection status. For each clonal line n=10 replicates. Different bold uppercase letters indicate significant differences between genotypes.

4.4. *Aphidius ervi* genetic diversity and parasitism efficiency against the potato aphid

4.4.1. Barcoding results (Cytochrome-oxidase -subunit-I gene)

Samples barcoded for this study were classified under 13 different COI haplotypes. All haplotypes from this study were similar (>99% sequence identity) to publicly available COI sequences for *A. ervi* in GenBank, which agreed with the morphological identification. Among the COI haplotypes, 29% of the individuals were pooled under haplotype H12, 12% under haplotype H04 and 8% under haplotype H03. The most differentiated haplotypes (H07, H10, H13), based on the number of mutations, included 15% of all individuals.

Parasitoids obtained from commercial suppliers were not structured rigidly by their source, with parasitoids originating from different commercial companies sharing haplotypes. About 47% of commercial *A. ervi* belonged to haplotype H12, which was not present amongst non-commercial sources, as well as 6 other haplotypes in minor proportions (H03, H05, H06, H07, H08, H10). Only H08 was detected both in commercial suppliers and non-commercial sources of insects, in this case coming from the field population. Parasitoids from non-commercial sources belonged to seven haplotypes, with haplotype H02 only represented amongst field samples, and haplotype H09 and H13 only represented amongst naturally occurring ("wild") samples. All other haplotypes that were detected in each non-commercial source were also not detected in commercial sources, except H08 which, as described above, was detected amongst only two commercial supplier sources (Figure 6.1).

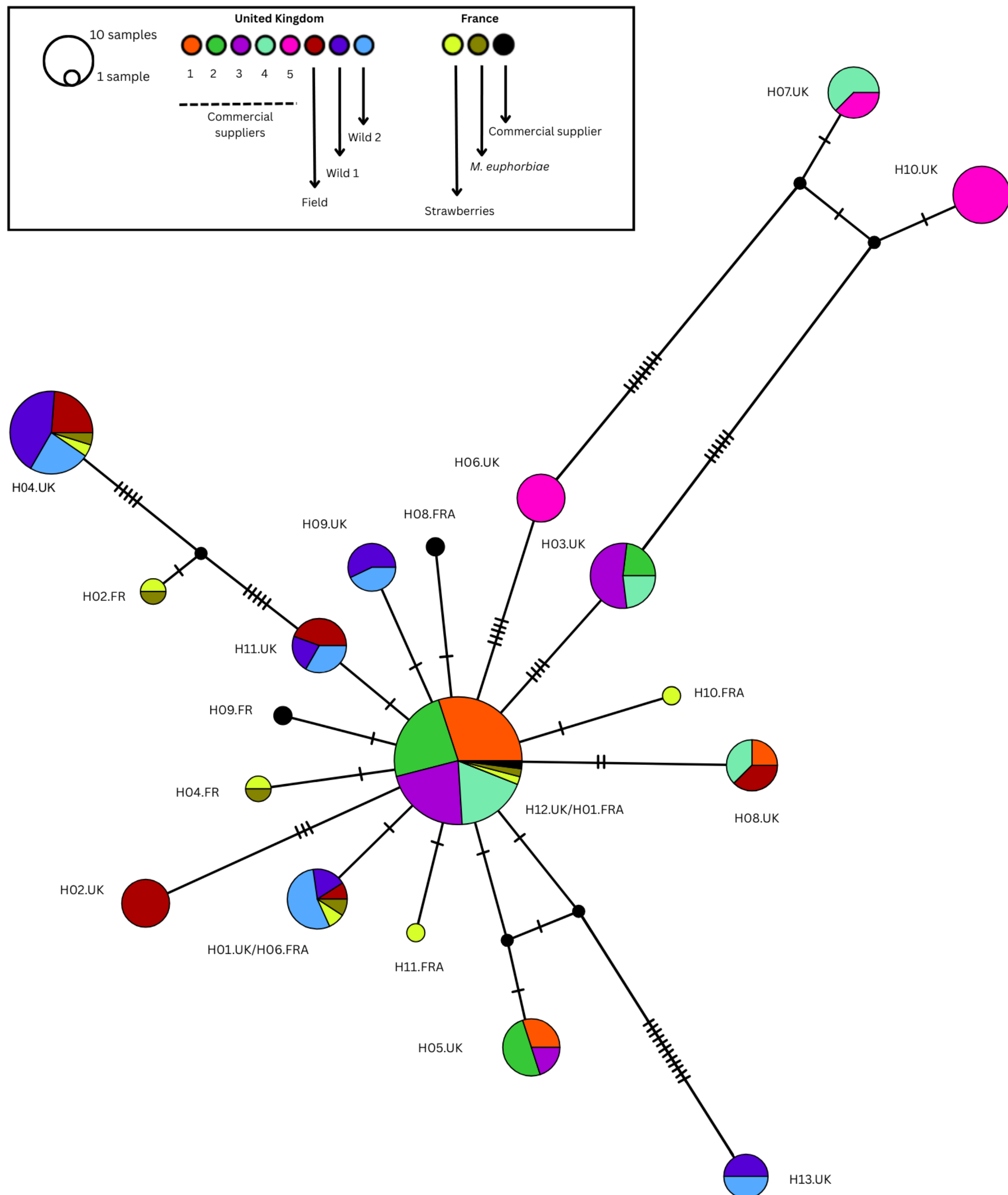


Figure 6.1. Median This is Joining Network of *Aphidius ervi* individuals based on COI sequences obtained from this study and samples from France (Postic *et al.*, 2021). The hatch marks indicate individual mutations between haplotypes. Number of samples per UK source = 20.

4.4.2. Genetic diversity based on microsatellite information

The number of effective alleles (**Ne**) ranged from 1.7 to 3.3 depending on the source. **Ne** was lower in commercial parasitoids (2.6) in comparison to *A. ervi* individuals obtained from the wild locations (3.7) but was similar to the field source (2.6). Individually, commercial parasitoids had a lower **Ne** compared with field-collected parasitoids except for commercial supplier 5 which had a higher **Ne** estimate (Table 6.3).

The observed heterozygosity (**Ho**) ranged from 0.35 to 0.77 in the different sources. **Ho** was lower in commercially available individuals (0.48) in comparison with both field (0.55) and "wild" *A. ervi* (0.72). However, **Ho** standard errors overlapped between commercial *A. ervi* sources and non-commercial sources. Unbiased expected heterozygosity (**uHe**) was higher than **Ho** for all sources except commercial supplier numbers 4 and 5, and wild location 1. Inbreeding was only detected for *A. ervi* originating from commercial suppliers (F_{is}=0.20) but not from field (F_{is}=0.04) or wild sources (F_{is}=0.00). Individually, inbreeding was only detected for commercial suppliers 2 (F_{is}=0.22) and 3 (F_{is}=0.28), and moderate outbreeding was detected for wild location 1 (F_{is}=-0.13).

When comparing the three main groups of sources of *A. ervi*, the differentiation indices between commercial, field and wild populations was low (F_{st} value<0.05) indicating minimal genetic differentiation between these three main groups of sources. Pairwise comparisons showed different levels of genetic differentiation, with the greatest difference between commercial suppliers 2 and 4 (F_{st} value=0.231), and the lowest between wild locations 1 and 2 (F_{st} value=0.008). However, pairwise comparisons between commercial supplier 5 and samples from the field, and between wild locations 1 and 2, were not statistically supported (probability > 0.05).

Table 6.3. Measurements of genetic variability of *Aphidius ervi* using six microsatellite loci for each source. Number of samples per source = 10.

Source		**Number of effective alleles (Ne) ± SE**	**Observed heterozygosity (Ho) ± SE**	**Unbiased expected heterozygosity (uHe) ± SE**	**Fixation index (F_{IS}) ± SE**
Commercial supplier	**1**	1.7 ± 0.2	0.35 ± 0.12	0.37 ± 0.09	0.00 ± 0.19
	2	2.1 ± 0.2	0.40 ± 0.11	0.50 ± 0.07	0.22 ± 0.22
	3	2.6 ± 0.3	0.42 ± 0.01	0.60 ± 0.07	0.28 ± 0.05
	4	2.2 ± 0.4	0.53 ± 0.18	0.46 ± 0.12	-0.09 ± 0.16
	5	3.0 ± 0.6	0.68 ± 0.14	0.62 ± 0.09	-0.07 ± 0.12
All commercial		**2.6 ± 0.3**	**0.48 ± 0.06**	**0.60 ± 0.04**	**0.20 ± 0.06**
Field		2.6 ± 0.3	0.55 ± 0.09	0.61 ± 0.06	0.04 ± 0.13

Wild location 1	3.3 ± 0.3	0.77 ± 0.04	0.72 ± 0.03	-0.13 ± 0.09
Wild location 2	3.1 ± 0.4	0.67 ± 0.17	0.66 ± 0.08	0.04 ± 0.19
All wild	**3.7 ± 0.5**	**0.72 ± 0.09**	**0.72 ± 0.04**	**0.00 ± 0.10**

4.4.3. Efficiency of different parasitoid populations against an array of clonal lines of *M. euphorbiae*

Using a black-box assay as a first approach the levels of parasitism success between the different commercial suppliers providing *A. ervi* in the UK when interacting with different clonal lines of potato aphids was assessed. For control purposes an additional host species was added, *A. pisum*, and two populations of *Praon volucre*. The highest total parasitism success proportion was recorded for the pea aphid with a mean of 63.7%, while the lowest was recorded for potato aphid clonal line HAU/02 with a mean of 25.3%. In terms of parasitoids, the population with the best performance across all aphid lines was commercial supplier 5 with a mean of 53.1% parasitism, and the one with the worst performance was commercial supplier 1 with a mean of 22.9% (Figure 6.4).

The statistical analysis showed that both aphid line (χ_2= 629, *df*=4, p<0.001) and parasitoid population (χ_2= 253, *df*=7, p<0.001) had a significant effect on total parasitism success. In addition, the interaction between aphid line and parasitoid population was also statistically significant (χ_2= 212, *df*=28, p<0.001). The post-hoc analysis showed that total parasitism success differed strongly among aphid lines-parasitoid population combinations with some signs of host specificity. In general, all parasitoid populations had a higher total parasitism success in pea aphid, followed by *A. ervi* from commercial supplier 5 against potato aphid clonal lines HAU/05 and HAU/10. Lower total parasitism success was recorded for aphid clonal lines HAU/02 and HAU/07 for almost all parasitoid populations, except *Praon volucre* against HAU/07, and *A. ervi* from commercial supplier 5 against HAU/02.

For aphid line only, the post hoc analysis showed that all aphid lines differed significantly between each other in their total parasitism success. When comparing parasitoid populations, *A. ervi* from commercial supplier 1 and 5 differed significantly between each other and from the other populations in their total parasitism success. On the other hand, *A. ervi* from commercial suppliers 2, 3, 4 and *A. ervi* from the laboratory culture did not show statistically different total parasitism success proportions. The same was true for *A. ervi* from commercial suppliers 3, 4 and both populations of *P. volucre*.

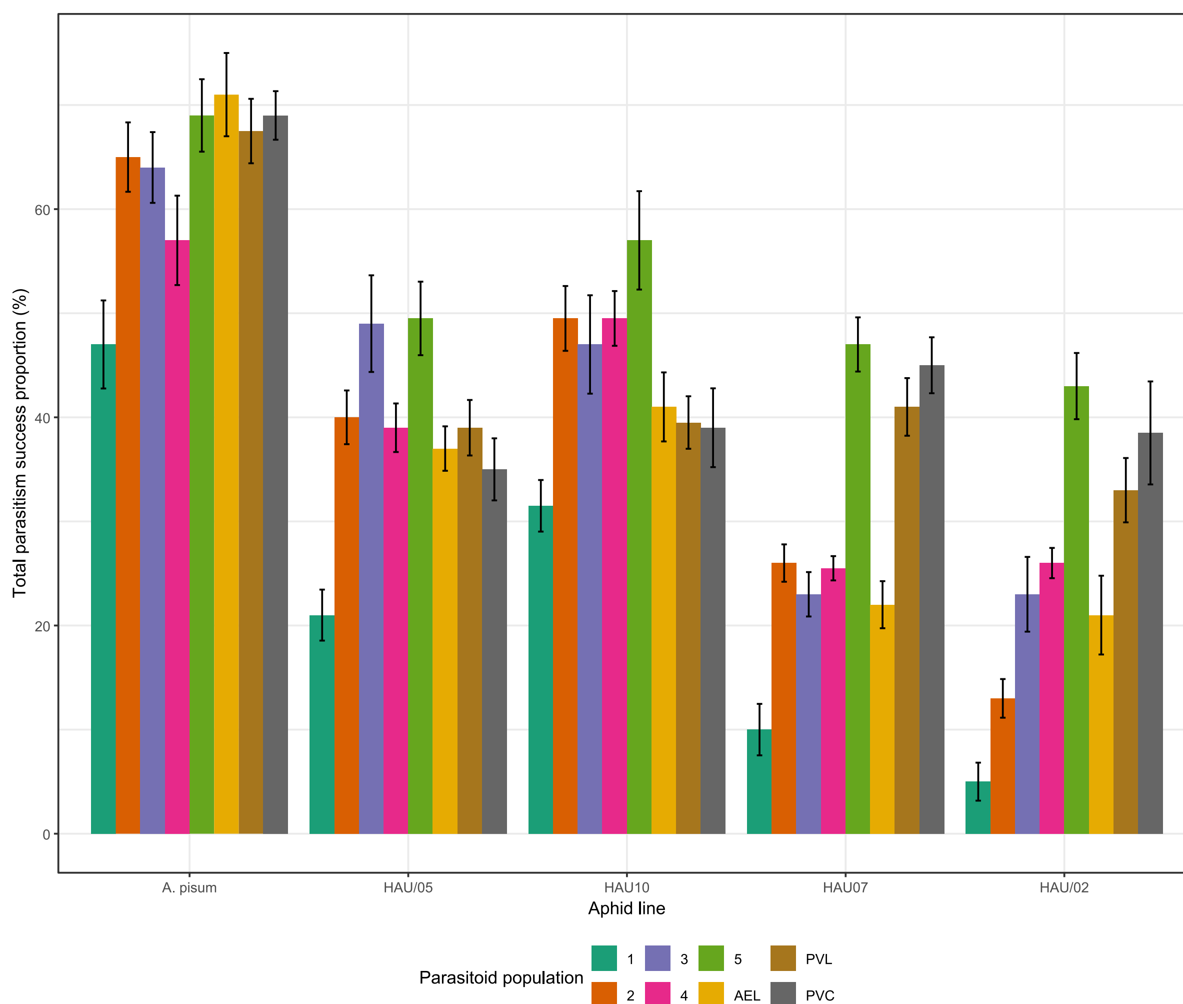


Figure 6.4. Total parasitism success (percentage of aphids parasitised) of the different parasitoid populations used in the black-box type assay. Parasitoid lines are filled with different colours. Parasitoid populations are described with numbers for commercial suppliers of *Aphidius ervi*, and letters for *A. ervi* population from the laboratory (AEL), *Praon volucre* population from the laboratory (PVL) and *P. volucre* from a commercial supplier (PVC). 10 replicates per combination of aphid line-parasitoid population. Bars represent standard error (S.E.).

4.5. Field sampling

4.5.1. Potato aphid characterisation

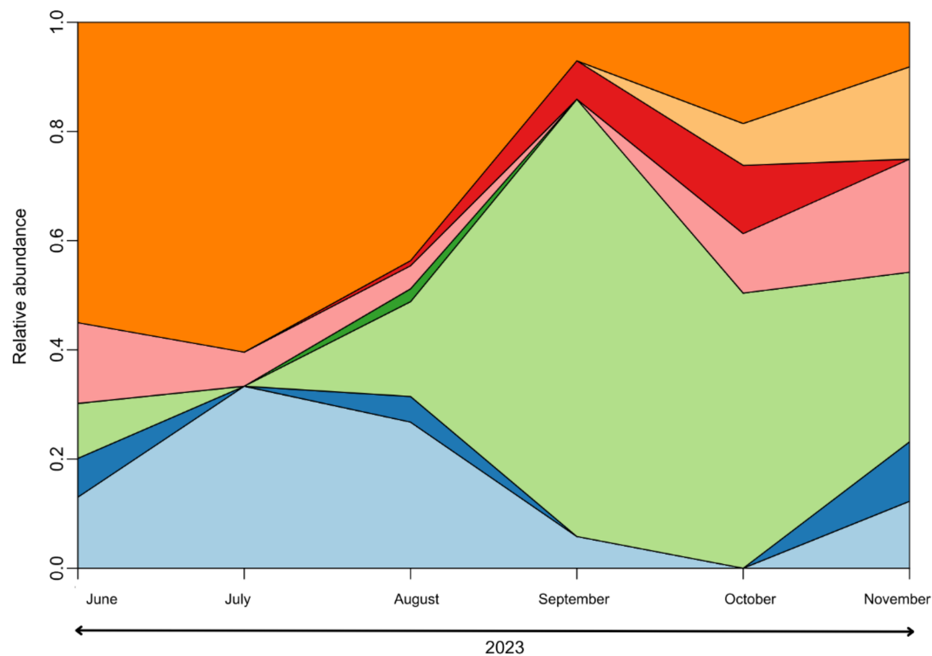
A total of 65 potato aphid genotypes were recorded over the two growing seasons based on four microsatellite loci. Eight potato aphid genotypes were found at a higher prevalence with abundances over 50 individuals (H, I, J, K, N, F1, J2 and K1) with the maximum recorded for genotype J with 499 individuals (20 %).

The endosymbiont results were dominated by the not-infected status with any of the three main endosymbionts (25.0 %) and by infections with *R. insecticola* (24.0 %). Infection status by potato aphid genotypes also varied, with some genotypes being exclusively associated with one single infection (e.g., genotypes T and F2), with none of the main endosymbionts (e.g., genotypes W1 and D), with double infections (e.g., genotypes W and K2) or with triple infections (e.g., genotypes Z and L2). However, considering the main eight genotypes, all of them were found to present multiple infection statuses. For genotypes J, I and N the most prevalent infection status was *R. insecticola* (44 %; 62 % and 87 % respectively). For genotype H the most prevalent infection was with *S. symbiotica* (46 %), and for genotype K with *H. defensa* (28 %). Genotype K2 was mostly double infected with *S. symbiotica* and *R. insecticola* (43%) and genotype J2 was mostly tripled infected (72 %).

In terms of temporal variation of the presence of potato aphid genotypes over the study period, the only genotype present in all sampling dates was genotype J. However, other genotypes also had interesting temporal trends, for example genotype F1, was first recorded at the end of the 2023 season and was present throughout most the 2024 records. Genotype H was also recorded in 6 different collection dates between both seasons, and genotype K2 was first recorded at the end of 2024 but was present again in the spring of 2025. These patterns are consistent with the perception that some genotypes are more commonly present on strawberry plants and that they could also be overwintering. In terms of genotype prevalence, genotypes J and I were particularly prevalent in the summer of 2023, and genotype J was again prevalent in the autumn of the same year. For 2024, the more prevalent genotype was genotype F1 at the end of the summer, nevertheless genotype J also had a moderate prevalence in over the same period. For the spring of 2025, genotypes J and K2 were the most prevalent.

All aphids that were genotyped were also screened for the main facultative endosymbionts that have been linked to parasitoid resistance in aphids using diagnostic PCRs. Considering the abundance of infection statuses in the overall data set, 49 % of samples were positive for *R. insecticola,* 19 % were infected with *H. defensa*, 19 % were infected with *S. symbiotica* and 25 % of the samples were free of the three main endosymbionts. For 2023, 70 % of the aphids were free of endosymbionts in June, however that proportion reduced to around 10 % by September when 80 % of aphids were infected with *R. insecticola* (Figure 7.5). The peak of infections with *R. insecticola* was followed by an increase of other infection statuses in October, although single infections with *R. insecticola* remained prevalent comprising around 30 % of the abundance towards the end of the season. Infections with *H. defensa* reached a maximum in July with a relative abundance of 23 %. There was a relatively small number of *S. symbiotica* infections recorded in this season, with single infections only reaching a maximum of 7 % in November (Figure 7.5).

For 2024, approximately 20 % of aphids were free of endosymbionts in June, however that proportion increased to 42 % of the samples in July. By September, again, the proportion of endosymbiont free samples was reduced to 10 % and infections with *R. insecticola* (50%) and double infections with *S. symbiotica* and *R. insecticola* (25%) were prevalent (Figure 7.5). The peak of infections with *R. insecticola* reduced after the summer and was followed by an increase of aphids with an array of infections in October such as single infections with *H. defensa,* double infections with *S. symbiotica* and *R. insecticola* and triple infections. Infections with *H. defensa* reached a maximum in August with a relative abundance of 20 % and reached the same level in October after a reduction in September. Contrary to the 2023 season, the abundance of *S. symbiotica* peaked at around 30 % in August but then crashed to 0% for the rest of the season. For the spring of 2025, 58 % of samples were free of endosymbionts but approximately 20 % were positive for *S. symbiotica* (Figure 7.5).



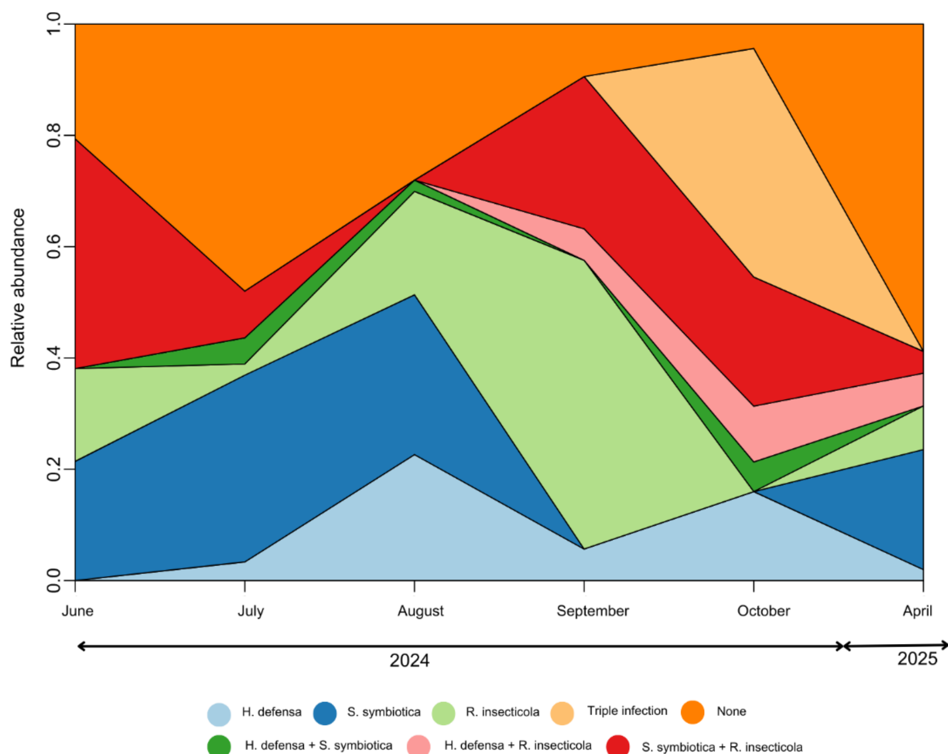


Figure 7.5. Relative abundance of the potato aphid infection statuses tested for over the study period.

4.5.2. Parasitoid characterisation

In terms of primary parasitoid species attacking potato aphids on strawberry plants, the specimens were obtained from either already mummified aphids collected directly from the field or from the representative samples of alive aphids that were brought to the laboratory and that mummified under controlled conditions. Most of the individuals recovered were identified as *Aphidius ervi* (67 %) and *Praon volucre* (26 %).

In terms of the variation of parasitoid species over time, both growing seasons showed overall similar trends. In 2023, 82 % of the parasitoids were identified as *A. ervi* for June, however the proportion of *P. volucre* increased over the summer reaching a maximum of 50% by September. By the end of the season, the prevalence of *A. ervi* increased reaching again pre summer levels, and *P. volucre* abundance fell to 20 %. Concerning other primary parasitoid species (*A. matricariae, A. colemani, P. volucre* and *A. abdominalis*), their relative abundance remained stable from June to September accounting for ~ 10 % of the parasitoid community, but no individuals were recovered after October and until the end of the season (Figure 7.6). For 2024, 100% of the parasitoids were identified as *A. ervi* in June, however the proportion of *P. volucre* increased again over the summer reaching a maximum of 75 % by September. By the end of the season, the prevalence of *A. ervi*

increased reaching a prevalence of 80 %, and *P. volucre* abundance declined to 0 %. The relative abundance of the other primary parasitoids slowly increased over the summer and reached their maximum in October accounting for around 18 % of the parasitoid community. In spring of 2025, only *A. ervi* individuals were recovered (Figure 7.6).

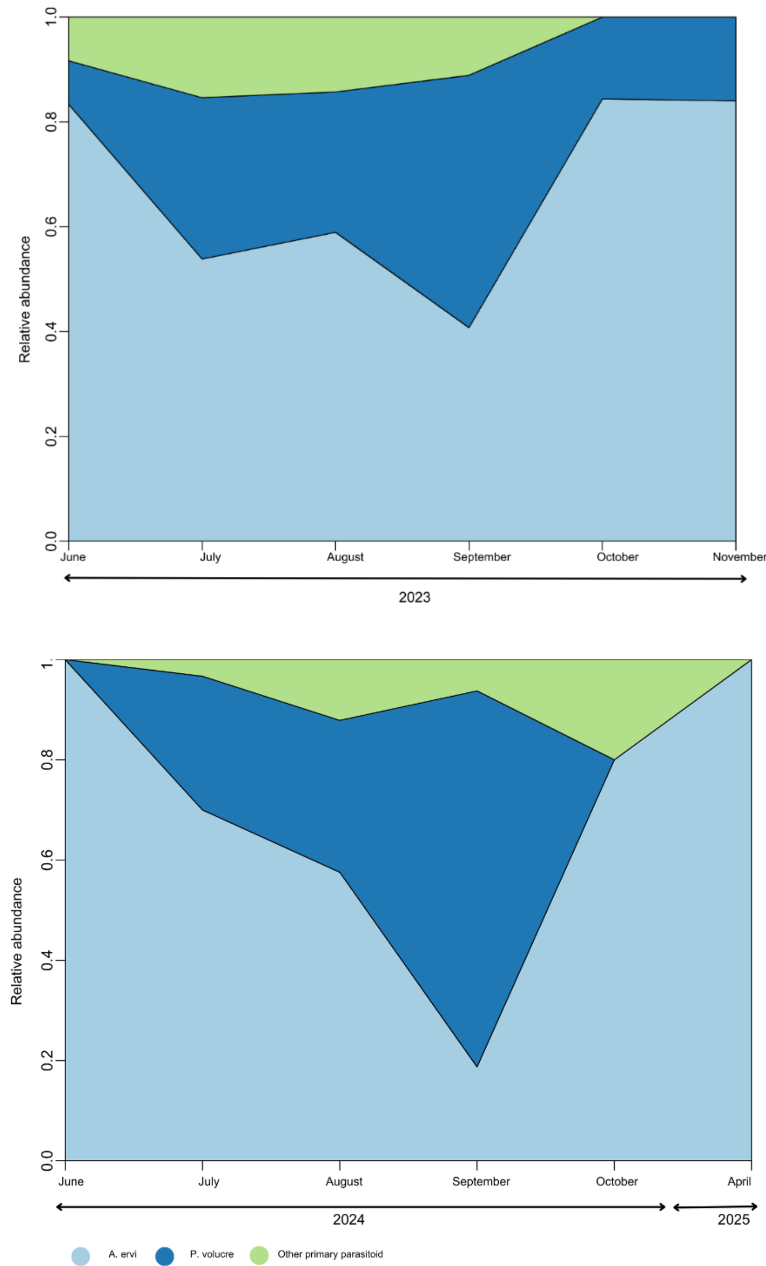


Figure 7.6. Relative abundance of the primary parasitoids attacking potato aphids over the study period.

4.5.3. Link between potato aphid genotypes, endosymbionts and parasitism pressure

In order to test whether there is an association between genotypes, endosymbionts and parasitism pressure the focus was on the results of two-way interactions between any of them, or the three-way interaction between all of them. Analysis showed a significant two-way interaction between the frequency of potato aphid genotypes and of the aphids infected with a secondary endosymbiont (χ_2 = 12.9, *df*= 6, p=0.044) and a marginally significant two-way interaction between the frequency of the infected aphids and of parasitism pressure (χ_2 = 3.74, *df*= 1, p=0.05). When analysing individuals infected with only *R. insecticola*, a significant two-way interaction between the frequency of potato aphid genotypes and of the infected aphids with that specific endosymbiont (χ_2 = 13.7, *df*= 6, p<0.05) was identified. However, when considering this is for the infection with *H. defensa* no significant two-way interactions were found and a marginal significance was reported for the three-way interaction (χ_2 = 5.7, *df*= 2, p=0.05).

When only the parasitism due to *A. ervi* was considered, analysis showed a significant two-way interaction between the frequency of potato aphid genotypes and of the aphids infected with a secondary endosymbionts (χ_2 = 12.4, df= 6, p<0.05) and a marginally significant two-way interaction between the frequency of the infected aphids and of parasitism (χ_2 = 3.74, df= 1, p=0.05). When testing for specific endosymbionts, the only significant two-way interaction was recorded between the frequency of potato aphid genotypes and of the infection with *R. insecticola* (χ_2 = 13.7, df= 6, p<0.05). In the case of the other main parasitoid, *P. volucre*, none of the interactions were significant either considering all endosymbiont infections or specific bacteria.

5. Discussion

5.1. Initial characterisation of *Macrosiphum euphorbiae* clonal lines

All the genotypes described in this study have not been described in previous work (Clarke *et al.*, 2017; Whitehead, 2019). This might be related to differences in the plant host previously sampled. In the case of (Clarke, 2013), only potato crops were included and this was also the host plant sampled in commercial crops and allotments by (Whitehead, 2019). Although the potato aphid is a polyphagous species, it has been reported that this species can show strong preference for certain plant species of the genus *Solanum* (Srinivasan, 2011). Adaptation of insects to different plant species can lead to population genetic differentiation and host preferences that results in the development of biotypes, host races and sub-species (Birkle and Douglas, 1999; Ferrari *et al.*, 2006). Biotypes have been described for multiple aphid species such as the pea aphid, the raspberry aphid and the English grain aphid (Birch *et al.*, 2002; Ferrari *et al.*, 2006; Wang *et al.*, 2019), but has not yet been described for the potato aphid. Nevertheless, strong genetic differentiation, based on microsatellite markers, between host plants has been also described for

A. gossypii (Charaabi *et al.*, 2008). Thus, the new genotypes reported here might indicate that certain populations of *M. euphorbiae* are associated with certain plant hosts, although more research is needed to establish differences in terms of fitness and behaviour.

This study identified single and multiple secondary endosymbiont infections in the potato aphid. Infections of the potato aphid with bacteria such as *H. defensa*, *R. insecticola* and *Rickettsia* have been described before for this species (Moran *et al.*, 2005; Francis *et al.*, 2010; Clarke, 2013; Yang *et al.*, 2023). On the other hand, the infection with *S. symbiotica* is novel for the potato aphid. *Serratia symbiotica* has been described infecting other aphid species, such as the pea aphid, in which it can play a role in terms of high temperature resistance, parasitoid resistance and protection against predators (Guo *et al.*, 2017). The characterisation of the secondary endosymbiont diversity was done by testing for the main facultative bacteria that have been previously described using diagnostic PCR targeting 16S rDNA. Thus, is still possible that other endosymbiotic species could exist in these potato aphid clonal lines but were not characterised. Other technologies, such as DNA metabarcoding, could be useful to elucidate other possible bacterial infections.

In terms of sex-ratio, the only clonal lines with sex-ratios significantly higher than 1, and thus producing more males than females, were HAU/02, HAU/05, HAU/07 and HAU/13. These clonal lines belonged to three different genotypes with HAU/02 and HAU/05 sharing the same genetic background; in addition, they all harboured single or multiple infections with secondary endosymbionts. Different factors can influence offspring sex ratio decisions in female parasitic wasps, including herbivore host factors, plant host factors, parent health and female previous experience conditions and environmental characteristics (King, 1987). One of the most discussed reasons why biased sex ratios can happen is the haplodiploid sex determination system in the sub-family Aphidiinae allows female wasps to select the proportion of the eggs that are fertilized and therefore developed as females, therefore influencing the sex ratio of the progeny (Heimpel and De Boer, 2008). It was noticed that the same clonal lines with male biased sex ratios, also had longer development times for both males and females. The maximum developmental time was recorded for the clonal line HAU/02 with a mean of 15.1 days for males and 23.1 days for females. Development times of *A. ervi* are typically described between 13-15 days at 20 °C, however this can vary depending on aphid host species, aphid size and host plant species and quality (Sequeira and Mackauer, 1992). Differences in development times between sexes of the same parasitoid species reared on the same host have been linked to limitations in the nutrition available to the egg and larvae leading to more time needed to achieve the specific mass gain for each sex, which usually is higher for females.

5.2. The interplay of aphid genotype and confinement method on potato aphid fitness

Aphid clonal/genotypic differences in fitness were observed under each containment treatment. Although aphid facultative symbionts have been reported to affect aphid fitness (Kaech, Jud and Vorburger, 2022; Liu *et al.*, 2023), there was insufficient replication of clonal lines with different symbiont infections to detect any effects on potato aphid fitness in this study. However, genotypic/clonal variability in aphid fitness was particularly pronounced when using clip cages. This might be explained by clonal/genotypic differences in the aphid response to plant/leaf stress status (Srisakrapikoop, Pirie and Fellowes, 2021) or the ability to exploit a specific feeding site. Although there is no information available on either genotypic or endosymbiont influence on feeding site preference for any aphids, it could be hypothesized that if feeding sites differ physically and chemically, and the insects are restricted to a specific part of the plant (i.e., the leaf in the case of clip cages), intraspecific variation in aphid preference could greatly impact aphid fecundity and body mass as observed in this study. Another possible explanation is that potato aphid genotypes may differ in their propensity to react to external stimuli, as has been shown for the presence of natural enemies in pea aphids (*A. pisum*) (Braendle and Weisser, 2001; Muratori, Rouyar and Hance, 2014). For potato aphid, typical defence behaviours centre around cessation of feeding followed by walking away (Humphreys, Ruxton and Karley, 2021a), but no information on clonal variation in defensive behaviours has been reported to date. As opening and closing clip cages can result in excessive leaf movement and vibrations detectable by an aphid as sensory information related to the presence of predators or parasitoids (Nelson, 2007), the use of clip cages could cause feeding cessation and could also trigger energetically costly defensive behaviours like dropping or producing alarm pheromones (Harrison and Preisser, 2016). Vibrations have been linked to a higher probability of an aphid withdrawing its stylet and dropping from the plant (Gish, 2021), which can have an impact on aphid fitness as finding and accepting another feeding site is energetically costly (Nelson, 2007). This can be exacerbated if the disturbance is repeated almost daily like in the case of experiments using clip cages. However, it is important to note that a limitation of this study is that due to the experimental design, it is not possible to tease apart the physical disturbance from checking the clip cages and possible damage to plant tissue from the spatiotemporal confinement of the aphid to a single part of the host plant. This disturbance might interact with genotypic variation in physiological or biochemical traits. Aphid salivary effector proteins, for example, have been described to vary in their fitness effect on aphids by modulating reproduction (Elzinga, De Vos and Jander, 2014); little is known, however, about intraspecific variation in these proteins in the potato aphid and any effects on the aphid-plant interaction (Jonckheere *et al.*, 2016). Other genes thought to relate to aphid sensory functions and detoxification pathways have been shown to vary in representation between potato aphid

genotypes, but their functional role remains to be confirmed (Whitehead, 2019). On the other hand, aphids maintain relationships with secondary endosymbionts that can confer fitness benefits or costs to themselves. For example, the presence of *H. defensa* has been proven to increase the fitness of the Indian grain aphid (*Sitobion miscanthi* Takahashi), but the presence of both *H. defensa* and *R. insecticola* leads to significant fitness costs in corn leaf aphid (Liu *et al.*, 2023). Therefore, clonal fitness variation in response to confinement method may have significant repercussions in studies using clip cages, especially if the populations are not characterized (genotyped and screened for endosymbionts) or conclusions are drawn from a single aphid clonal background.

5.3. The aphid-parasitoid interaction: behaviours and outcomes

The results for both experiments indicate that the behavioural profiles varied depending on endosymbiont infection status with a significant separation of behaviours associated with non-infected aphids and those infected with *H. defensa* or *S. symbiotica.* Potato aphid genotype, however, did not significantly affect the behavioural profile of either potato aphid or its counterpart *A. ervi.* The aphid-parasitoid interaction is a counter-adaptative relationship in which adaptative strategies from both sides are in constant exhibition and evolution. Most of the research in this topic has been focused on physiological adaptations, which from the aphid perspective take the form of intrinsic or endosymbiont-based resistance and from the parasitoid perspective as virulence developments in response to aphid resistance (Le Ralec *et al.*, 2010). However, here the adaptative strategies exhibited by aphids and parasitoids before oviposition happens and how they can also be shaped by aphid holobiont variation were investigated for the first time.

The influence of secondary endosymbionts in insect behaviour has been also largely recognised. The most (in)famous example is the bacteria *Wolbachia* which can manipulate different aspects of their host biology, including its reproduction (Lewis and Lizé, 2015). Other secondary endosymbionts such as *H. defensa* have been linked to a reduction in defensive behaviours when attacked by parasitoids compared with their uninfected counterparts, as an energy-saving and redundancy strategy as this endosymbiont can also provide physiological resistance (Dion *et al.*, 2011; Polin, Simon and Outreman, 2014; Martinez *et al.*, 2018). The complicated effect of secondary endosymbionts and different strains in the variation of aphid defensive behaviours has already been reported for *A. pisum*, for which the effect of *H. defensa* and *R. insecticola* was shown to depend on the specific aphid genetic background (Sochard *et al.*, 2021). The effect of aphid endosymbiont infection in parasitoid searching behaviours have been mostly linked to the effect of endosymbionts in aphid chemical cues. For example, it has been

shown that female *A. ervi* wasps prefer to orientate themselves towards uninfected aphid hosts (versus infected with *S. symbiotica*) and spend more time foraging patches containing them (Attia *et al.*, 2022). It has also been shown that female wasps are able to discriminate different levels of pheromones produced by infected and uninfected aphids and adapt their behaviour accordingly (Oliver *et al.*, 2012; Frago and Zytynska, 2023). Previous studies have also found that both aphid genotype and endosymbiont infection with *H. defensa* and *R. insecticola* can change the composition of aphid honeydew (Schillewaert *et al.*, 2017). Although honeydew has been considered more as a food complement for parasitoids, the volatiles released with them can also act as host finding kairomones and stimulate oviposition (Leroy *et al.*, 2011).

The lack of a direct effect of genotype on any of the interaction outcomes contradicts previous work that has shown significant differences in host acceptance and parasitism proportion of *A. ervi* between potato aphid genotypes collected from potato crops (Clarke, 2013). This could suggest that a different array of aphid genotypes could be associated with strawberry versus potato crops and that genotypic effects might be context dependent. In addition, it should be noted that as the potato clonal lines were infected with multiple endosymbionts when the genotype focused experiment was undertaken, this could have masked weaker genotypic effects. The prominent role of aphid endosymbiont infection, which was already seen in the behavioural profiles, is also shown here as a direct effect on certain key behaviours (number of probes and number of ovipositions) and on parasitoid acceptance proportion and parasitoid efficiency. In addition to this, the analysis showed a direct effect of endosymbiont infection on parasitism success proportion. For all *outcomes* variables measured, aphids infected with *H. defensa* and *S. symbiotica* showed lower values. This highlights the mediation role of key behaviours on parasitoid acceptance proportion and parasitoid efficiency but also the independent role of endosymbiont infection on physiological resistance.

5.4. *Aphidius ervi* genetic diversity and parasitism efficiency against the potato aphid

The COI haplotype network analysis showed that the *A. ervi* commercially supplied in the UK were mostly classified under an exclusive haplotype (H12) which was not present in the wild or field parasitoid samples. However, the same haplotype was also found in samples of *A. ervi* commercially supplied and from field samples in France. This could be explained by the structure of commercial biological control companies in Europe, in which most production facilities are centralised in specific countries (in order to increase economic viability) and distributed to different markets. It also highlights the possibility of different commercial suppliers sharing parasitoid

material, either for the foundation of their *A. ervi* rearing system or more regularly. Another hypothesis that could explain this result is that unwanted selection has happened in rearing conditions if haplotype H12 is the *A. ervi* haplotype that it is better adapted to the mass rearing controlled environment or to the host species used (Sørensen, Addison and Terblanche, 2012). However, the fact that this haplotype is not found in samples coming from the field or from wild locations raises concerns about its performance against potato aphid once released in the field. However, this concern can be generalised, as only one haplotype (H08) found in commercially supplied samples and was also found in field or wild locations. In addition to this, it is important to note that most haplotypes detected in material from commercial supplier 5 were not represented in other commercial or non-commercial samples, indicating that this supplier might have a different production system, or used different *A. ervi* founders, or has limited material exchange with other companies.

Lower levels of genetic differentiation were found between most commercial suppliers and between some commercial populations and with non-commercial ones (pairwise F_{st} results). In addition, populations from different commercial suppliers also showed different levels of heterozygosity, all showing lower levels than non-commercial populations, except commercial supplier 5. The presence of inbreeding was detected when pooling results from all commercial suppliers together, but when analysed separately only commercial suppliers 2 and 3 were positive for inbreeding. On the other hand, inbreeding was not detected in either field samples or wild samples. Both lower levels of heterozygosity and the presence of inbreeding suggest that the mass-rearing techniques are affecting genetic diversity in those commercial populations either by genetic drift (if the populations are not too big), founder effects or unintentional selection by the rearing environment (Fauvergue *et al.*, 2012). Parasitoids from wild location 2 showed signs of outbreeding, which is expected from natural populations that usually have higher levels of gene flow than isolated populations such as mass-reared ones (Fauvergue *et al.*, 2012; Hoffmann *et al.*, 2021). These results contradict what was found before for *A. ervi* in France, in which "wild" populations were found to have signs of inbreeding, however, in that case field samples from agricultural crops were also considered as "wild" populations (Postic *et al.*, 2021), which might have influenced the results.

Considering the performance of the *A. ervi* parasitoids sourced from different companies in general, the ones from commercial supplier 1 showed the lowest parasitism success proportion across all potato aphid lines, and the ones from commercial supplier 5 the highest. Taking into account the results from the genetic diversity assessment, the *A. ervi* population from commercial supplier 1, had the lowest COI haplotype diversity and differentiation, while the population from commercial supplier 5, although not more diverse had the highest number of different COI

haplotypes recorded. Microsatellite results also showed that commercial supplier 1 was the least genetically diverse, while commercial supplier 5 was more genetically diverse. A significant interaction of aphid line and parasitoid population was also detected, in addition to signs of specificity in some *A. ervi* and *P. volucre* populations to specific potato aphid lines in which they did better. This result supports the idea of species-specific interactions between aphid and parasitoids but also the hypothesis that parasitism outcomes in the potato aphid-*A. ervi* system follow an intraspecific interaction pattern, with some parasitoid lines doing better than others depending on the aphid line. Genetic diversity is essential for enabling populations to adapt to change (Maia *et al.*, 2024), a more genetically diverse population of parasitoids could be linked to a higher probability to the population to harbour specific genotypes that are adapted to resistant hosts (Poirié, Carton and Dubuffet, 2009). However, it is important to consider that for this experiment, parasitoids were selected at random from a pool of greater or lesser genetic diversity (depending on commercial supplier) and genotypes of the females used were only tested *a posteriori* (results not included in this dissertation). In addition, this is a snapshot of the continuous co-evolutive processes that are happening between the potato aphid as hosts and parasitoids, and that higher virulence from certain parasitoid lines can also drive host resistance and vice versa (Fors *et al.*, 2016).

5.5. Field sampling

This study identified that a population of potato aphid feeding in a strawberry commercial crop was composed of 67 genotypes that carry up to 7 secondary endosymbiont combinations. Most aphid species reproduce by cyclical parthenogenesis, which allows for the generation of genetic diversity via only one event of sexual reproduction a year which is followed by a rapid succession of clonal generations (Blackman and Eastop, 2006). However, potato aphid is thought to reproduce completely asexually in the UK (Blackman, 2022), which has popularised the hypothesis that this species should only be composed of a handful of genotypes in the region. However, even if true clones are expected in each generation, genotypic differences can be created when mutations, chromosomal rearrangements or recombination occur (Kanbe and Akimoto, 2009). In addition, it is well known that insect species which live in non-permanent hosts, such as crop plants, have a population advantage by not having host-specific genotypes as they are forced to look for a different host in their environment when the crop is removed (Fenton, Woodford and Malloch, 1998). This then can favour the maintenance of generalist genotypes in the population that have a broad tolerance for different host plant species and don’t become established and can come and go from the crop to other hosts making their appearance in only specific data points of a long-term sampling. The rapid turnover of genotypes throughout the season has been reported before for other aphids (Hemiptera: Aphididae) such as the melon and cotton aphid *Aphis gossypii* Glover on cucurbits, the

peach-potato aphid *Myzus persicae* (Sulzer) on potatoes and *Sitobion avenae* on maize (Fenton, Woodford and Malloch, 1998; Fuller *et al.*, 1999; Haack *et al.*, 2000). It is important to note that 9 genotypes were described as prevalent due to their abundance over the study period. The main hypothesis that could explain these population dynamics could be that the strawberry crop might be playing a differential selective pressure on the different potato aphid genotypes, with some genotypes (i.e., prevalent genotypes) having a better fitness when feeding on this crop than others (i.e., transient), something that has been described for the English grain aphid on maize (Haack *et al.*, 2000). Differences in potato aphid clonal lines fitness were described in Chapter 4, when it was showed that they differed in their fitness proxies when reared on this plant. Some genotypes might also be more or less susceptible to natural enemies, chemical products used in the crop or other environmental conditions in the polytunnels (Simon and Peccoud, 2018; Musaqaf *et al.*, 2022). Genotype J is the most abundant potato aphid genotype in this strawberry crop but also the most persistent over the seasons. Highly persistent aphid clones can be maintained by selection in temporarily variable environments, these clones are usually denoted as “superclones” (Harrison and Mondor, 2011). Aphid “superclones” have been described to be highly successful in agroecosystems, sometimes due to their high plasticity to connect with different endosymbionts and their high environmental adaptability that can arise from that (Vorburger, Lancaster and Sunnucks, 2003; Figueroa *et al.*, 2005, 2018).

In terms of endosymbiont infections, potato aphids in this study were infected with three main secondary endosymbionts (*H. defensa, S. symbiotica, R. insecticola*), with all the double infection combinations possible and even triple infections. To date, natural infections with *H. defensa* and *R. insecticola* in potato aphids have been described before in potatoes and strawberry crops, however co-infections rates varied between studies (Clarke, 2013; Postic *et al.*, 2020; Donner, Beekman, *et al.*, 2024). Thus, it seems that the *M. euphorbiae* bacterial community might be strongly dominated by those two secondary endosymbionts. In addition, the genus *Macrosiphum* has been linked also to *Fukatsuia symbiotica*, *S. symbiotica*, co-infections and triple infections (Donner, Slingerland, *et al.*, 2024) on non-crop related host plant species. For all the studies previously mentioned, uninfected aphids also accounted for most of the abundance, as in this case. This suggests that potato aphid populations in general might be more frequently not infected with the three main endosymbionts, both on strawberry and non-crop plants. Concerning the fact that co-infections were common in this study, no other research has found similar results, in fact it has been described for the pea aphid *Acyrtosiphon pisum* (Harris) that none of the three endosymbionts studied here usually co-occur over a year period (Mathé-Hubert *et al.*, 2019), but that could modulated by the aphid host.

No two-way relationship between genotype and parasitism was found in this study, however a marginal significant three-way relationship between aphid genotype, *H. defensa* infection and

parasitism was described for all parasitoids and for only *A. ervi*. Genetically encoded resistance in aphids has been described for multiple species, including the pea aphid and the potato aphid (Clarke, 2013; Martinez *et al.*, 2014). However, even less research has been done on this topic. Indeed, most of the studies that have described intrinsic resistance to parasitoids in aphids have been done under laboratory conditions (Martinez *et al.*, 2018; McLean and Parker, 2020), but intrinsic resistance has not been described from field conditions. This could suggest that intrinsic resistance is less common that endosymbiont mediated resistance, but also that they might be genotype by endosymbiont interactions that make predictions difficult from field samples. Although, some of the three-way interactions in this were marginally non-significant, it is important to notice that interactions between aphid genotypes and endosymbionts have been described from controlled environment experiments for the pea aphid involving both *H. defensa* and *R. insecticola* (Hansen, Vorburger and Moran, 2012; Martinez *et al.*, 2018), indicating a strong relationship between the three organisms usually resulting in variation in parasitoid susceptibility in aphids. But there is no information for other aphid species under natural conditions where other environmental factors can come into play.

6. References

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