Tritrophic genetic interactions in *Acyrthosiphon pisum*

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Abstract

Anthropogenic climate change is putting ecosystems under threat and so it has become increasingly important to understand community structure in order to protect the integrity of remaining global ecosystems. One understudied element of community structuring is the role of intraspecific variation (ITV) and how it interacts with indirect genetic effects (IGEs). IGEs can act across multiple trophic levels and by altering these effects, ITV can have a cascading role across ecosystems. A small community across several trophic levels is therefore ideal to study the effect ITV has on IGEs and the well-studied pea aphid (*Acyrthosiphon pisum*) provides this. *A. pisum* is entirely dependent on its host plant for nutrition. The aphid also has a parasitoid wasp (*Aphidius ervi*) and a bacterial symbiont (*Hamiltonella defensa*) providing protection against the wasp. Strong coevolutionary pressures within this community would be expected.

To explore the effects of intra- and interspecific variation across different trophic levels I looked at aphid fecundity and resistance to *A. ervi* using a variety of aphid, *Hamiltonella* and host plant genotypes as well as plant species. Host plant species altered innate aphid parasitoid resistance as well as symbiont mediated protection and plant genotype significantly interacted with aphid genotype to affect A. ervi susceptibility. Aphid fecundity was affected by host plant species and *Hamiltonella* genotype but surprisingly there was no variation in fecundity between aphid clones. *Hamiltonella* also offered a different protective effect across two different aphid genotypes.

The observed role of host plants in parasitoid resistance was novel and raises interesting questions about the evolutionary pressures governing plant-aphid-symbiont-parasitoid interactions. Aphid host plants altering the aphid genotype effect on *Hamiltonella* mediated protection provides evidence for the importance of multitrophic ITV in communities. Thus, future work into understanding the role native and irregular plant hosts have on aphid-parasitoid defences would be insightful.

Author's declaration

I declare that this thesis is a presentation of original work, and I am the sole author. This work has not previously been presented for an award at The University of York, or any other University. All sources are acknowledged as references.

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List of Contents

List of Tables

List of Figures

1 Introduction

1.0 Introduction to the topic

It is widely accepted that humanity is causing a mass extinction event (Cafaro, 2015; McLellan et al., 2014). Species within a community are interconnected with direct and indirect relationships and so the loss of one or multiple species within communities may cause future extinctions and begin the destabilisation of entire ecosystems (Kehoe et al., 2021). This anthropogenic danger to ecological communities has led to much research hoping to explore and understand the important underlying mechanisms behind community structure in order to preserve and potentially even restore them. It is well established from classic studies that removing 'key-stone' species (those which have a disproportionately large effect on communities relative to their abundance) from communities has severe consequences (Brooks and Dodson, 1965; Power et al., 1996). More recently, a meta-analysis from Roches et al. (2018) found the removal of non 'key-stone' species has direct ecological consequences too. As a result, plenty of time and resources have been devoted to protecting important species within their communities (Miller et al., 2002).

It is not only species richness that is important to ecological integrity. In the last 15 years the importance of intraspecific variation to community structure has become apparent, and the negative consequences of losing population diversity and structure (Roches et al., 2018; Albert et al., 2010; Crutsinger et al., 2009). Anthropogenic climate change is a major threat to global ecosystems, and it may disproportionately reduce genetic diversity (Ceballos et al., 2017); the current extinction rate of populations is far greater than for species (Roches et al., 2018; Hughes et al., 1997). Considering the ubiquitous spread of climate change, genetic diversity is likely to decline. Therefore, it is important to fully understand the role of intraspecific variation within communities in order to predict and potentially prevent damage to ecosystems and this present study aims to augment this understanding.

1.1 Intraspecific variation within communities

Despite a long-held assumption to the contrary, intraspecific effects can equal and even overshadow interspecies effects on community structure and properties (Roches et al., 2018). In their meta-analysis, intraspecific variation (ITV) was found to be more important than species differences for indirect community effects (e.g., ladybird abundance affecting plant fecundity), although species interactions were still more important for direct community effects (e.g., consumption). Siefert (2012) added ITV into a model which predicts community structure and patterns from species-mean plant trait data. The addition of ITV strengthened patterns previously produced and predicted non-random assemblages within species highlighting the potential importance of ITV. Using field data, Messier et al. (2010) found that the amount variation in several plant traits explained by genetic differences is similar to the amount explained by species differences. Using a mathematical model, they also found evidence that this result will remain constant across a variety of ecosystems. This suggests that work focusing on species interactions with mean traits of single genotypes may be only considering half the picture.

Patterns in ITV are often explained using coevolutionary theories (e.g., Red Queen), however, it may be helpful to also consider processes previously limited to explaining interspecific structures. In this way, ITV may alter community structuring processes. For example, genetic diversity may provide the starting blocks for resource partitioning between species to occur and increase species richness (Herrera et al., 2015). Equally in other systems, high ITV may allow resource partitioning within species, allowing these species to become more generalist and potentially more dominant (Hart et al., 2016) which would

reduce species richness. ITV does also play a role in co-evolutionary dynamics (Shipley et al., 2016) by altering individual and species interactions. Individuals within populations can differ in parasite resistance (Ganz and Ebert, 2010; Pearl et al., 2008), resource use (Bolnick et al., 2003) and competitive ability and defences to predation (Duffy, 2010) which means ITV has the potential to alter a wide range of interactions indirectly and directly. Community structure and properties can come about due to processes such as environmental filtering and niche differentiation. Environmental filtering is a type of directional selection wherein abiotic factors drive selection towards certain traits. This leads to a convergence of traits within habitats (Grime, 2006) and potentially even trait convergence between similar but geographically distant habitats. Niche differentiation, represents a density dependent selection pressure, driven by competition, which leads to interspecific differences and resource partitioning (Stubbs and Wilson, 2004). As such these processes work antagonistically. These forces may act upon different genotypes as well as species (Siefert, 2012) and may help explain population structures.

Both Bolnick et al. 's (2011) mathematical study and Fajardo and Siefert's (2019) empirical rainforest study conclude that ITV plays an important role in community structures, and I propose indirect genetic effects are a tangible mechanism for ITV to play this important role. Direct genetic effects are where the genotype affects the phenotype of the individual it occupies, whereas indirect genetic effects (IGEs) describe genotypes altering the phenotype of different individuals. Thus, with IGEs there is a genetic basis to an individual's phenotype that originates from another individual; (Wolf et al., 1998). Classically IGEs have been considered to occur between socially interacting organisms (Moore et al., 1997) but may also arise indirectly whereby one individual alters the environment experienced by others (Wolf, 2003). In a study of the important forest understory coverage species, *Trifolium repens* (white clover) and *T. pratense* (red clover), Awmack et al. (2007) examined how intra- and interspecific differences in high CO_2 adaption affected plant growth and so understory composition. *T. pratense* populations were increased under high CO_2 conditions but *T. repens* populations were not affected, thus changing the vegetative composition of the understory community. In this case, genetic intraspecific variation had no effect. Many insects frequent forest understory plant populations for refuge and food (Hamilton et al., 2004). Therefore, although this was not quantified in this study, there were likely IGEs between the genes controlling high CO_2 adaption in red clover and forest invertebrates. It would be expected in the case of competition that IGEs would be negative on focal individuals, however this is often not the case. Competition based IGEs have been found to promote increased foliage, deemed shade avoidance strategy (Ballare, 1994; Botto and Smith, 2002), and even allelochemicals designed to inhibit competitors growth may have neutral or even positive effects on focal individuals (Mahall and Callaway, 1991; Nilsson, 1994). Competition based IGEs were examined in the classic model organism *Arabidopsis thaliana* (Mutic and Wolf, 2007) and QTLs were looked for to explain these counterintuitive results. Amongst others, QTLs associated with ethylene production were found to be responsible for altering competitors' growth. Ethylene is often used by plants to trigger their own growth but is highly diffusible, and so these IGEs may be accidental (Mutic and Wolf, 2007). These IGEs were across one trophic level, but multitrophic effects have been quantified. Aphid fecundity has been found to be reduced by the presence of onions growing nearby their host plants, and even when the host plant soil has just been trained with onions (Khudr et al., 2018).The reduction in aphid fecundity is thought to occur via anti-herbivore biochemicals derived from onions or onion-associated microorganisms (Khudr et al., 2018). This effect was altered by genetic variation in the host plants and aphids. This demonstrates the potential for IGEs to affect ecosystem dynamics and a role for ITV in that process. The potential effects of IGEs may not even be limited to within ecosystem interactions. Intraspecific variation in the leaves of riparian species *Alnus rubra* (red alder) causes structural shifts in aquatic macroinvertebrate communities (Jackrel and Wootton, 2014). Red alder leaves from nearby or upstream riparian habitats are broken down much faster by aquatic detritivores than leaves from further away or downstream (leaves may flow from

upstream downwards but not the other way around). ITV in red alder leaves may be due to varying ontogenetic and herbivory responses in different areas as well as genetic variation, so a genetic map of red alder trees could help confirm IGEs are the driving factor behind this. If trees closer together are more likely to be related, it would confirm that genes coding for compositional changes in red alder leaves are affecting the fitness and phenotypes of macroinvertebrates in a separate ecosystem, which indicates the potential reach of IGEs and how ITV may impact communities.

IGEs may describe both direct and indirect ecological effects and explain a multitude of species interactions. For example, within the same system (Johnson, 2008) genetic variation in aphid host plants was found to have a direct ecological effect on aphid growth rate, while plant ITV also explained 30% of the variation in mutualistic ant abundance which is an indirect ecological effect. In this present study, I am considering the interactions between pea aphids *(Acyrthosiphon pisum*), their host plants, their symbiotic bacterial species *Hamiltonella defensa* and their parasitoid wasp *Aphidius ervi*. There are multiple examples of IGEs here, including direct ecological relationships as found between the aphids and plants, as well as indirect relationships such as between plants and parasitoid wasps. I have added genetic intraspecific variation in aphids, plants and bacteria to this model to explore how ITV may affect complex IGEs and so community assemblage and functioning. In a recent review of plant-based ITV literature, Westerband et al., (2021) concluded ITV is key to understanding species interactions and therefore community structure and properties. Despite the importance, the review concluded that the effects of ITV on communities have not been well studied and my own literature review found little research outside of plant-based ecology. IGEs are key to understanding interspecies interactions (Costa e Silva et al., 2013), kin and multilevel selection (Costa e Silva and Kerr, 2012) and community genetics (Whitham et al., 2006). This study looks to add to the limited existing work and will consider the effects genetic variation has on IGEs in terms of direct and indirect ecological effects.

1.2 Symbionts

Although symbiosis and therefore symbionts are terms applicable to a wide range of cohabitating organisms, in this study I will use the term to refer to (often conditionally) mutualistic endosymbionts. The model used in the present study includes the pea aphid *Acyrthosiphon pisum*, and its facultative symbiont *Hamiltonella defensa* which offers protection against the parasitoid wasp *Aphidius ervi* making it a conditional mutualist. While no other facultative symbionts are used in this study, *A. pisum* individuals contain an obligate symbiont (*Buchnera aphidicola*) which is not explored experimentally in this thesis but is ever present in the aphids by definition. Symbionts are introduced with their binomial nomenclature but thereafter referred to by genotype (e.g., *Hamiltonella*) as is often used in the literature (Smee et al., 2021; Parker et al., 2017).

The majority of arthropods contain heritable bacterial symbionts (such as *Hamiltonella*) (Duron and Hurst, 2013) although interestingly 33% of the insects Duron and Hurst studied to determine this contained at least one out of four bacterial symbiont genera. As well as a wide range of hosts, symbionts also have a wide range of functions. For example, the symbiont *Wolbachia* has been found to increase the fecundity of *Drosophila melanogaster* when the flies are exposed to nutritional stress (Brownlie et al., 2009), while *Wolbachia* has also been found to provide protection against pathogens and parasites in *D. melanogaster* by interfering with the replication and transmission of natural enemies (Hedges et al., 2008; Teixeira et al., 2008). In a similar vein, the symbiont *Rickettsia* increases host survival and reproduction in *Aleyrodidae* (whitefly) as well as creating a female-biased sex ratio (Himler et al., 2011) and thus acting as a reproductive parasite. Symbiont functions can be vital for their host's survival, useful or even just conditionally beneficial. In *A. pisum* (pea aphids), the symbiont *Buchnera*

provides essential nutrients which are lacking from the pea aphids' plant sap diet (Douglas, 1998). Whereas, a strain of the symbiont *Fukatsuia* was found to act as a pathogen in its pea aphid host unless *Pandora neoaphidis* (a pathogenic fungus) was present, against which *Fukatsuia* confers protection (Smee et al., 2021).

Symbiont mediated protection is a common symbiont trait and has potentially wide-reaching consequences.This trait has been found across a wide range of host taxa including plants (Arnold et al. 2003), mammals (Barton et al. 2007), and of course invertebrates (Teixeira et al. 2008; Oliver et al. 2014; Parker et al. 2017; Smee et al. 2021). Symbiont mediated protection can come in many forms. In *Paederus* beetles, the symbiont *Pseudomonas* produces a toxin which is used to protect the host eggs from predation (Kellner, 2001). *Spiroplasma* is a common symbiont in arthropods (Regassa et al., 2006) and in *Drosophila neotestacea* it has been found to rescue fertility after infection with a nematode parasite (*Howardula aoronymphium*) which usually sterilises female fruit flies (Jaenike et al., 2010) by inhibiting the worm's growth. In the case of *Spiroplasma*, this protective effect has allowed the bacterium to rapidly spread throughout North America (Jaenike et al., 2010). Symbiont mediated protection is thought to occur in vertically transmitted symbionts as increasing host fitness will also increase the potential hosts and so symbiont fitness (Lively et al., 2005).

Vertically transmitted symbionts are tied to their host fitness which creates an interesting evolutionary dynamic between 2 selfish 'allies'. Alongside protection against natural enemies, vertically transmitted facultative symbionts also use a range of reproductive manipulation to spread throughout populations (Charlat et al., 2003). Although the mechanisms vary, the goal of reproductive manipulation is to increase the number of offspring containing the symbiont compared to offspring without. This often therefore leads to symbiont mediated increases in the host female to male birth ratios as vertical transmission is typically maternal (Duron et al., 2008), making females more valuable to the symbiont (Engelstadter and Hurst, 2009). This behaviour may therefore be classed as reproductive parasitism (Cosmides and Tooby, 1981). Unlike reproductive manipulators, symbionts which confer protection against natural enemies act as (often conditional) mutualists and increase host fitness in order to spread throughout populations (Lively et al., 2005). However, the evolutionary pressures on protective symbionts are just as selfish, as are those of the insect hosts, so a similar 'Red Queen' race will still occur between protective symbionts and their hosts. For example, population density within the host is highly variable (McLean et al., 2016) and this is partially down to a selection pressure in protective symbionts to increase bacterial density at the expense of their host (Bennet and Moran, 2015). The relationship between host and protective symbiont is therefore just another coevolutionary arms race, for which intraspecific variation is often key (Thrall et al., 2012).

The ecological importance of protective symbiont traits create potential for interactions with not just their host but the wider community. These interactions are increased by complex genetic webs which may form around symbionts (Duron and Hurst, 2013). Genetic material may be exchanged between symbiont populations, symbiont species and even between hosts and symbionts (Dunning et al., 2007). This is possible as even vertically transmitted symbionts frequently horizontally transfer between hosts (Henry et al., 2013) and sometimes this introduces symbionts into new host lineages and even new species although this does require either direct or indirect contact between host species (Ahmed et al., 2013). Transfer of genetic material between symbionts from different host lineages may counteract the genome degradation Bennet and Moran (2015) supposed to be inevitable in vertically transmitted symbionts and prevent the extreme genomic evolution termed as the 'symbiosis rabbit hole'. Horizontal transfer of symbionts has allowed *A. pisum* to colonise and adapt to new niches by diversifying its host plants (Henry et al., 2013) which could have profound impacts on communities. Furthermore, if this principle can be applied to natural enemy resistance in *A. pisum* and other insects then there is potential for multitrophic genetic interactions centred around symbionts. This seems possible as the aphid symbiont *Hamiltonella* confers resistance to the parasitoid wasp *A. ervi* with a bacteriophage (Oliver et al., 2009) which has been found

in a parasitic wasp (*Nasonia vitripennis*) bacterial symbiont *Arsenophonus nasoniae* (Wilkes et al., 2010), showing genes for protective traits can move between symbiont species and interact with hosts in diverse functional groups. Overall, the ecological importance of protective traits and the infidelity of horizontal transfer give symbiont genes both the potential and the means to interact with genetic variation across multiple trophic levels in communities.

1.3 The pea aphid community

Pea aphids (*A. pisum*) are a sap-feeding *Hemipteran* insect species which feeds on several species of *Fabaceae.* During Spring/Summer they reproduce asexually and thus consist of populations of female clones, with sexually reproducing morphs only developing at the end of the season in order to produce cold resistant eggs (Simon et al., 2002). *A. pisum* can be split up into distinct genetic clusters (Biotypes) which each specialise on a specific host plant species within *Fabaceae* (Ferrari et al., 2012) although the domestic broad bean (*Vicia faba*) is a universal host (Ferrari et al., 2008). For example, in this present study we are using two biotypes: *medicago* which feed on *Medicago sativa* and *trifolium* which use *Trifolium pratense* as a host. Pea aphids are widely spread across Eurasia and North America and are considered a source of major economic damage due to their phytophagy (Golawska et al., 2010) and their ability to act as a vector for plant pathogenic viruses (Paudel et al., 2018). They are thus an influential part of many communities across multiple continents.

Aphids (*Aphidoidea)* make excellent model systems to look for the effects of species level traits within communities for a number of reasons. They have natural enemies from several species rich taxa (Dixon, 1998) and are relatively easy to manipulate both in a lab setting (Koga et al., 2007)) and the field (Dixon, 1998). Aphids are attacked by two large clades of parasitoids (*Aphidiinae and Aphelinus*) and these parasitoids are attacked themselves by specialised hyperparasitoids (Sullivan and Volkl, 1999). In addition, aphids have emerged as an effective model system for the dynamics of both obligate (Koga et al., 2012; Bennet and Moran, 2015) and facultative symbionts (Oliver et al., 2005; Ferrari et al., 2007; Oliver et al., 2008). In essence therefore, aphids represent communities within communities with a collection of symbiont and aphid genetics contributing towards aphid phenotypes. Their usefulness as a model is not just due to directly and indirectly interacting with a large number of species; the clonal nature of most aphids allows a degree of untangling between genetic effects of aphids and their exo and endosymbionts.

This study uses a parasitoid wasp of *A. pisum*. Parasitoid wasps have evolved to attack the majority of insect species (Godfray, 1994) and have been proposed as the most important natural enemy of insects, even more so than predators and pathogens (Hawkins et al. 1997). This ubiquitousness, has led to much research focus centering on them (Clark et al., 2010) although it must always be considered that the research focus may have given an exaggerated impression of their omnipresence in comparison with other taxa. Aphid parasitoids are solitary koinobionts, allowing their host to develop and increase in size after parasitism, and all are endosymbionts: ovipositing and completing larval development inside their living aphid host (Mclean et al., 2016) which is eventually killed before the adult parasitoid emerges. *Aphidius ervi* is the species of parasitoid wasp used in this study and attacks *A. pisum* aphids as well as several other *Aphidoidea* species (Henter and Via, 1995). *A. ervi* injects venom along with an egg into its aphid host to aid parasitoid development. The venom stops the development of aphid ovarioles and thus blocks oogenesis (Digilio et al., 2000). A group of extraembryonic cells (teratocytes) attack and digest any preexisting aphid embryos (Falabella et al., 2000), extract nutrients from host tissues, and even redirect nutrients from the aphid's obligate symbiont (*Buchera*) with all these released nutrients being directed to the developing wasp larva (Caccia et al., 2005). *A. pisum* has some innate resistance to this which varies by

genotype (Ferrari et al., 2001; Ferrari et al., 2004) although the majority of any protection against *A. ervi* comes from a host of facultative symbionts (Oliver et al., 2005).

The pea aphid has been found to host at least eight different facultative symbiont species (Tsuchida et al., 2011; Russel et al., 2013), in addition to its obligate symbiont (*Buchnera*), with anywhere between zero and four bacterial species regularly coexisting in a single host (Ferrari et al., 2012; Henry et al., 2013). While *Buchnera* is strictly vertically transmitted (Van Ham et al., 2000), the pea aphid facultative symbionts may be spread via both vertical and horizontal transmission. Symbionts are capable of switching between species as well as between aphid lineages (Henry et al., 2013) although it is thought a partially shared life-history may be required (Russel et al., 2013). Indeed, Henry et al. (2015) proposes life-history traits may be a crucial factor in explaining the patchy nature of symbiont presence within different aphid lineages. Thus, symbionts may be influenced by the community interactions of their host just as they influence those interactions themselves. For example, aphid species which are involved in mutualisms with ants are less likely to host symbionts that offer protection against natural enemies (Henry et al., 2015). This is likely due to protection offered by the ants reducing the fitness benefit of harbouring the protective symbionts.

The nutritional obligate symbiont *Buchnera aphidicola* is found in almost all aphids and synthesises essential amino acids missing in the sap diet of its host (Douglas, 2009). *Buchnera* are vertically transmitted with the bacteria exocytosed from the maternal bacteriocyte and then immediately endocytosed by the embryo (Koga et al., 2012). In this way, *Buchnera* are highly fidelitous to their aphid lineage which can lead to genome degradation and indeed *Buchnera* genome size is quite small at 657 kbp (Shigenobu et al., 2000) compared to *Escherichia coli* which has an average genome of ~ 5 mb (Bergthorsson and Ochman, 1998). *Buchnera* also has a high mutation rate even for bacteria (Moran et al., 2009), and thus genetic variation in the primary symbiont may explain some differences between aphid lineages. For example, *Buchnera* is damaged by high temperatures which leads to reduced host fitness and is thought to partially explain the geographical ranges or temperate aphids (Wernegreen, 2012; Dunbar et al., 2007). This reduction in fitness can be reduced or even reversed by the facultative symbionts *Serratia symbiotica* and *Candidatus Fukatsuia symbiotica* (*Fukatsuia*) (Heyworth and Ferrari, 2015) and indeed *Serratia* is found more frequently in aphids in arid regions (Henry et al., 2013). Variation in *Buchnera* may also lead to differences in the host plant responses triggered by aphids. Despite being contained in bacteriocytes, *Buchnera*-derived proteins are secreted in aphid saliva (Chaudhary et al., 2014). One of these proteins (Gro-EL) is recognised by host plants and triggers an immune reaction that lowers aphid fecundity (Chaudhary et al., 2014). High host fidelity may prolong maladaptive traits such as this in aphid lineages and lead to intraspecific differences within aphid species.

In comparison to obligate symbionts, facultative symbionts have been studied far less across insects even though it has been estimated over one-third of insects contain a facultative symbiont (Duron and Hurst, 2013), therefore *A. pisum* has emerged over the last 15 years as an important model (McLean et al., 2016). Facultative symbionts of *A. pisum* have been ascribed a number of ecologically important roles. For example, Tsuchida et al. (2004) cured pea aphids of the symbiont *Regiella insecticola* and found the aphid had a reduced ability to feed on its host plant *Trifolium pratense*. When the same *Regiella* strain was introduced to another aphid host, an improved performance on *T. pratense* was noted (Tsuchida et al., 2011). However, these results have not been repeated elsewhere (Mclean et al., 2011; Ferrari et al., 2007; Leonardo, 2004) suggesting the effect may have been specific to symbiont strain, aphid clone or the interaction of a specific coupling. Phylogenetic evidence has also found a link between symbiont acquisition and host shifts in pea aphids (Henry et al., 2013) although it is unclear whether this is due to the symbionts helping the aphids to adapt to new hosts or simply due to aphids being exposed to new symbionts after switching hosts (Mclean et al.,

2016). There is some evidence that the protection offered by *Serratia* against heat shock may be due to the symbiont compensating for the lost metabolic function provided by *Buchnera* (Koga et al., 2003) and perhaps additionally to the production of proteins that may reduce the damage incurred to *Buchnera* (Koga et al., 2003). As previously discussed, facultative symbionts are often involved in protection against natural enemies (Duron and Hurst, 2013) and this is found in pea aphids as well. *Regiella* has been established as having a protective effect on pea aphids against the pathogenic fungus *Pandora neoaphidis* for over 15 years (Ferrari et al. 2004; Scarborough et al. 2005). Heyworth and Ferrari (2015) also recently found *Fukatsuia* and possibly *Spiroplasma* to provide a protective effect against the entomopathogenic fungus. *Hamiltonella* has been known to protect against the parasitoid wasp *A. ervi* for a long time also (Oliver et al., 2003) and is the pea aphid symbiont involved in the present thesis. Although not used here, Serratia (Oliver 2008) and Fukatsuia (Heyworth and Ferrari, 2015) have also been found to provide protection against A. ervi and even Regiella was found to confer protection against parasitoids in a single clone of the peach potato aphid (Myzus persicae) (Vorburger et al., 2010).

Hamiltonella has been found to have multiple ecologically important roles in aphids including subverting host plant defences but perhaps the most important trait is protection against *A. ervi*. Oliver et al. (2003) concluded previously assumed differences in aphid clones' susceptibility to *A. ervi* were in fact caused by different infection statuses of *Hamiltonella* and Ferrari et al. (2004) found *Hamiltonella* increased resistance to *A. ervi* and its congeneric *Aphidius eadyi* in 41 aphid clones. A more recent study found some *Hamiltonella* isolates to provide complete protection against *A. ervi* (Smee et al., 2021) demonstrating the ecological importance of this bacterial trait. Over the last 20 years the mechanisms behind this protection have been somewhat elucidated. A bacteriophage named bacteriophage 1, *A. pisum* Secondary Endosymbiont (ASPE-1) was found in *Hamiltonella* by Van der Wilk et al. (1999) and five years later a second phage (ASPE-2) was found in *Hamiltonella* that contains a gene for a cytolethal distending toxin (cdtB) that interrupts the eukaryotic cell cycle (Moran et al., 2005). This toxin has been proposed (Moran et al., 2005; Oliver et al., 2009) as the element responsible for the protection conferred by *Hamiltonella* against *A. ervi*. In a multi-locus analysis of five phage and ten bacterial loci, Degnan and Moran (2008) found high levels of recombination in the phage despite small differences elsewhere in the *Hamiltonella* genome which suggests differences in the phage may partly be responsible for differences in the protection conferred by different *Hamiltonella* strains (Parker et al., 2017; Smee et al., 2021). Possibly, alongside interactions between *Hamiltonella* (including ASPE-2), aphid genotypes, and even host plant genotypes which I will explore further below.

1.4 Symbionts in aphid-plant and aphid-parasitoid interactions.

The interaction between host plant and endosymbiont genotypes with aphid genotypes explains much of the global distribution of aphids. Henry et al. (2013) found aphids' host plant to explain a significant amount of the variation in *Hamiltonella* and *Regiella* genotypes across aphid lineages and even found different clades of *Hamiltonella* associated with different host plants. It is difficult to tell however, whether this is due to symbiont-plant interactions or simply increased horizontal transmission between aphids that share host plants. Particular strains of *Hamiltonella* and *Regiella* increase the likelihood of aphids colonising certain host plants (Henry et al., 2013). For example, one clade of *Regiella* is associated with a higher rate of aphid colonisation on *T. pratense* and the same clade is lost at a much faster rate in aphid host lineages associated with other plants (Henry et al., 2013). The same pattern was found in aphids infected by one clade of *Hamiltonella* on the host plant *Medicago sativa*. Previous to this, *Regiella* had been found to increase pea aphid fecundity on *Trifolium repens* and decrease fecundity for aphid hosts kept on *M. sativa* (Leonardo and Muiru, 2003). Follow up studies found a variety of symbiont mediated effects on aphid host adaptability (Leonardo,

2004; Ferrari et al., 2007) and suggested this range is down to interactions between symbiont and aphid genotypes (Ferrari et al., 2007). These results combined suggest symbiont genotype is an important factor in aphid host plant adaptability which will have community wide effects. With further research we may find there are also interactions between the symbionts and host plants genotypes as well as plant species. In this present study, I explore the interaction between *Hamiltonella, A. pisum* and *M. sativa* genotypes and the effect on aphid fecundity and parasitoid resistance. To a lesser extent this will also be explored in *T. pratense* again with *Hamiltonella*.

One potential set of mechanisms for the interactions between symbiont, aphid and host plant is the ability of symbionts to both activate and neutralise host plant defences and immunity. Many of the roles insect symbionts have on mediating plant defences remained hidden until recently (Frago et al., 2012) but symbionts are now recognised as being pivotal in aphid-plant interactions (Oliver et al., 2014). *Diabrotica virgifera virgifera* (Western corn rootworms) infected with the symbiont *Wolbachia* were found to suppress defence related genes in their maize host (Barr et al., 2010). Closer to home, *Hamiltonella* symbionts infecting the whitefly *Bemisia tabaci* suppress jasmonic acid (JA) in tomato host plants which increases whitefly growth and survival (Su et al., 2015). Both JA and salicylic acid (SA) are used by plants as part of their anti-herbivore defences and these compounds act in an antagonistic fashion (Takahashi et al., 2004; Niki et al., 1998). In the whitefly, *Hamiltonella* was only capable of suppressing JA with clones containing an intact SA pathway (Su et al., 2015) suggesting *Hamiltonella* was manipulating crosstalk between the SA and JA pathways. In a study on wheat aphids (*Sitobion miscanthi*) however, *Hamiltonella* suppressed both the wheat plants' SA and JA related genes which decreased defence-related enzyme activity in the wheat and subsequently improved in aphid fitness compared to uninfected aphids (Li et al., 2019). In addition to this, insect symbionts have been found to detoxify plant secondary metabolites and even chemical pesticides (Oliver et al., 2010; Berasategui et al., 2016; Cheng et al., 2017).

Another source of interaction between symbionts and the community of their insect host is with the interference of signalling between plants and natural enemies of herbivorous insects hosting symbionts. Predators and parasitoids frequently use volatile chemicals to locate herbivorous insects which may be otherwise concealed (Schoonhoven et al., 2005). The insect-associated symbionts may influence these volatile secretions in two ways (Frago et al., 2017). First, symbionts may produce chemicals that attract natural enemies. For example, symbiotic fungi in bark beetles (*Scolytinae*) release volatiles that attract parasitic wasps (Adams et al., 2008; Boone et al., 2014). This is maladaptive and will likely reduce the fitness benefits offered by a symbiont to its insect host. Alternatively, insect symbionts may change or reduce host plant signalling to insect natural enemies. This signalling takes the form of volatile compounds produced by the plant upon predation by herbivorous insects with the aim of attracting the insect's natural enemies (Dicke and Baldwin, 2010). By interrupting this, symbionts may counteract the host plant's defence and increase insect fitness. This has been observed in my model species *A. pisum*. The universal pea aphid host, *V. faba*, is known to release volatiles that attract the wasp *A. ervi* upon sensing pea aphid feeding (Du et al., 1996; Du et al., 1998; Powell et al., 1998; Guerrieri et al., 1999). Frago et al. (2017) used a combination of behavioural experiments and volatile analysis to determine *Hamiltonella* reduces parasitic wasp recruitment by *V. faba* host plants via systematic changes in the volatiles induced by pea aphid feeding. It is not known whether the change in volatiles released prevents the wasps from finding their aphid hosts or signals that the aphids are infected with *Hamiltonella* and thus poor targets (Frago et al., 2017). Since volatile emissions are significantly reduced as well as changed (Frago et al., 2017), it is more likely the former. The ability of *Hamiltonella* to reduce its host's visibility to *A. ervi* may explain an interesting finding from Mclean et al. (2016). They found that while *Hamiltonella* impeded parasite larval development, aphid survival rates were not affected by the symbiont, suggesting no increase in direct fitness. The authors proposed some aphids may have survived just long enough to

reproduce a little and this in combination with fewer *A. ervi* detecting the aphids in the wild may provide the direct fitness benefit of *Hamiltonella*.

There is conflicting evidence whether parasitoid success in aphids is primarily a function of parasitoid-aphid, symbiont-parasitoid or symbiont-genotype genetic interactions. I hypothesise parasitoid success is driven by a combination of all these interactions with the inclusion of the aphid host plant genotype as well. Interactions will be tested in this present study between aphid, symbiont and host plant genotypes. However, there is evidence for less complicated parasitoid dynamics. In one of the first studies to firmly establish the role of *Hamiltonella* as a protective symbiont against *A. ervi*, Oliver et al. (2005) concluded variation in parasitoid success is down to *Hamiltonella* genotype alone and found no difference in performance when symbionts infected different aphid host clones. In the black bean aphid *Aphis fabae* the protective effects of *Hamiltonella* against a parasitoid were measured across different aphid and parasitoid clones (Vorburger et al., 2009). Interaction between symbiont presence and parasitoid genotype was found whereas no interaction between aphid and parasitoid genotype in uninfected aphids occurred and so the authors concluded in support of Oliver at al. (2005) that the symbiont-parasitoid dynamic best explains parasitoid success. However, this study was limited and could not distinguish between aphid and symbiont genotype effects in infected aphids. Back to pea aphids, and Heyworth and Ferrari (2015) found a more complex picture. Using the symbiont *Fukatsuia*, they found the symbiont increased protection against *A. ervi*, but this protection significantly interacted with host aphid genotypes. This study was also limited however, as *Spiroplasma* was present in the aphid hosts, so interactions between *Fukatsuia* and *Spiroplasma* may be mistaken for symbiont-host interactions. In a slightly different system, a *Regiella* strain was found to be a significant factor in explaining the variation in protection against the entomopathogen *P. neoaphidis* (Parker et al., 2017). As was the interaction between *Regiella* and pea aphid genotypes (Parker et al., 2017). Furthermore, symbiont clade or host biotype did not affect either aphid fitness, or the symbiont-host interactions, suggesting the genetic interactions are idiosyncratic (Parker et al., 2017). This may indicate antagonistic coevolution between host and symbiont or frequency dependent selection on resistance genes in both symbiont and host in response to natural enemies.

Overall, symbiont genotype clearly affects the protection conferred to the aphid host from natural enemies, however, the role of host genotype in symbiont-parasitoid interactions is unclear. The role of host plant in these genetic interactions has not been considered as of yet despite evidence host plant genotype affects symbiont density in soybean aphids *Aphis glycines* (Enders and Miller, 2016) and, as mentioned earlier, in a system with the aphid *Aphis oestlundi*, host plant (primrose) genotype was found to directly affect mutualistic ant abundance independently of aphid density (Johnson, 2008) showing a multitrophic effect.

Thus, throughout this thesis, I aimed to explore whether, as hypothesised, aphid genotype interacted with symbiont genotype to impact both parasitism and fecundity. I found that one *Hamiltonella* genotype did offer different levels of protection in two clones. I also aimed to test whether host plant genotype or species identity could interact with either symbiont strain or aphid clone identity to affect both parasitism and fecundity. I also tested whether the host plant genotype or species would affect these traits in its own right. These are all examples of indirect genetic effects. Overall, I found evidence that host plants have a role in aphid parasitism and fecundity outcomes as well as interact with host and symbiont genotypes.

Table 1. Summary of the three experimental sections and the hypotheses each section allowed me to test.

Section name	Aphid lines used	Experimental design	Study aims tested
Hamiltonella (H) genetic variation	There was one aphid genotype used : 218. 218 aphids were infected with 5 *Hamiltonella* genotypes: H1 through H5.	Aphid susceptibility to *A. ervi* was measured across the 6 lines. As was aphid fecundity. 2 host plant species were used comprising 6 different genotypes.	In this section I tested whether the aphid host plant species or indeed genotype can have an effect on protection against *A. ervi* as well as aphid fecundity. I also tested whether *Hamiltonella* genetic variation has an impact on the protection conferred against *A. ervi* and fecundity cost in the aphids as has previously been found. Combining these 2 factors I tested whether the plant genetic or species effect may interact with *Hamiltonella* genotype effects on *A. ervi* resistance in the aphid and costs to fecundity.
Aphid (A) genetic variation	There were 4 aphid genotypes used here: 218, 200, md10 and 313. 2 genotypes are from the *Medicago* biotype and 2 are *trifolium* All were uninfected with facultative symbionts.	Aphid susceptibility to *A. ervi* was measured across the 4 lines. As was aphid fecundity. 3 host plant species were used comprising 8 different genotypes.	Here, I tested whether aphid host plant species or genotype could impact innate resistance to *A. ervi* and fecundity in uninfected aphids. Whether genetic variation in aphids has an effect on innate resistance to *A. ervi* and fecundity was also tested here. This design also allowed me to test whether genetic variation in the aphids can interact with the effects of genetic and species differences in the host plants on the susceptibility of aphids to *A. ervi* Parasitism and fecundity.
H5 interaction in 2 aphid hosts	There were 2 aphid genotypes used here: 218 and md10. H5 was introduced to both genotypes creating 218 H5 and md10 H5. These were used in this section alongside uninfected 218 and md10 aphids.	Aphid susceptibility to *A. ervi* was measured across the 4 lines. As was aphid fecundity. 3 host plant species were used comprising 8 different genotypes.	In this section the susceptibility to *A. ervi* and fecundity of each aphid line was measured on each host plant genotype and species. This allowed me to test whether the protection conferred by the H5 symbiont is affected by the genotype of the aphid host. The difference in fecundity cost incurred by H5 across the 2 genotypes was also tested. Interactions could also be looked for between the effects of host plant species/genotype and the effect of aphid genotype on the performance of H5 in terms of aphid fecundity and susceptibility to *A. ervi*.

2 Methods

2.1 Host plant, aphid and Hamiltonella choices

2.1.1 Host plants

Throughout the experiments the effects on fecundity and parasitoid resistance of feeding aphids on different host plant species and genotypes were measured and interactions along with aphid and *Hamiltonella* genetic variation (table 1).

Each aphid biotype is only viable on one native host plant (J Ferrari, personal communication) and the universal host *V. faba*. Therefore, to test the effect of host plant species I used *M. sativa* for *medicago* aphids, *T. pratense* for *trifolium* aphids and *V. faba* for both.

To test genetic variation in host plants I selected three varieties of each species from various seed suppliers. Each genotype had different listed traits from the suppliers so are assumed to vary genetically. The lineages and relatedness of each genotype are unknown however as it would not have been financially viable to undertake sequencing. Seeds for one genotype of *T. pratense* never arrived, so for trifolium aphids I could only draw a comparison between aphids kept on two native host plant genotypes. The *V. faba* varieties are “Grano violetto”, “Sutton dwarf” and “Masterpiece green longpod”. The *M. sativa* varieties are “Ezzelina”, “Marshal” and a variety listed as suitable for being grown as edible sprouting seeds “Sprouting seeds”. For *T. pratense*, “Essex broad leaf” was used as well as a variety described by the supplier Moles seeds as a wild flower variety “Moles wild flower”.

2.1.2 Aphid clones

Four aphid clones were used to explore the effects of aphid genetic variation on fecundity, parasitoid resistance and any interactions with *Hamiltonella* and host plant. Two clones were chosen from each biotype with 218 and 200 from *medicago* and 313 plus md10 from *trifolium*. The 218 clone was chosen to allow comparison between this work and a study from Smee et al. (2021) which used the same *Hamiltonella* strains in 218 aphids. The other genotypes were available to the lab group and were not known to be particularly poor performing (J Ferrari, personal communication). All clones were taken from their native host plant.

2.1.3 Hamiltonella clones

Five *Hamiltonella* strains were used to allow testing of the effects of genetic variation in the symbiont on parasitoid resistance and aphid fecundity. Again interactions between aphid genotypes and host plants were also looked for. The five strains used were sequenced and characterised by Smee et al. (2021) which allowed me to ensure I had genetic variation in my strains and indeed there are two distinct clades across my symbionts with H1, H2 and H3 belonging to one clade and H4 plus H5 another (Smee et al., 2021). All strains were taken from aphids collected on *M. sativa*.

2.1.4 Initial choices, issues and changes

The initial experimental goals were to explore G_{aphid} x $G_{Hamiltonella}$ x $G_{host\ plant}$ interactions more thoroughly and so a factorial design was proposed with two strains in all four aphid genotypes. To create this set up, aphids needed to be infected with the desired strains with microinjections from infected donor haemolymph. Unfortunately, the entire lab had issues with microinjections over the course of the time allotted to experimental set up and only one successful infection

was achieved. Therefore, I designed an altered experiment involving five *Hamiltonella* strains instead of two, but with a heavily reduced G_{aphid} x $G_{Hamiltonella}$ section.

Shortly before the fecundity experiment some aphid lines were exposed to high heat due to a heatwave creating issues with the air conditioning unit in the temperature-controlled room. The uninfected 218 aphids were kept on plants which were especially damaged by this high heat and the combination reduced the uninfected 218 aphids' condition for multiple generations. Subsequently 218 aphids were often visibly in poor condition. They were initially included in the experiment but uninfected 218 aphids had significantly higher mortality than any other line. Additionally, this line's fecundity was significantly lower than the fecundity of other uninfected aphids and several lines of 218 with *Hamiltonella*. With the high mortality and poor appearance, these data were excluded from the analyses unless otherwise stated. It is possible damage to *Buchnera* can occur across three generations simultaneously and this has been well established to reduce aphid fecundity (Smee et al., 2021; Heyworth and Ferrari, 2015; Dunbar et al., 2007).

2.2 Experimental set up

2.2.1 Curing protocol

The aphid clone md10 was infected with *Regiella* which is a common symbiont in *trifolium* aphids. To identify *Hamiltonella* and aphid genotype sources of fecundity and parasitoid resistance variation with certainty, therefore, md10 aphids needed to be free of other facultative symbionts. Thus, they were cured using the protocol below. Curing aphids of facultative symbionts in this way does not affect the densities of obligate host *Buchnera* (Li et al., 2019). All other aphid clones had symbiont free representatives prior to my experimental work.

This protocol was adapted from Smee et al. (2021) and is standard to the field. Two second or third instar aphids were placed and allowed to feed on a *V. faba* leaf for 5 days with its petriole inside an Eppendorf tube containing an antibiotic cocktail (1% Ampicillin, 0.5% Gentamicin and 0.5% Cefotaxime). Aphids were kept in a temperature-controlled room at 20 degrees Celsius with a 16h light cycle. Survivors were kept and then allowed to reproduce. The first 10 offspring were discarded and the next few offspring were kept and grown until adults. When adults, the aphids were separated and again allowed to reproduce. The offspring were kept alive and in separate parental groups while the adults were tested for symbionts using the PCR protocol below. The offspring of adults free of *Hamiltonella*, *Fukatsuia*, *Regiella* and *Spiroplasma* were then kept and these lines were tested to check they were facultative symbiont free every few generations until 8 generations had passed since the initial antibiotic exposure. The verified uninfected md10 lineages were then combined and used for the experimental work. Some md10 aphids were then infected with H5 with the microinjection protocol below

2.2.2 Microinjection protocol

Although this protocol was used in the attempted infection of all four aphid genotypes with H4 and H5, the only successful application was the infection of md10 aphids with H5 from the 218 H5 line which existed in the lab previous to my work. Prior to the use of this microinjection protocol, the symbiont status of each aphid line was ascertained with the below PCR protocol and the success of any injections was determined in the same way.

Haemolymph was removed from a donor aphid using a glass capillary tube sharpened into a needle and connected to a paraffin filled syringe to provide suction. The needle point pieced the aphid thorax in one of the thoracic leg junctions. The removed donor haemolymph was

then immediately injected into a recipient aphid with the needle piercing the same point on the recipient. This was the easiest location to avoid a large wound in the aphid which would lower survival chances and cause suffering in the aphid.

The surviving recipient aphids were kept separately on *V. faba* leaves in petri dishes with their petrioles sealed in agar and aphids were allowed to reproduce. Aphids were kept in a temperature controlled room at 20 degrees Celsius with a 16h light cycle. As in the curing protocol, the first 10 offspring were discarded and the next several were separated and allowed to become adults. Those adults were allowed to reproduce and the first few offspring were kept alive and separate while the adults were tested for the desired symbiont. Any offspring from parents which had been successfully infected were kept and re-tested until the eight generation after the initial injection. This was done to ensure symbiont populations were stable in their new host. Several other successful injections of symbionts into different aphid hosts occurred but only one remained stable during the eight generation period. Lineages of md10 H5 which kept the donor symbiont for 8 generations were combined and used in the appropriate experiment .

2.2.3 PCR protocol

Two protocols for DNA extraction were used as a faster method was adopted after some testing had already taken place. Both methods were found by me and a member of the lab group (C Fitzpatrick, personal communication) to produce identical results.

Initially the QIAGEN DNeasy protocol was used: “Purification of total DNA from insects using the DNeasy® Blood & Tissue Kit” (Qiagen, Aug-06). This was accurate but costly and time consuming so I switched to a diagnostic PCR protocol used by Smee et al. (2021).

Adult aphids were used immediately after collection and cooled in ice or stored in the freezer for up to 1 week. Aphids were homogenised in a 200 µl 5% Chelex solution made in distilled water. 10 µl of proteinase K (Promega, 10 mg/ml) was added per sample, and samples were incubated overnight at 56°C to facilitate digestion. They were then ‘boiled’ at 100°C for ten minutes before being centrifuged at 13,000 rpm for 3 minutes and the supernatant containing the DNA pipetted into a clean 1.5 ml Eppendorf tube which was stored at -20°C until use, or used immediately.

No matter which of the two DNA extraction protocols used, DNA was added to the same PCR mix and exposed to the same PCR reaction. The PCR mix comprised 6.25 µl BioMix (Bioline), 0.1 µl (20 µM) of forward and 0.1 µl (20 µM) reverse primer (table 3), 5.55 µl distilled water and 0.5 µl sample DNA. The PCR reaction for all symbionts was performed at 94°C for 2 minutes, followed by 35 cycles of: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. It concluded with 6 minutes at 72°C and then cooled the sample to 4°C indefinitely. PCR products were run on a 1% agarose gel and the presence of a band confirmed the presence of the symbiont.

2.3 Experimental methods

2.3.1 *A. ervi* susceptibility method

As discussed, *A. ervi* is a parasitoid wasp which attacks *A. pisum* and was used here to measure the protective effects of symbionts, the innate immunity of aphids and how host plants may affect these defences against natural enemies.

The wasps were purchased as pupae on the verge of emerging as adults from the supplier Koppert. The supplied wasps were of mixed sex although only females were used. They arrived in cold storage and were refrigerated until the day before exposure to aphids. The day before use, the wasps were released into a cage kept in a room with 16h light and kept at 20 degrees Celsius. Upon release into the cage wasps were fed by cotton wool balls soaked with a honey and water solution. Immediately before exposure to aphids, females were identified and collected in Eppendorf tubes. All females thus had no previous exposure to aphids but had likely mated with males prior to the experiments. Different individual wasps were used for each combination within a replicate and a different shipment of wasps for each replicate.

One to three adult aphids from each line were placed on a 92mm assay dish (~75g of leaf matter with petioles enclosed by a 2% agar gel). This was done for each combination of plant genotype for each aphid line. After three days, the first 10 offspring (or as many had been produced if below 10) were placed on a new assay dish. The following day the 1st/2nd instar aphids were exposed to *A. ervi* females by releasing the wasp into the assay dish and re-sealing. Each wasp was left in the assay dishes for exactly four hours which has been ascertained as a time period wherein the wasp has sufficient time to inject each aphid with 1 egg (C Fitzpatrick, personal communication). No precautions were taken to avoid multiple injections of eggs into the same aphid, however, there would be equal probability of this occurring throughout the treatment and *A. ervi* would benefit from avoiding double injections.

Aphids were placed on new dish assays of the same host plant variety after five days to keep aphids in a healthy condition. Successfully parasitized aphids will develop into ‘mummies’ which are caused by developing *A. ervi* larvae. After 10 days, allowing sufficient time for wasp development (Smee at al., 2021), the number of healthy aphids and ‘mummies’ were counted and measured and a susceptibility proportion ascertained. This was repeated for each aphid line and host plant variety combination and these combinations were replicated eight times each. Aphids were stored in an air conditioned room with sixteen hours of light per day and a temperature of 20C. All aphids within replicates were kept on trays next to each other and at the same height to ensure maximum consistency of conditions.

2.3.2 Fecundity experiment methods

As in the parasitoid experiment one to three adult aphids from each line were placed on a 92mm assay dish (~75g of leaf matter with petioles enclosed by a 2% agar gel). This was done for each combination of plant genotype for each aphid line.

After 3 days, the first 10 offspring (or as many had been produced if below 10) were placed on a new assay dish. These ~ 10 offspring were kept on a new assay dish for a week to allow development into adults. After seven days, one healthy adult aphid was taken and placed on a new assay dish. The proportion of the 10 (or however many) aphids which survived the week was measured and a mortality count generated. The adult aphid on a new assay dish had fecundity measured over the next seven days. If no adults had developed in time, then a 4th instar aphid was taken and this was noted on the dish.

After three to four days, the offspring and the length of time elapsed were counted to create a fecundity count per day. The aphids were given fresh leaves and then left for the remainder of the seven days. Any 4th instar aphids that had become adults had their fecundity count begin with the change of leaves. If any aphids were still not adults they were removed from the experiment as it would not be possible to generate a per day fecundity count. Any aphids which had died during the three to four days were removed from the data as it would not be possible to ascertain a per-day fecundity count.

After the remainder of the seven day period, the fecundity per day was measured again for all aphids. Fecundity was measured as an average per day instead of total so I could keep data from aphids which died part way through the experiment or developed into adults midweek. The fecundity data for each aphid at each of the two counts were kept for use in sense checks. For example, these data were used to check treatment groups were not disproportionately represented by aphids aged 0-4 days or 4-7 days which may have different reproductive rates. Aphids were stored in an air-conditioned room with sixteen hours of light per day and a temperature of 20C. All aphids within replicates were kept on trays next to each other and at the same shelf height to ensure maximum consistency of conditions.

2.4 Analysis

All analysis was done in R version 4.2.0, using R studio. All figures were made using ggplot2 in the same version of R also.

The parasitoid data was a binary outcome dataset as aphids are either turned into ‘mummies’ or not. Therefore, a binomial family GLM was used to analyse the data.
The function multcomp:glht (general linear hypothesis) was used to make pairwise comparisons within the dataset using the Tukey method. The response variable was a weighted paired variable composed of the number of healthy aphids and those turned into ‘mummies’. The explanatory variables are listed in table 3.

After conducting Shapiro-Wilk normality tests, Bartlett’s test of homogeneity of variances, as well as looking at normal QQ plots and the fitted vs residual values, I concluded the fecundity dataset fits the assumptions of normality. As such I used two-way ANOVA tests to analyse this dataset . Tukey’s HSD test was used to make pairwise comparisons. The response variable was simply the average fecundity per day of each combination from each replicate. The explanatory variables are also listed in table 3.

Host plant genotype and species are nested factors as separation by genotype is just added complexity to separation by species. Therefore, significance of host plant genotype effects were ascertained by a Chi squared test between models using either genotype or species as the explanatory variable. This was done like this: anova(species_mod, genotype_mod, test = “Chisq”) also in the same version of R studio as the rest of the analyses.

Table 2. Primer sequences for PCR reactions.

Symbiont	Forward	Sequence	Reverse	Sequence	Reference
Hamiltonella	10F	5'-AGTTTGATCATGGCTCAGATTG-3'	T419R	5'-AAATGGTATTSGCATTTATCG-3'	Ferrari et al, 2012
Fukatsuia	10F	5'-AGTTTGATCATGGCTCAGATTG-3'	X420R	5'-GCAACACTCTTTGCATTGCT-3'	Ferrari et al, 2012
Regiella	10F	5'-AGTTTGATCATGGCTCAGATTG-3'	U433R	5'-GGTAACGTCAATCGATAAGCA-3'	Ferrari et al, 2012
Spiroplasma	10F	5'-AGTTTGATCATGGCTCAGATTG-3'	TKSSsp	5'-TAGCCGTGGCTTTCTGGTAA-3'	Fukatsu & Nikoh, 2000

3 Results

3.0 Overall Findings

I found both aphid and *Hamiltonella* genotypes have a significant effect on the susceptibility of *A. pisum* to parasitism from *A. ervi*. The host plant species which aphids were kept on during these experiments also had a significant impact on parasitism by *A. ervi*. There was also strong evidence for an effect of plant genotype on parasitism outcome in some clones although it didn't quite reach the 95% significance threshold. I also found significant interactions between host plant species and *Hamiltonella genotype* as well as between aphid genotype and host plant genotype. Finally, although this was limited to one *Hamiltonella* strain, the host clone did significantly affect the symbiont mediated resistance to *A. ervi* (table 3).

I also found *Hamiltonella* genetic variation to significantly affect aphid fecundity and so likely fitness. However, within the 3 genotypes tested here, aphid genetic variation did not have a significant effect. The aphids' host plant species also explained a significant amount of variation in aphid fecundity. The host plant genotype had a significant effect on fecundity in aphids infected with *Hamiltonella* but not in those without. There were significant interactions in the effects on aphid fecundity between host plant genotype as well as species and *Hamiltonella* isolate, but not with aphid genotype (table 3). The fecundity experiments were limited as one line (218 00) suffered low fecundity due to damage sustained in a heat wave.

Table 3. Summary of results from all experimental sections. Asterisks represent significance levels. Analysis done in R.

Experiment	Effect measured	Proportion of variance/deviance	Distribution	P value
Hamiltonella (H)	H genotype	0.33	Binomial glm	<0.001***
genetic variation	H presence	0.26	""	<0.001***
Parasitoid	Plant species	0.03	""	<0.001***
	Plant genotype	0.01	""	0.996
	G_H x Plant species	0.05	""	0.116
	G_H x $G_{host\ plant}$	0.02	""	0.601
	H pres. x plant species	0.01	""	0.0495*
	H pres. x $G_{host\ plant}$	0.03	""	0.053'
Hamiltonella (H)	H genotype	0.21	ANOVA	<0.001***
genetic variation	H presence	N/A	""	N/A
Fecundity	Plant species	0.12	""	<0.001***
*218 00 excluded	Plant genotype	0.14	""	<0.001***
	G_H x Plant species	0.09	""	0.002**
	G_H x $G_{host\ plant}$	0.15	""	0.015*
Aphid (A) genetic	Aphid genotype	0.10	Binomial glm	<0.001***
variation	Plant species	0.03	""	<0.001***
Parasitoid	Plant genotype	0.01	""	0. 099'
	G_{aphid} x Plant species	0.00	""	0.239
	G_{aphid} x $G_{host\ plant}$	0.03	""	<0.05*
Aphid (A) genetic	Aphid genotype	0.02	ANOVA	0.131
variation	Plant species	0.55	""	<0.001***
Fecundity	Plant genotype	0.04	""	0.211
*218 00 excluded	G_{aphid} x Plant species	0.01	""	0.159
	G_{aphid} x $G_{host\ plant}$	0.03	""	0.175
H5 interaction	H presence	0.00	Binomial glm	0.183
in 2 aphid hosts	H pres. x G_{aphid}	0.02	""	0.003**
Parasitoid	Plant species	0.02	""	0.01*
	Plant genotype	0.03	""	0.065'
	H pres. x plant species	0.01	""	0.063'
	H pres. x $G_{host\ plant}$	0.02	""	0.244
H5 interaction	H presence	0.01	ANOVA	0.228
in 2 aphid hosts	H pres. x G_{aphid}	N/A	""	N/A
Parasitoid	Plant species	0.44	""	<0.001***
*218 00 excluded	Plant genotype	0.03	""	0.083'
	H pres. x plant species	0.00	""	0.87
	H pres. x $G_{host\ plant}$	0.02	""	0.627

3.1 *Hamiltonella* genetic variation

3.1.1 Parasitoid experiment

To explore the effects of symbiont genetic variation on aphid susceptibility to *A. ervi* I used 5 different *Hamiltonella* genotypes in 1 aphid clone as well as uninfected individuals from this clone to measure the overall effect of *Hamiltonella* presence. This section was designed to allow for effects of genetic variation in the bacterial symbiont to be observed, using protection against *A. ervi* to measure these effects. The effects of host plant species and genotype on *A. ervi* parasitism was also measured by keeping the aphids on 6 plant genotypes across 2 species. I also looked for interactions between plant genotype/species and *Hamiltonella* genotype.

The species of host plant the pea aphids were kept on significantly affected the parasitoid success rates ($\chi2 = 10.3$ df=1, p = 0.001) (**Figure 1**). Host plant genotype did not overall add to this species effect ($\chi2 = 1.7$, df=5, p > 0.9) (**Figure 2**) and there were no interactions between host plant and *Hamiltonella* genotype effects in this section or between host plant species and symbiont genotype. However, I did find a significant interaction between *Hamiltonella* presence and host plant species ($\chi2 = 3.9$, df = 1, p = 0.049) and a near significant interaction with host plant genotype as well ($\chi2 = 7.0$, df =5, p = 0.053). Host plant effects on parasitism are thus affected by *Hamiltonella* presence. In the absence of *Hamiltonella*, the proportion of aphids susceptible to *A. ervi* is higher when they're kept on *V. faba* (mean = 0.63) than *M. sativa* (0.45, p>0.05). Whereas, in the presence of *Hamiltonella* strain 4 (Vicia = 0.0, Medicago = 0.1, p >0.05) and strain 5 (Vicia = 0.16, Medicago = 0.53, p = 0.054), this effect seems to be reversed (**Figure 1**). It is unknown what effect the other 3 strains may have as they offered almost complete protection by themselves.

As expected, overall *Hamiltonella* presence reduced parasitism from *A. ervi* ($\chi2 = 83.3$, df =1, p < 0.001). *Hamiltonella* genotype also had a significant effect on pea aphid susceptibility to wasp attack ($\chi2 = 105.8$, df =4, p < 0.001) (**Figure 1**). *Hamiltonella* effects accounted for the majority of deviance reduced by the model with genotype explaining 33% and presence another 26%. Host plant species (3.2%) and genotype (0.6%) are thus much less powerful explanatory variables in this model.

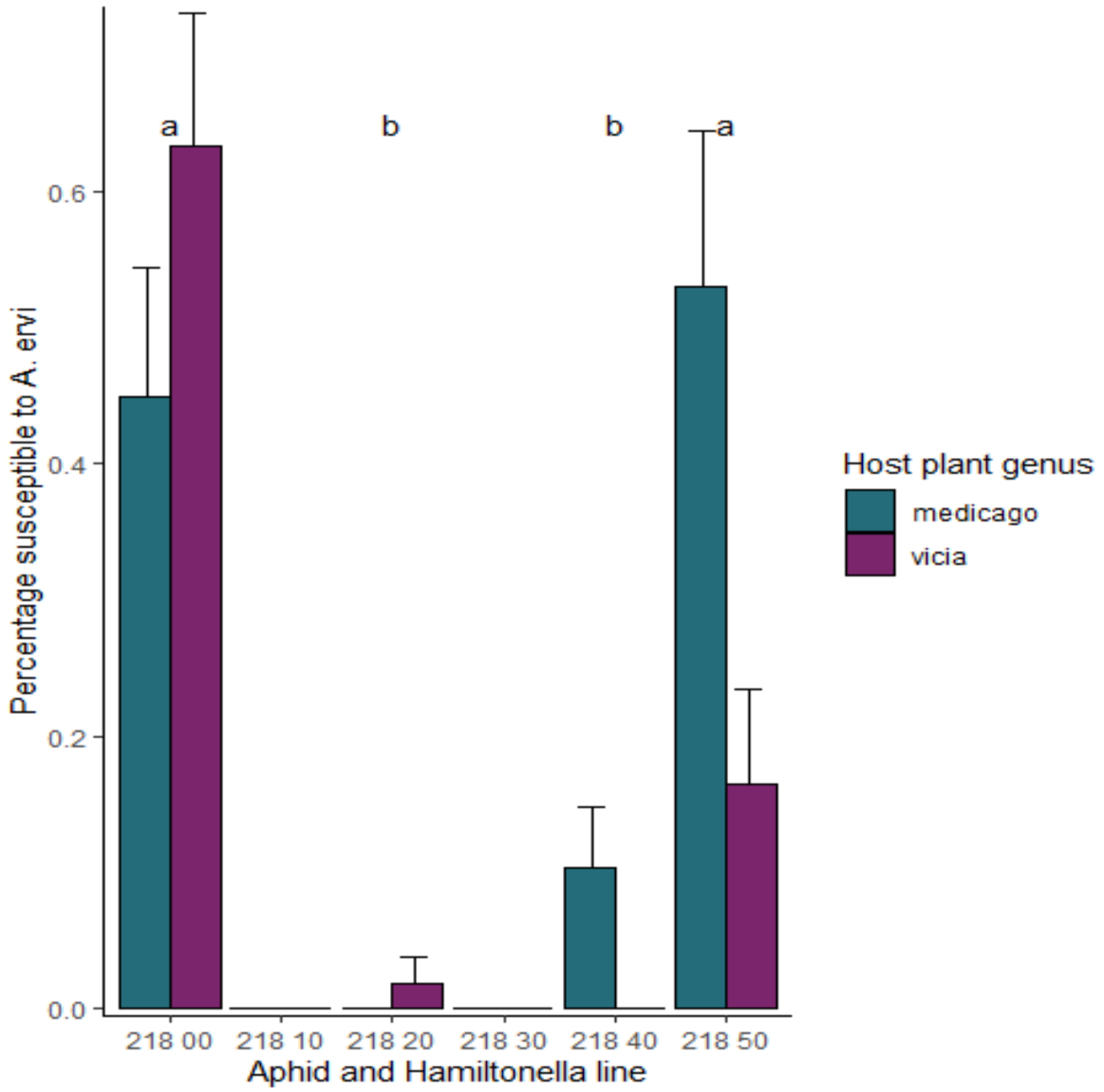

Figure 1. Effect of *Hamiltonella* genotype and host plant species on aphid susceptibility to *A. ervi*. On the X axis, 218 10 represents H1 etc. Bar whiskers show standard error and letters show significance groups between 'lines'. 218 10 and 30 showed no significant pairwise comparisons as they only produced 0% susceptibility results so sign tests were used to determine pairwise significance.

The significant genetic based variation in protection conferred by *Hamiltonella* was principally driven by H4 and H5 (**Figure 1**) as H1, H2 and H3 all offered close to or complete protection against *A. ervi*. These 2 *Hamiltonella* genotypes are part of the same clade (Smee et al., 2021). P values could not be calculated for 'glht' pairwise comparisons involving H1 and H3 as they had no values above 0. Therefore, sign tests were used to determine significance (J Ferrari, personal communication). H4 and H5 only differ significantly in their protection against *A. ervi* to uninfected aphids when aphids are kept on *V. faba* however (**Figure 1**). The strong protective effect of *Hamiltonella* presence was therefore principally driven by H1, H2, and H3 which gave aphids almost complete protection against *A.* ervi compared to very high susceptibility without the symbiont (mean proportion susceptible on *Vicia* = 0.63, on *Medicago* = 0.45).

The near complete parasitoid protection conferred by H1,H2 and H3 and very high protection given by H4 (**Figure 1**) may in some part explain the low plant deviance values as there were limited data where plant effects could actually be seen.

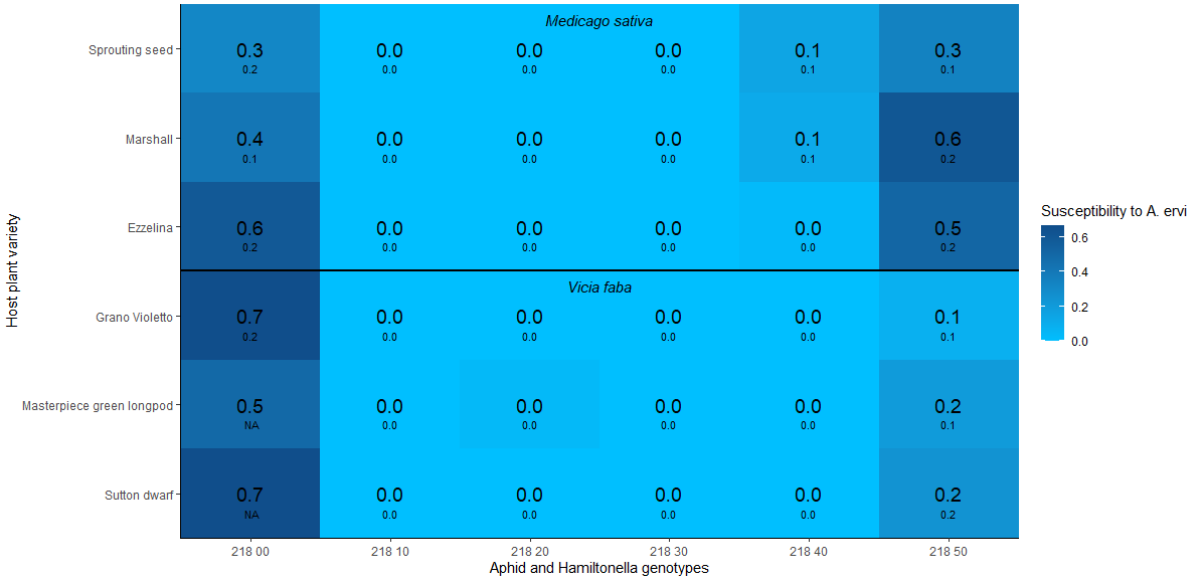

Figure 2. Effect of both *Hamiltonella* genotype and host plant variety visualised with a heatmap. The shade indicates susceptibility to *A. ervi*. There is variation present between different genotypes and species. The central values in each tile are the mean susceptibility, with the standard error beneath. Host plant varieties are separated into their two different species to aid comparisons.

3.1.2 Fecundity experiment

Facultative symbionts sometimes incur a cost as well as conferring protection against a natural enemy. Here, I am testing the effect *Hamiltonella* genotype has on aphid fecundity as well as host plant species and genotype. Additionally, any interactions between these effects have been tested. As in the parasitoid experiment in this section on *Hamiltonella* genetic variation, 1 aphid genotype was separated into lines infected with 5 symbiont genotypes or left uninfected and the aphids are kept on 6 genotypes of 2 host plant species.

Unfortunately, as discussed in the methods, this experiment was disrupted by high external temperatures which caused some lines to suffer reduced health and in particular 218 00. This is the case in all 3 fecundity experiments. Therefore, in the fecundity experiments, 218 00 aphids have been removed from the analysis which has limited the experimental power and the analyses possible.

Across this section host plant species (**Figure 3**) significantly affected aphid fecundity ($F = 24.5$, $df = 1$, $p < 0.001$) and host plant genotype (**Figure 4**) had a significant additional effect ($F = 13.3$, $df = 5$, $p < 0.001$) as well. There are significant interactions between plant and symbiont effects here. Both the effects of plant species ($F = 4.6$, $df = 4$, $p = 0.002$) and genotype ($F = 2.0$, $df = 20$, $p = 0.015$) on aphid fecundity are significantly affected by *Hamiltonella* genotype but not by *Hamiltonella* presence in the 218 aphids. Fecundity is significantly higher in aphids infected with H2, H4 and H5 and kept on Vicia compared to those on Medicago, but the same is not true aphids infected with H1 and H3 where fecundity looks evenly matched between the 2 host plants (**Figure 3**). At the host plant genotype level, the significant interaction between *Hamiltonella* and plant genotype effects was again caused by H1 and H3 aphids. For example, in one plant variety, H1 had 0.6 offspring per day and H3 0.2, while H2, H4 and H5 had 4.7, 6.9 and 6.5 respectively (**Figure 4**).

Overall, there was a significant genotype effect of *Hamiltonella* on aphid fecundity($F = 10.7$, $df = 4$, $p < 0.001$) (**Figure 3**). The relative importance of *Hamiltonella* and host plants on aphid

fecundity was somewhat balanced here with ~21% of the variance was explained by *Hamiltonella* genotype, host plant species explaining ~12% and genotype accounting for an additional ~14%.

Interestingly aphids infected with the two strains (H4 and H5) that offered the lowest protection against *A. ervi* have significantly higher fecundity than H1 and H3 which offered the most parasitoid protection (**Figure 3**). Due to the exclusion of data from uninfected 218 aphids, it is not possible to determine whether *Hamiltonella* presence overall reduced fecundity in 218. However, average fecundity across H1 and H3 was 3.2 (95% CI [2.4, 4.0]) offspring per day while the average offspring of the 3 other aphid genotypes without *Hamiltonella* was 5.1 (95% CI [4.4, 5.8]) offspring per day. Therefore, there is some evidence H1 and H3 *Hamiltonella* strains reduced aphid fecundity.

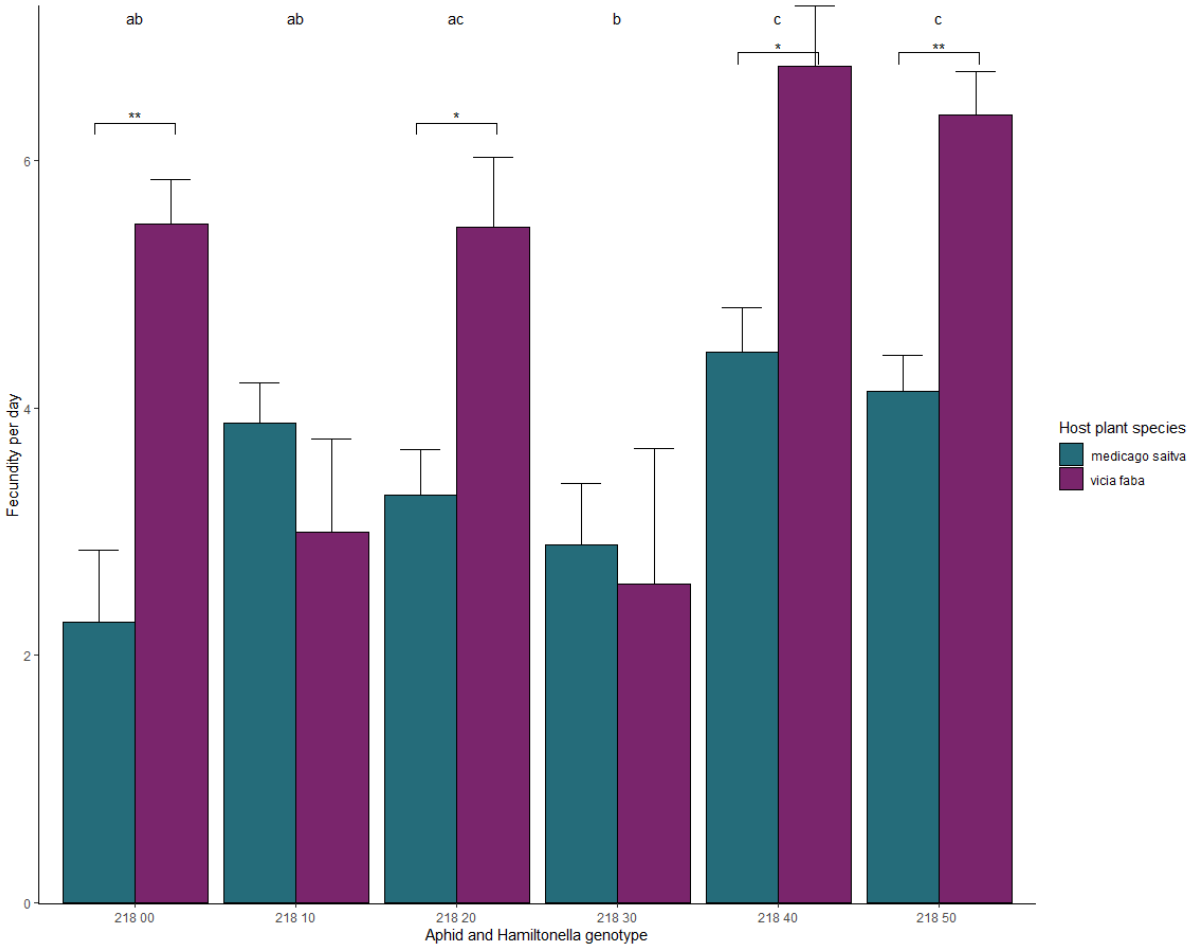

Figure 3. The fecundity of 218 aphids kept on 2 host plant species with no symbiont and 5 different strains of *Hamiltonella*. The whiskers represent standard error. Significance groups between lines are denoted by letters and significant differences between species are denoted with asterisks.

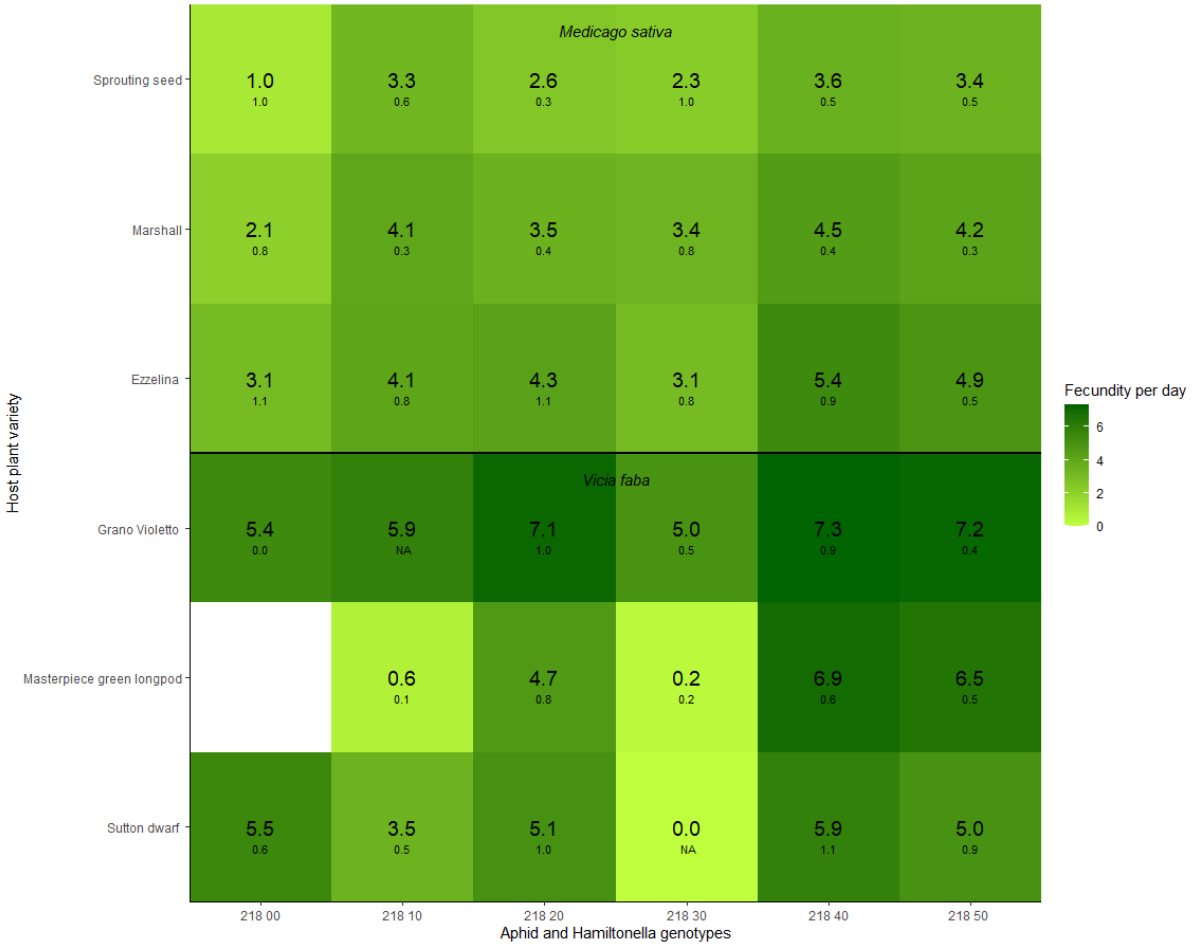

Figure 4. The effect of *Hamiltonella* genotype as well as host plant genotype on 218 aphid fecundity. The *V. faba* genotype 'Grano violetto' has higher fecundity than either 'Sutton dwarf' (p = 0.006) or 'Masterpiece green longpod (p = 0.003). The *M. sativa* genotype 'Ezzelina' led to higher aphid fecundity than the genotype 'Sprouting seed' as well (p = 0.011). This is a heatmap with the shade of green representing average fecundity per day. The central figures are mean fecundity per day and the figures beneath the mean in each tile are the standard errors where possible to calculate. Blank tiles represent missing data.

3.2. Aphid genetic variation

3.2.1 Parasitoid experiment

This parasitoid experiment was designed to test whether host plant species or genotype affect the innate resistance of aphids to *A. ervi* and whether any effect varied by aphid clone. Whether aphid genotype is important in determining parasitoid resistance was also explored here. 4 aphid genotypes were used consisting of aphids from *trifolium and medicago* biotypes. Therefore, 3 host plant species were used with aphids being kept on their native host and 8 genotypes were used: the 6 previously used and 2 *T. pratense* strains.

The host plant species once again significantly affected parasitism across these uninfected aphids ($\chi 2 = 20.8$, df = 2, $p < 0.001$) with aphids kept on *V. faba* being more susceptible to *A. ervi* than those kept on the other species (**Figure 5**). This effect of plant species did not interact with aphid genotype. There is sufficient evidence to suggest a possible separate effect of host plant genotype on aphid parasitism (**Figure 6**) although this effect was not significant ($\chi 2 = 4.3$, df = 7, p = 0.099). The effect of host plant genotype did significantly interact with aphid genotype however (df = 11, $p < 0.0496$) (**Figure 6**). This significant interaction may be caused partially by 1 *Vicia* variety having beyond a standard error higher susceptibility than the other

2 varieties in only 1 (313) out of 4 aphid clones. Similarly, 1 *Medicago* variety has over a standard error more susceptibility than the others in 218 aphids but not 200 and 1 *Trifolium* variety in md10 but not 313 aphids (**Figure 6**). None of these differences are statistically significant though.

The aphid genotypes differed significantly in their innate resistance to *A. ervi* ($\chi 2 = 59.6$, df = 3, $p < 0.001$) with 1 clone (md10) having particularly high susceptibility (mean proportion = 0.69) and 1 clone (200) having much lower susceptibility (0.34) (**Figure 5**). There was no difference in parasitism between the 2 biotypes. Overall, aphid genotype explained ~9.7% of the reduction in deviance with host plant species (~3.4%) and genotype (~0.7%) explaining less. Quite a large proportion of the deviance in the data is unexplained by our model suggesting unknown factors may be important or this set up generated high stochastic variation. Within these genotypes however, it does seem clear *Hamiltonella* strain is a more important predictor of aphid parasitoid resistance.

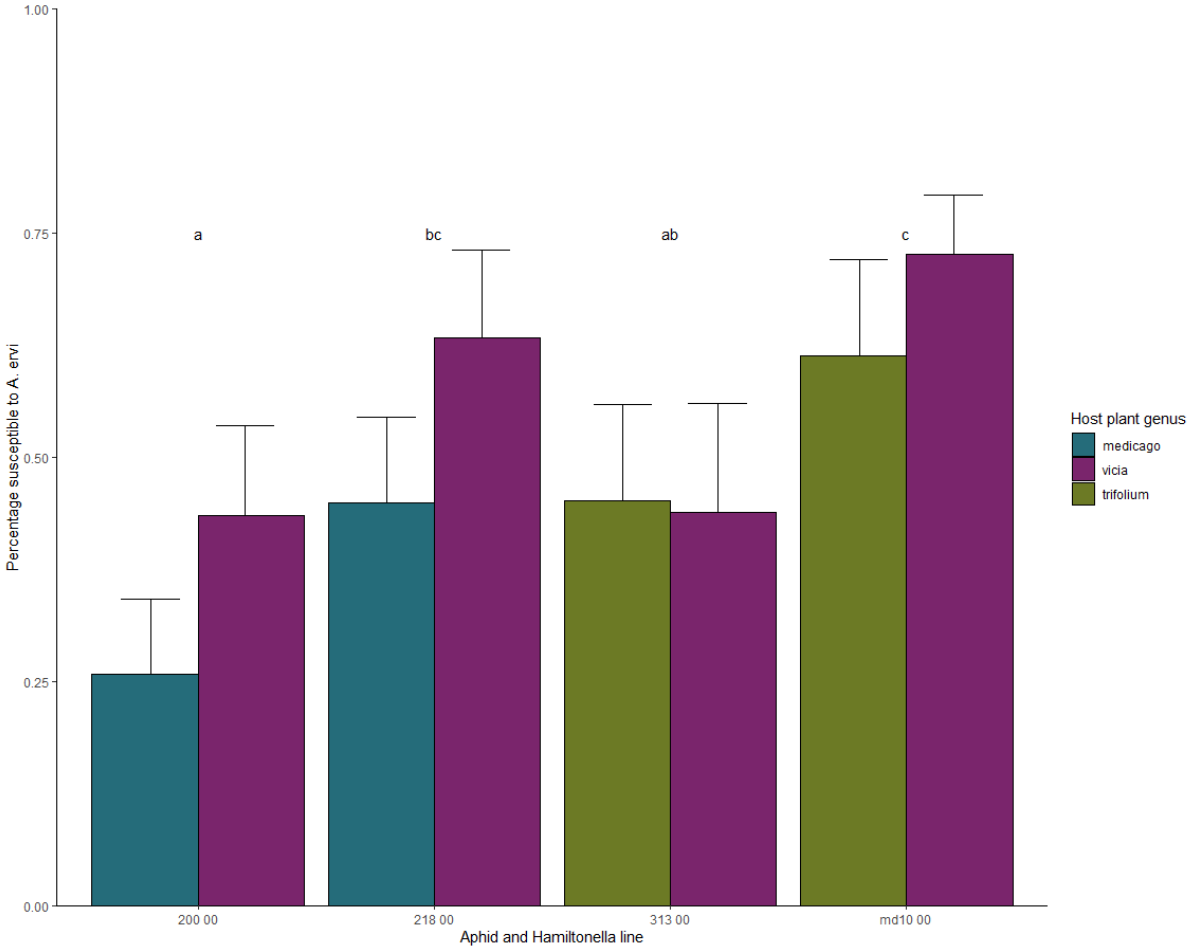

Figure 5. The effect of aphid genotype and host plant species on aphid susceptibility to *A. ervi*. The whiskers on each bar represent an individual standard error. Significance groups between lines have been notated with letters.

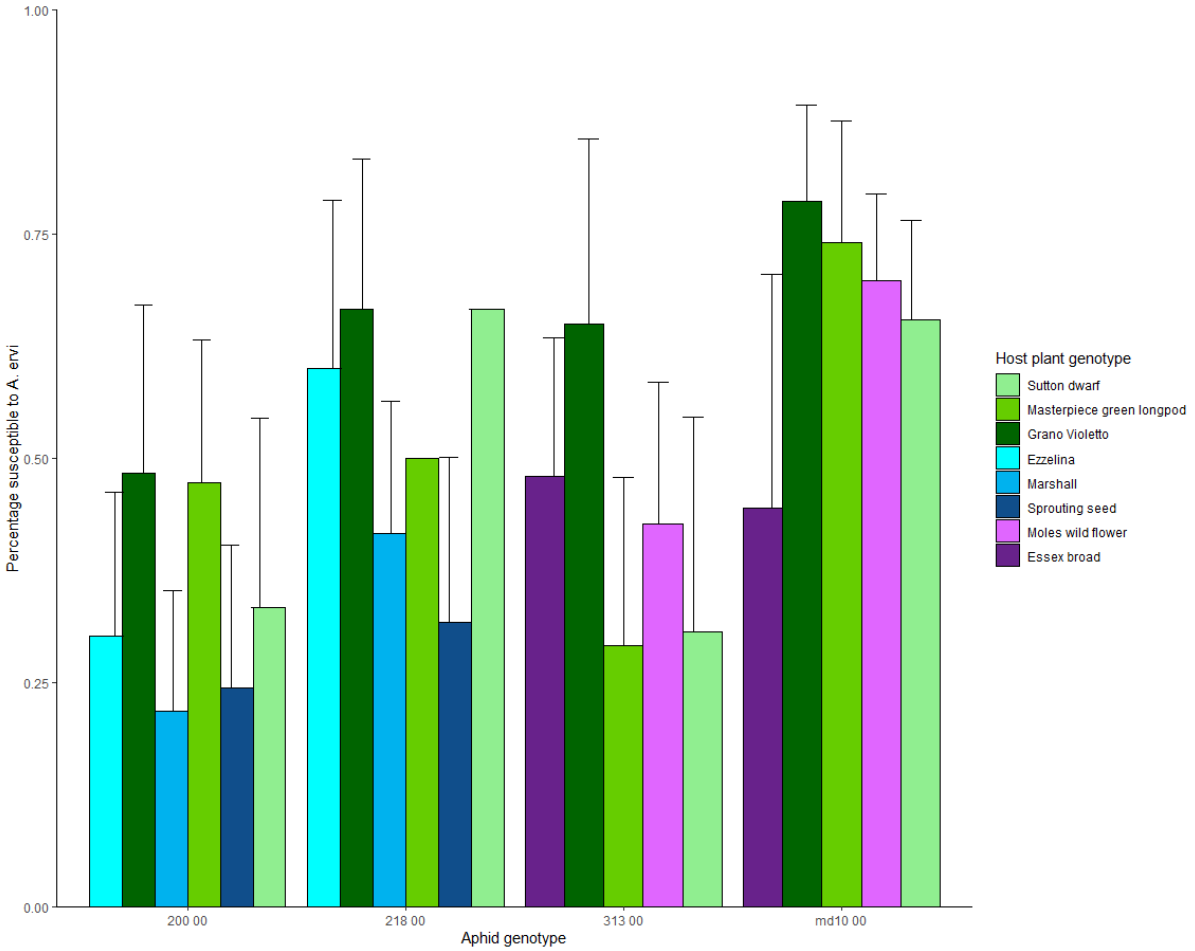

Figure 6.

The effect of plant genotype on susceptibility to *A. ervi*. Species are separated by colour and variety by shade. The *V. faba* genotypes 'Grano violetto' ($p<0.01$) and 'Masterpiece green longpod' ($p=0.01$) both lead to higher parasitisation than the *M. sativa* genotype 'Marshall'. In addition, although not statistically significant, 'Grano violetto' is associated with an increase in susceptibility compared to the other *V. faba* genotypes in all 4 aphid lines and this increase is above 1 standard deviations in 313 and md10. The bar whiskers represent standard error and where absent standard error was not possible to calculate.

3.2.2 Fecundity experiment

Here, I am exploring the effect of host plant species and genotype on pea aphid fecundity and whether this effect varies with aphid clone. The same 4 aphid clones across two biotypes were used, with 8 plant species across the 3 previously used species of plant. However, the data from 218 uninfected aphids were removed from analyses as previously discussed.

Host plant species had a significant effect on aphid fecundity ($F = 36.5$, $df = 2$, $p < 0.001$) (**Figure 7**). Aphids kept on *V. faba* had a higher fecundity per day (5.84) than those kept on either *T. pratense* (4.54) or *M. sativa* (3.18) (**Figure 7**). In the aphid clone 313, this species effect did not occur (**Figure 7**) and indeed when the 218 data are added to the model, a significant interaction between plant species and aphid genotype is found ($F = 30.5$, $df = 3$, $p <0.001$). However, there is no interaction without the 218 aphids and the 218 clone fecundity data are potentially unreliable, so there is insufficient evidence to state whether a significant interaction between host plant species and aphid genotype exists. Host plant genotype had no additional effect on aphid fecundity ($F = 11.9$, $df = 7$, $p = 0.21$) and there were no plant and aphid genotype interactions (**Figure 8**).

Aphid genotype had no effect on fecundity in this experiment (F = 2.3, df = 2, p = 0.13) (**Figure 7**). Excluded clone 218 had significantly lower fecundity than all 3 included clones but due to high mortality and poor health it is impossible to determine whether the low fecundity is driven by environment or genotype. Unsurprisingly given the data, host plant species explained the majority (~55%) of the variance in aphid fecundity while host plant genotype explained ~4%. Aphid genotype only explained ~1.8%. Host plant species appears to be the biggest driver behind differences in uninfected aphid fecundity here.

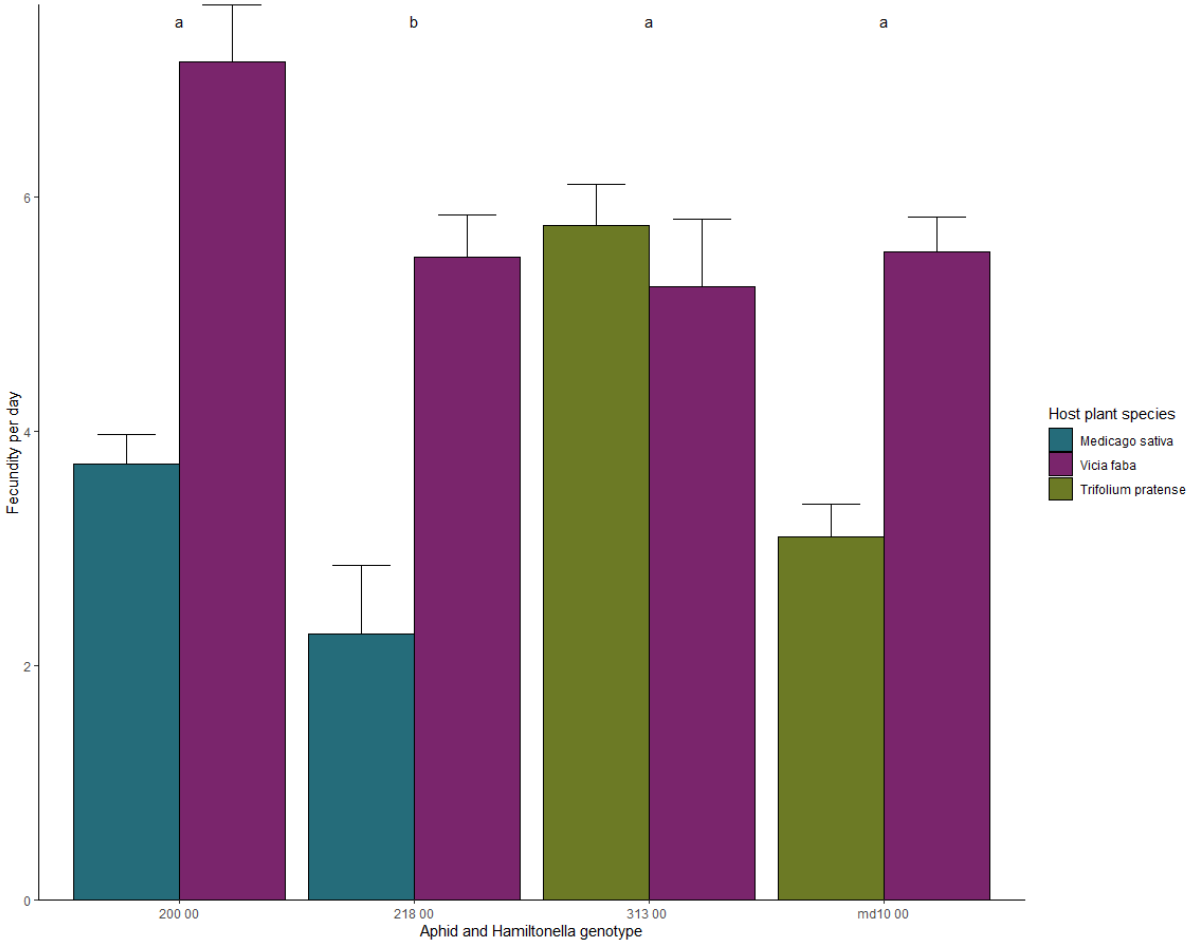

Figure 7. The effect of aphid genotype and host plant species on fecundity. The whiskers represent standard error. Significance groups between lines are denoted with letters and significant differences between species with asterixis.

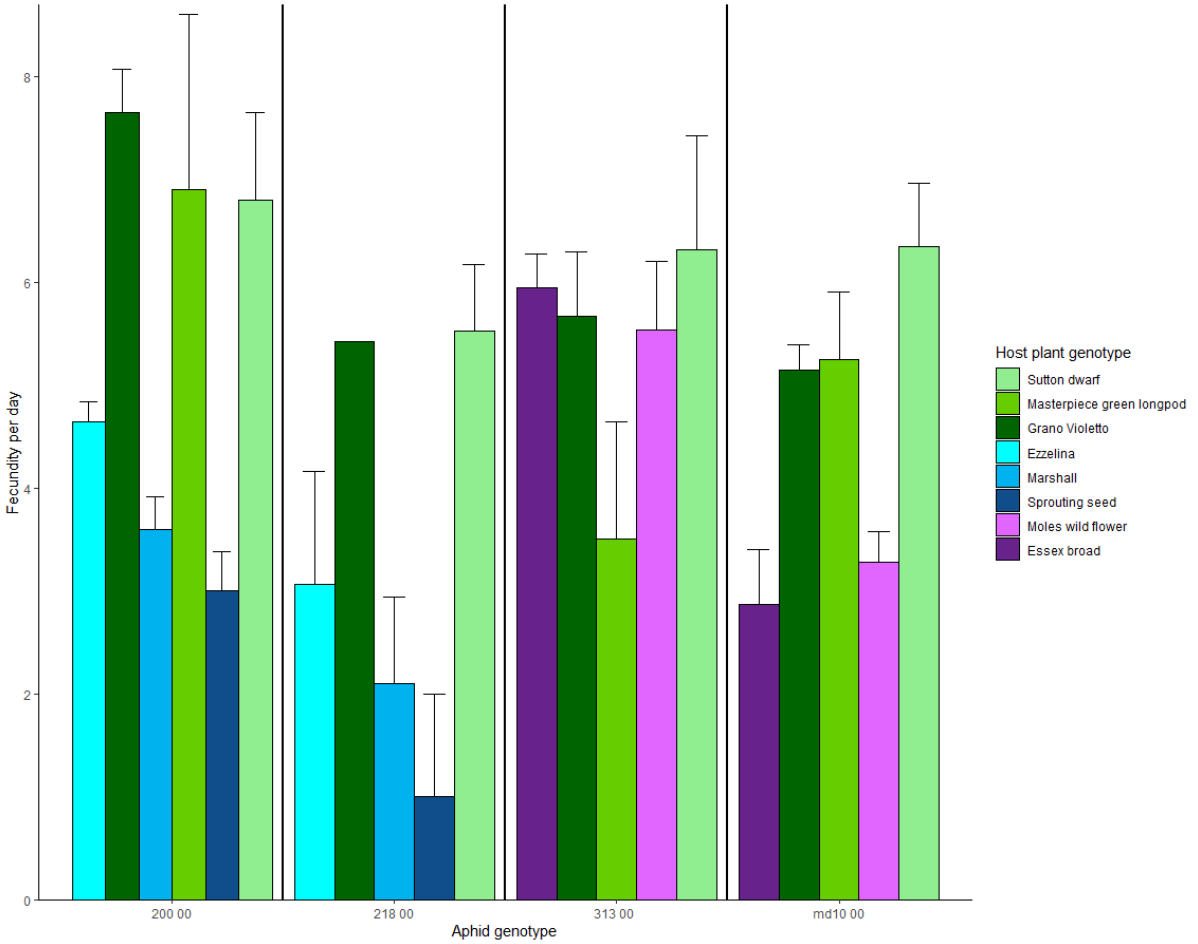

Figure 8. The effect of host plant genotype on aphid fecundity. Species are represented by colour and genotype by shade. The whiskers represent standard error.

3.3 H5 interaction in two aphid hosts

3.3.1 Parasitoid experiment

In this section I aimed to test whether the protective effect of a *Hamiltonella* strain (H5) would be altered by occupying a different aphid host (md10). I also tested whether host plant species/genotype would interact with any potential aphid and symbiont genetic interactions to form tritrophic genetic interactions. General effects of plant host species and genotype across this data were also examined along with the overall effect of *Hamiltonella* presence.

Overall, *Hamiltonella* presence had no significant effect in this dataset ($\chi2 = 1.8$, df = 1, p = 0.183). This is because of a significant interaction between H5 presence and aphid genotype ($\chi2 = 8.6$, df = 1, p = 0.003). In this analysis, H5 presence significantly decreases parasitoid susceptibility in the 218 clones compared to uninfected aphids, but has no effect in the md10 aphids (**Figure 9**).

The host plant species also significantly affects aphid susceptibility to *A. ervi* ($\chi2 = 9.2$, df = 2, p = 0.01). This was driven in the *trifolium* biotype with aphids kept on *V. faba* having lower resistance to wasp attack than aphids kept on *T. pratense* (**Figure 9**). In the *medicago* biotype, there is no real difference between the two host plants as aphids kept on *V. faba* have higher susceptibility in uninfected 218 clones and the opposite in clones infected with H5 (**Figure 9)**. This makes it very likely an interaction between host plant species and Hamiltonella presence

exists, although in this case the interaction was just above the significance threshold ($\chi 2$ = 5.5, df = 2, p = 0.062). Therefore, despite the lack of significance, there is some evidence for tritrophic genetic interactions between host plant, symbiont and aphid in response to parasitoid attack. This is due to the fact that the possible interaction between *Hamiltonella* presence and host plant species is only apparent in 1 of the aphid genotypes, suggesting G x G x G interactions as a potential explanation.

There was insufficient evidence to state that host plant genotype had an effect on aphid susceptibility to *A. ervi* in this dataset as the p value was also just above the threshold ($\chi 2$ = 13.8, df = 7, p = 0.065) (**Figure 10**). There were also no interactions between host plant genotype and *Hamiltonella* presence. Aphids that fed on 1 variety of *T. pratense* ('Essex broad leaf') however, had a significantly lower susceptibility to *A. ervi* than 4 out of 5 other *trifolium* biotype host plant genotypes (**Figure 10**) and the p value was 0.056 for the 5th pairwise comparison. Therefore, although overall the effect of host plant genotype did not reach the significance threshold, it is likely that at least within md10 aphids, the host plant genotype is important in determining parasitoid resistance.

The model did not explain much of the null deviance. Interaction between *Hamiltonella* presence and aphid genotype explained ~2% but further interactions with plant species explained less than 1%. Overall, host plant species explained 2% and genotype 3%. Therefore, although the model leaves much deviance unexplained, the variables were fairly evenly matched in this section.

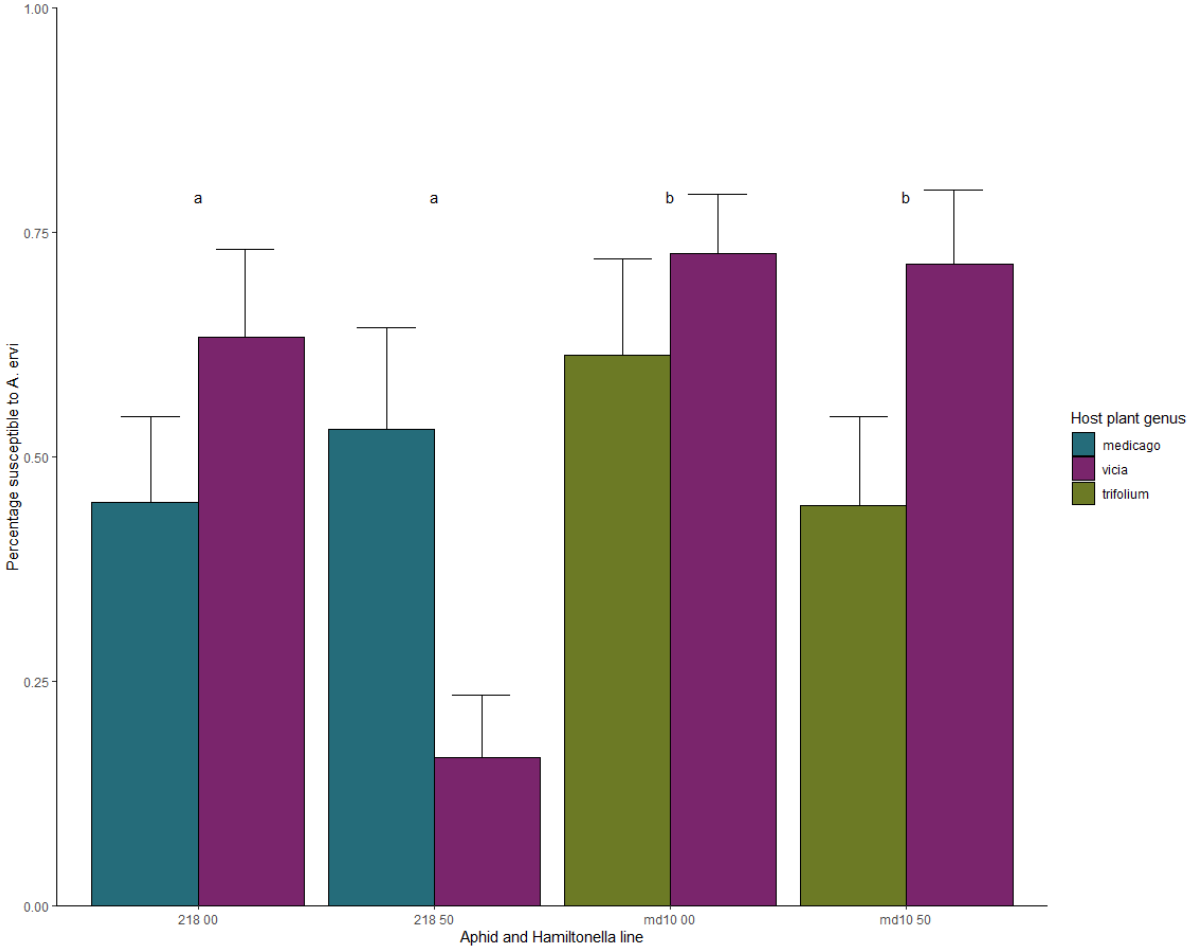

Figure 9. The susceptibility of two aphid genotypes to *A. ervi* with and without H5 and across different host plant species. The whiskers represent standard error calculations. Significance groups between aphid lines are denoted by letters.

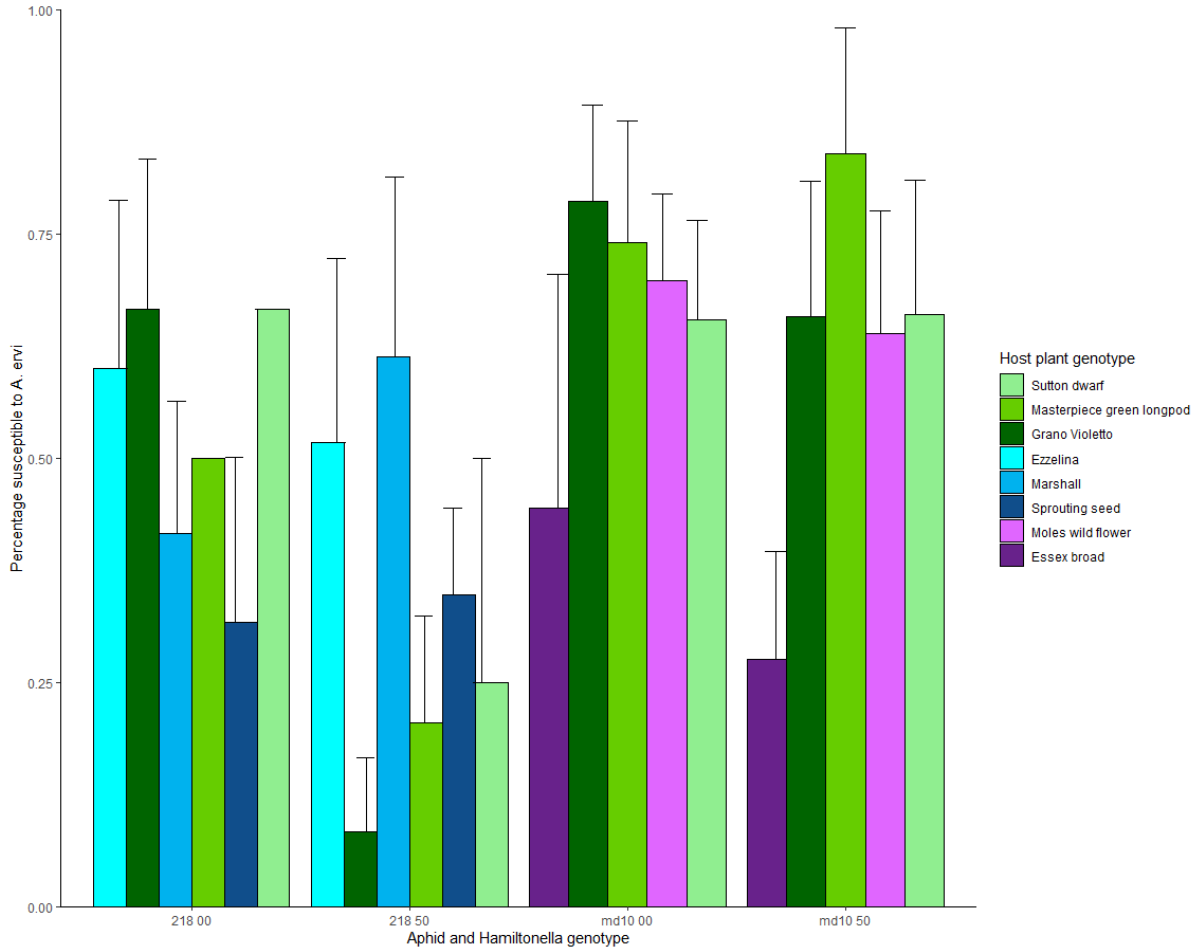

Figure 10. The susceptibility of two aphid genotypes to *A. ervi* with and without H5 and across different host plant genotypes. Species are represented by colour and genotype by shade. The whiskers on the bars represent standard error.

3.3.2 Fecundity experiment

This section originally aimed to examine the differing fecundity costs to 2 aphid genotypes harbouring the same symbiont and the host plant species/genotype interactions. Due to the exclusion of 218 00 data, this is not possible. However, I have still tested whether in this data overall H5 has affected aphid fecundity and the plant species and genotype effects in this dataset.

Host plant species once again has a significant effect on aphid fecundity ($F = 33.8$, $df = 2$, $p < 0.001$) although this is not a surprise as only the md10 H5 data are new here (**Figure 11**). There is no interaction between host plant species and aphid genotype or *Hamiltonella* presence as aphids kept on *V. faba* had higher fecundity in all 4 aphid lines (**Figure 11**). Host plant genotype did not have an additional significant effect on aphid fecundity (**Figure 12**) although there is some evidence of a plant genotype role ($F = 11.3$, $df = 7$, $p = 0.083$). There was no interaction between *Hamiltonella* presence and or aphid genotype and host plant genotype.

Overall, *Hamiltonella* had no effect on aphid fecundity here ($F = 1.2$, $df = 2$, $p = 0.228$). There is consistency with how H5 impacted aphid fecundity in both 218 and md10 aphids in comparison with other *Hamiltonella* strains in 218 aphids although no statistically relevant comparison can be drawn due to the loss of 218 uninfected aphids.

Here, host plant species explained ~44% of the variance in aphid fecundity with plant genotype explaining another 3%. *Hamiltonella* presence explained ~1%. As in the fecundity experiment with uninfected aphids, host plant species is the most important factor in determining aphid fecundity.

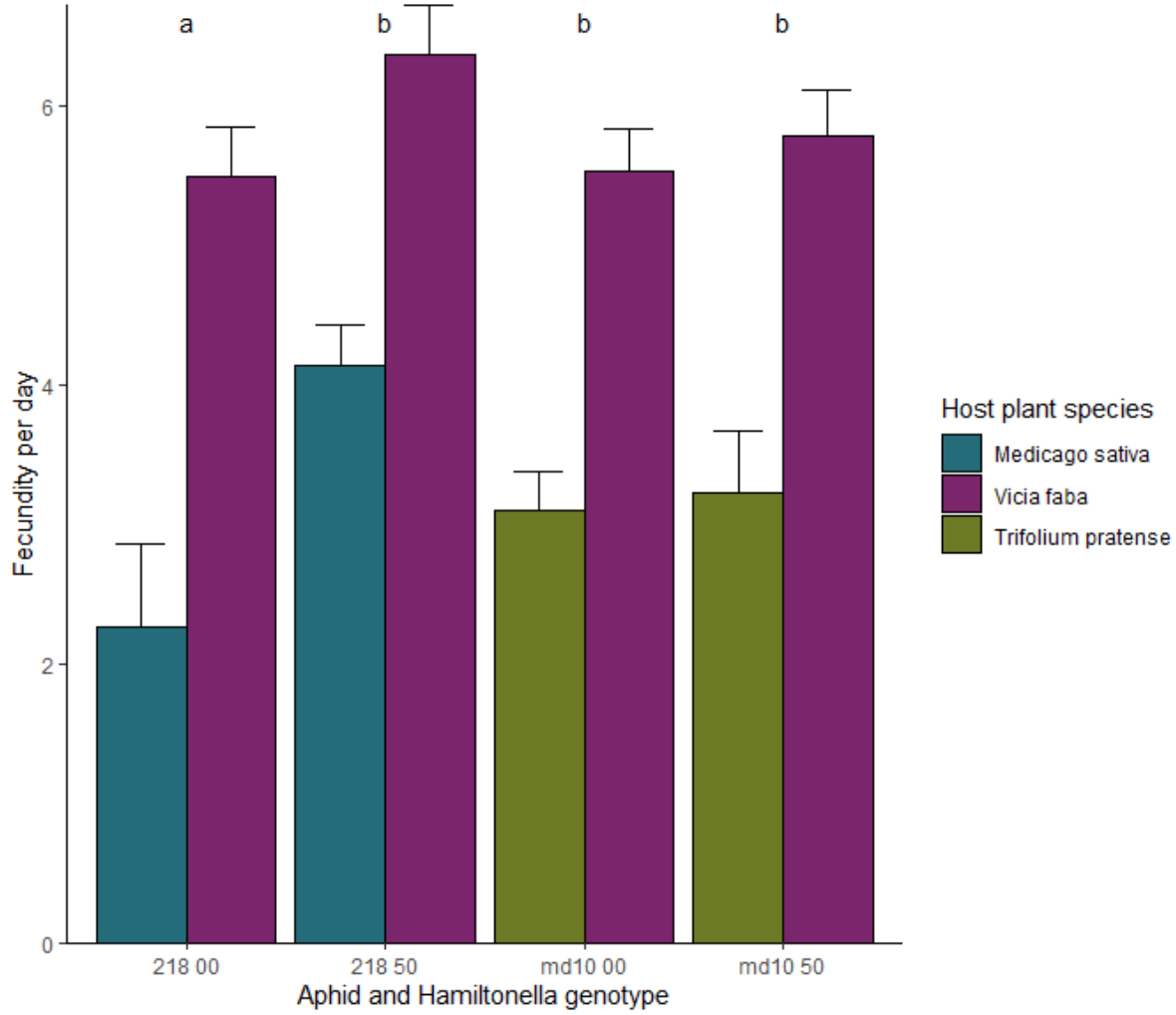

Figure 11. Aphid fecundity across different aphid lines and host plant species. The whiskers represent standard error. Differences between lines are denoted by significance groups.

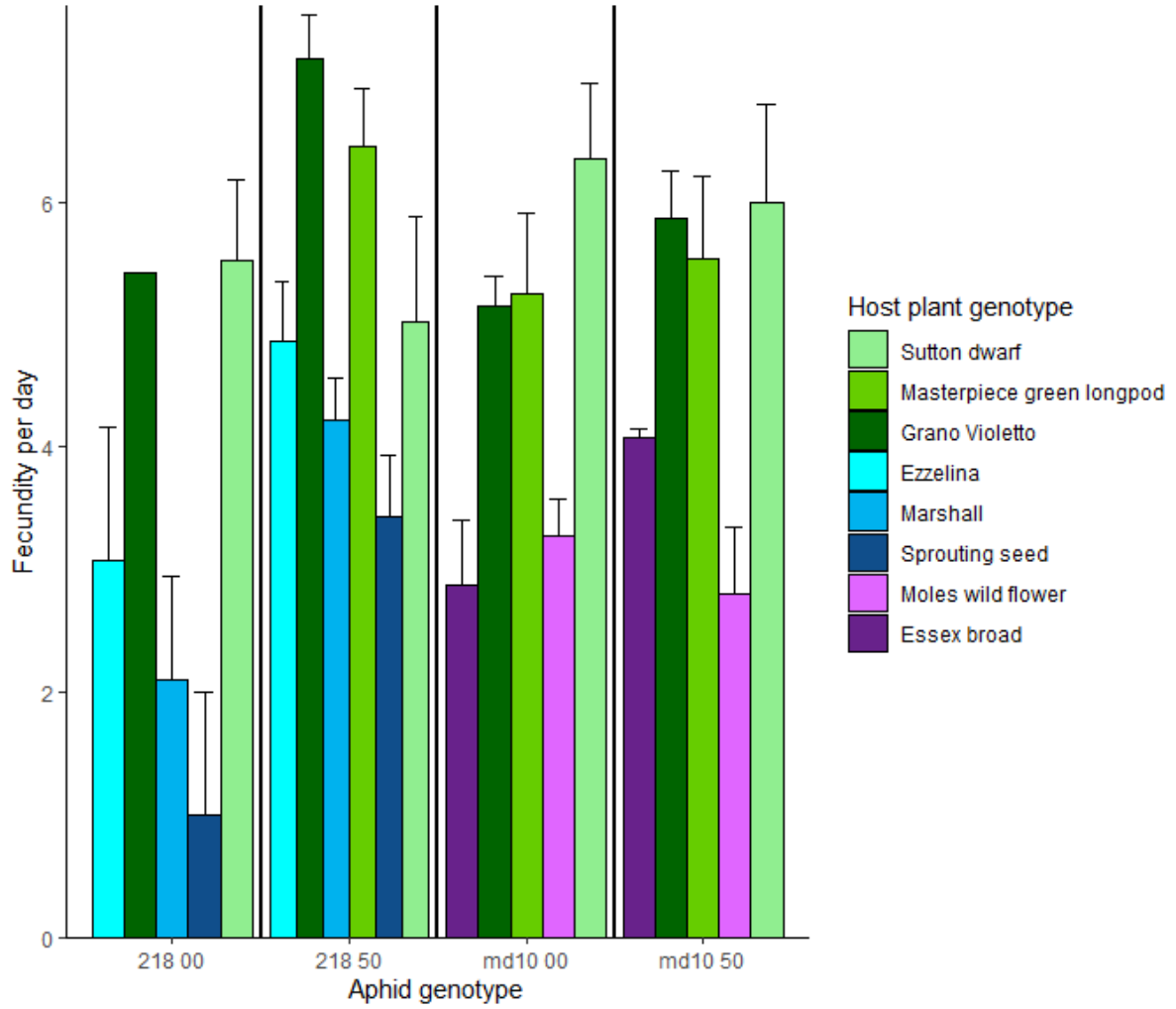

Figure 12. The effect of host plant genotype on aphid fecundity. Colour represents species and shade represents genotype. No pairwise comparisons were significant between genotypes within species. Whiskers represent standard error.

4 Discussion

4.0 Summary of results

Overall, the results showed a significant effect of aphids' host plant on the resistance to parasitism from *A. ervi*. Both plant species, and to a lesser extent genotype, impacted aphid susceptibility. There were also significant genetic interactions between uninfected aphids genotypes and the host plant genotype. Host plant species may also have interacted with $G_{Hamiltonella}$ x G_{aphid} effects on aphid parasitoid susceptibility to create tritrophic interactions although this was narrowly above the significance threshold. As far as I am aware these are novel findings. I also found both *Hamiltonella* and aphid genotype effects on resistance to *A. ervi* as expected and an interaction between *Hamiltonella* presence and aphid genotype. *Hamiltonella* genotype significantly affected aphid fecundity although there was no genetic derived variation in fecundity between uninfected aphid clones. Host plant species had a significant effect on aphid fecundity as expected and host plant genotype had an effect on *Hamiltonella* infected aphids from one clone.

4. 1 Host plant effects

4.1.1 Plant effects with different *Hamiltonella*

In the *Hamiltonella* genetic variation section, 218 clone aphids were infected with 5 different *Hamiltonella* strains or kept symbiont free and exposed to *A. ervi* to test the effects of symbiont presence, genetic variation and any interactions with host plant species and genotypes. The plant species aphids were fed on during the experiment significantly affected the rates of successful parasitism. This species effect did not interact with *Hamiltonella* genotype but did with *Hamiltonella* presence (Figure 1) suggesting the effect host plants have on *A. ervi* is altered by the symbiont. Overall, the genotype of the host plant did not have an additional effect on aphid resistance but there was strong (albeit not quite significant) evidence for interactions between host plant genotype and *Hamiltonella* presence (Table 3). Taken together, these results show that different host plant species and varieties may confer varying effects on aphid resistance to *A. ervi* depending on whether *Hamiltonella* is absent or present.

This interaction between symbionts and host plants in parasitoid resistance has not been looked for previously in the pea aphid system so these results are novel. Despite the ubiquity of herbivorous insect-parasitoid relationships in communities, the whole area remains understudied (Ode, 2019). In addition, bottom-up forces in community structure such as from insect-herbivore interactions have been found to be no more important than top-down forces such as parasitoid and predator attacks (Vidal and Murphy, 2018). Therefore, to get a complete picture of how species and populations interact within communities both bottom-up and top-down effects should be considered such as the interactions found here between plant, symbiont and parasitoid.

Despite separation by a trophic level, there is an evolutionary pressure in host plants to reduce aphid success in resisting parasitoid attacks which will indirectly reduce herbivory. Equally, in parasitoids, there is an evolutionary pressure to subvert plant defences to increase host health which will increase parasitoid fitness (Kagata and Ohgushi, 2006). For example, Ode (2019) proposes interactions between parasitoids and symbiont may alter SA and JA mediated defensive pathways in plant hosts of herbivorous insects such as had been found with symbionts in their host previously to increase host fecundity (Shikano et al., 2017). Therefore, the evolutionary relationship between parasitoids and host plants may be strong and resemble a Red Queen style of coevolution leading to variation in parasitism caused by interaction

between plants, symbionts, insects and parasitoids as hinted at by the present study's findings.

In this section aphids with H4 and H5 strains saw a large reduction in protection when kept on *M. sativa* vs *V. faba* (figure 1). Although not significant, the opposite appeared to occur in uninfected 218 aphids and indeed overall, uninfected aphids across the four genotypes experienced higher parasitism on *V. faba* which we will discuss later. In addition, Smee et al. (2021) found H5 to offer much higher levels of protection than found here and in that paper the aphids were kept on *V. faba*. This all suggests *M. sativa* may interfere with *Hamiltonella* mediated protection of pea aphids against *A. ervi*. This would of course be in the fitness interests of *M. sativa* plants. It would also make sense that this occurred in aphids kept on *M. sativa* and not *V. faba* as the aphids and symbionts used in this section were all collected from *M. sativa* in the field. Potentially a *M. sativa* derived toxin or metabolite interferes with the cdtB toxin produced by *Hamiltonella*. Although all *Hamiltonella* contain this toxin, the ASPE-2 phage is highly variable (Degnan and Moran, 2008) so perhaps a mutation in the H4/H5 clade makes it vulnerable. Future research could look at cdtB toxin expression in *medicago* aphids containing a *Hamiltonella* symbiont kept on either *M. sativa* or *V. faba*. Other members of the H4/H5 clade could be found and tested for this reduced protection in *M. sativa* to determine whether this is likely an interaction between symbiont genotype and host plant. Aphids from other biotypes which still regularly possess *Hamiltonella* (Ferrari et al., 2012) could be infected with H4 and H5 and parasitism recorded with aphids kept on native host plants and *V. faba* to explore whether my result is *M. sativa* or *V. faba* dependent.

It is worth noting that in this section and some others, although host plants have a significant effect on aphid parasitoid resistance and fecundity, the deviance or variance explained by host plants was quite low (table 3). Plenty of noise is generated in studies such as these (Smee et al., 2021) and factors such as high external heat will not have helped and this stochastic noise may have artificially decreased the explanatory power of tested variables a little. Nonetheless, the low deviance found indicates that the overall biological effect of host plants on aphid-symbiont-parasitoid relationships in real communities may be important but relatively low.

The fecundity was also measured in *Hamiltonella* infected 218 aphids. Plant species had a significant effect on fecundity and this effect significantly interacted with *Hamiltonella* genotype. The same was true for host plant genotype. Host plant has been thought to interact with symbiont to determine aphid fecundity on a species level (Henry et al., 2013) but not a genotype level previously. This is a novel insight into the complexity of plant-symbiont-aphid interactions before parasitoids are even considered. In addition, the relative benefits of symbionts may be altered by parasitoid frequency in a more complicated way than assumed. Prior research has suggested *Hamiltonella* would lose its fitness benefit in low parasitoid densities (Mclean et al., 2016) but some strains may offer varying benefits independent of parasitoid presence depending on the aphid host and the plant species it is feeding on.

4.1.2 Plant effects in different aphids

In this section four uninfected aphids were exposed to *A. ervi* and fed on eight different host plant genotypes across three species. Once again plant species had a significant impact on aphid resistance to *A. ervi* with aphids kept on *V. faba* suffering from higher rates of parasitism than aphids fed on *M. sativa.* (Figure 5). This effect did not significantly alter by aphid genotype although it appears to only happen in *medicago* aphids. However, while overall plant genotype offered no additional effect on parasitoid resistance, plant genotype did interact with aphid genotype (Figure 6). These are both novel results and show host plant species and genotype play a role in pea aphid innate resistance to *A. ervi* in these clones as well as *Hamiltonella* mediated resistance.

There has been insufficient research into this area to postulate the exact mechanism of interactions between *V. faba* and the inate immunity of aphids if such interactions exist as suggested by these results. However, members of the Lepidopteran insect order have been found to sequester potentially harmful secondary metabolites and toxins from their host plant in order to inhibit parasitoid growth (Nishida, 2002; Reudler and Nouhuys, 2018). As discussed, *medicago* aphids kept on their native host *M. sativa* were more resistant to parasitoid attack. This may occur as the longer evolutionary association between *medicago* aphids and their native host compared to *V. faba* will have given *medicago* pea aphids more time to adapt *M. sativa* derived toxins for defence against *A. ervi*. This could be further explored using additional *medicago* clones and testing whether parasitism is lower in their native host than *V. faba*.

Plant species also had a significant effect on aphid fecundity with aphids fairing better on *V. faba* than the 2 alternative host plants. There were no significant interactions between the effects of host plant species or genotype and aphid genotype on aphid fecundity and no significant effect of host plant genotype on fecundity in this section. *V. faba* is a universal host in pea aphids (Ferrari et al., 2008) and thus is likely to have lower specific defences against aphid biotypes and given its selective breeding as a crop plant it may have poorer anti-herbivore defences overall. This may explain the increased fecundity of both biotypes on *V. faba* and also explain the increase in parasitoid susceptibility. Lower host plant defences may be beneficial for aphids without natural enemies present but there would be less toxin to sequester to fight parasitoids. Insects in the field have been found to choose a less favourable host plant if there are also negative fitness consequences for its parasitoid (Singer et al., 2009; Smilanich et al., 2011; Milan et al., 2012). Insect host weight has been positively correlated with parasitoid fecundity (Karowe and Schoonhoven, 1992) and this form of evolutionary 'spite' (Gardner and West, 2004) could occur in largely clonal populations where a reduction in parasitoid fecundity at the expense of aphid fecundity could provide an indirect fitness benefit by decreasing parasitoid density.

4.1.3 Plant effects on H5 presence x aphid genotype interactions

In the third experimental set up two different aphid clones were infected with H5. I found an interaction between aphid genotype and *Hamiltonella* presence on parasitoid resistance which I will discuss below. Host plant species had a significant effect on *A. ervi* parasitism success overall in this section and there was a near significant (Table 3) tritrophic interaction between *Hamiltonella* presence, aphid genotype and host plant species.

Due to issues with microinjections as discussed in the methods this section was curtailed and thus lost a lot of experimental power. This could easily be fixed in future experimental work with a full factorial design involving several aphids and *Hamiltonella* genotypes along with the variation in host plants achieved here. This would of course take time to set up and run. However, the present set up still gives an insight into the complex nature of community interactions which was the central aim of this thesis

The behaviour of the H5 symbiont across both aphid genotypes provided more evidence for an antagonistic relationship between hoist plant and symbiont mediated protection against parasitoids (figure 9). In 218 aphids, as discussed, H5 confers far less protection against *A. ervi* when its hosts are fed on *M. sativa* which is the host plant H5 was collected from. Thus, there is more likely to be an evolutionary history between H5 and *M. sativa* than with *V. faba*. In md10 aphids, H5 confers less protection against *A. ervi* in aphids kept on *V. faba*, while performing better with *T. pratense*. *V. faba* is a potential host for *medicago* aphids and thus

H5 but *T. pratense* is not. Therefore, on both occasions, H5 confers less protection when its host aphids feed on the plant most closely linked with the symbiont. This suggests a model of host plant disruption to symbiont protection which would increase the fitness of host plants by reducing herbivore aphid density. Future work initially focussed on other members of the H4/H5 *Hamiltonella* clade could elucidate this potential pattern and shed insight into the evolutionary dynamics of this important model system.

4.2 Aphid genotype effects on parasitoid resistance and aphid fecundity

I found a significant effect of aphid genotype on the outcome of *A. ervi* parasitism. There is conflicting evidence in the literature as to the role of aphid genotype in defence against natural enemies (Oliver et al., 2005 and Vorburger et al., 2009) suggest a limited role and (Heyworth and Ferrari 2015; Parker et al., 2017) found evidence for a more important role. This thesis adds to the evidence for a significant role for aphid genotype in determining defence against *A. ervi*. Pea aphids have a complex innate immune system (Luo et al., 2021) so genetic variation in immune responses makes sense.

Only two out of the four aphid genotypes drove this genotype effect. This suggests that while genetic variation does affect parasitism it is not present in all aphid clones and may explain why aphid genetic variation has been missed by some research . In addition, aphid genotype did explain less deviance than *Hamiltonella* genotype suggesting across this set up *Hamiltonella* genotype was more impactful. This is supported by literature finding symbiont-parasite to be the key relationship in determining aphid susceptibility (Oliver et al., 2005; Vorburger et al., 2009; Mclean et al., 2016). These results, along with the host plant results, add evidence to the idea of a more complicated picture, but symbiont genotype seems to be the single largest factor in parasitoid success looking at the deviance explained in my results (table 3).

Future work could explore whether aphids which offer similar levels of resistance are more likely to be part of the same clade. If so, this could pave the way for GWAS type studies which could track down the chromosomes or even genes responsible. This would allow a better understanding of how innate resistance in insects may work against parasitoids and thus illuminate one of the most common symbiotic relationships in the animal kingdom. It may also help us understand how host plant species and genotype effects interact with this resistance.

Unexpectedly, aphid fecundity was not affected by aphid genetic variation. However, only three genotypes ended up as part of the analysis so it is possible that if we added more clones there would be aphid genetic based fecundity variation visible. Host plant species explained 55% of the deviance suggesting host plants play the largest role in aphid fecundity and not aphid genotype. The lack of interaction (table 3) between aphid genotype and plant species is surprising. This is especially surprising given that aphids from two biotypes both had lower fecundity on their native host. This may be due to being cured of symbionts. *Hamiltonella* has been associated with better aphid performance on *M. sativa* and *Regiella* with *T. pratense* (Henry et al., 2013). Without the symbionts aphids may find it more difficult to exploit their native host. Additionally, the native host plants may have acquired more successful anti herbivory defences as discussed above than *V. faba* which none of the aphids were collected from.

4.3 *Hamiltonella* effects on aphid parasitoid resistance and fecundity

4.3.1 *Hamiltonella* genetic variation effects

Across the five *Hamiltonella* strains that 218 aphids were infected with, there was a significant effect of *Hamiltonella* genetic diversity on parasitoid susceptibility. Both members of one clade

(H4 and H5) conferred significantly less protection than two members of the other clade (H1 and H3) (Figure 1). The behaviour of these strains was generally congruent with previous work looking at them (Smee et al., 2021) although H5 did offer less protection than expected in aphids kept on *M. sativa* as previously discussed. Overall, *Hamiltonella* reduced *A. ervi* success and its effect varied by strain which is in keeping with much of the literature on *Hamiltonella* (Oliver et al., 2005; Heyworth and Ferrari, 2015; Smee et al., 2021). While not novel, this is a nice sense check for the rest of the more interesting results.

Hamiltonella genotype also significantly affected aphid fecundity in 218 aphids. The ideal comparison to draw to determine a fitness cost of *Hamiltonella* would have been uninfected 218 aphids but these results are unreliable. Therefore, as different aphid clones did not differ in fecundity, a comparison was drawn with the average fecundity from uninfected aphids of other genotypes. The aphids that offered the most protection (H1 and H3) seemed to incur the highest fitness cost to their 218 host while H4 and H5 aphids did not appear to reduce fecundity. This is only an approximation however without 218 00 aphid data.

While imperfect, the comparison above could suggest a pattern between aphid cost to fecundity and symbiont mediated protection. This would require additional experimental work to confirm; ideally testing the effects of these strains on aphid fecundity in other aphid genotypes too. However, this has not been widely found (Mclean et al., 2016; Parker et al., 2017; Smee et al., 2021). This could occur with symbiont density. Higher density populations of *Hamiltonella* may produce more toxin, and thus parasitoid protection, but also incur a greater metabolic cost but again this is not supported by the literature. Alternatively, these results could simply be explained by the fact that symbiont and host genetic couplings may be idiosyncratic as Parker et al. (2017) suggest. Some combinations may be good for symbiont-mediated protection, some combinations may be good at reducing symbiont fecundity costs.

218 H1 is the only native symbiont-host line in the experimental setup. Neither Parker et al. (2017) or Smee et al. (2021) found evidence that symbionts incur lower costs or provide more protection in their native host. In fact, when looking at *Fukatsuia* the opposite was found (Smee et al., 2021) where the native *Fukatsuia* strain acted as a pathogen in its native host instead of a conditional mutualism. Here, H1 offers similar protection to its native host as other strains in the same clade and incurs a similar fecundity cost to 218 aphids as the related H3 strain. Thus, these data support the idea that symbionts are not particularly well adapted to their host. This suggests a possible mix of mutualistic and antagonistic selection pressures acting on this host symbiont relationship.

4.3.2 *Hamiltonella* role in H5 aphid x symbiont interactions

In this section H5 was added into two different aphid hosts and the effects on parasitoid success and fecundity were measured. H5 conferred significantly different levels of protection against *A. ervi* in the two aphid clones. This is further evidence that parasitoid success is more than just a function of symbiont-parasitoid interactions and is in fact affected by the aphids themselves as well as host plants as we have seen. Plant species had a significant interaction with these aphid genotype x *Hamiltonella* effects on parasitoid success. This is evidence for tritrophic interactions determining an ecologically important trait. Obviously, a fully factorial design could better test this as was initially planned for this thesis. Parasitoid genotype could also be included as Parker et al. (2017) showed parasitoid-host genetic interactions certainly play a role in parasitoid success.

Facultative symbionts (including *Hamiltonella*) reduce the expression of pea aphid innate immunity associated genes (Dubreuil et al., 2014) and reduce levels of phenoloxidase which is a key component of *A. pisum* immune response (Luo et al., 2021). This effect is dependent on the facultative symbiont species (Luo et al., 2021). This raises the possibility of *Hamiltonella*

and other symbionts altering expression of immune genes potentially involved in parasitoid response. It would require a fine balance for symbionts to reduce immunity towards them without compromising aphid health. This may be achieved in some aphid-symbiont genetic combinations better than others and be a source of variation. This could also be a source of plant based variation if symbionts also alter expression of genes involved in defence against toxins and other plant derived defence mechanisms. This seems like a potential mechanism for the host plant adaptability conferred by *Regiella* on *T. pratense* and *Hamiltonella* on *M. sativa* (Henry et al., 2013).

The fecundity aspect of this section was further curtailed by the loss of 218 00 aphid data. It was not possible to compare the effects of H5 in 218 and md10 properly without uninfected 218 aphids. However, it did not appear H5 caused a fecundity cost in 218 and there was definitely no fecundity cost of H5 in md10 aphids. Therefore, I found no evidence for *Hamiltonella* x aphid genotype effects on fecundity although this limited experiment does not provide much evidence against this sort of interaction occurring. This is surprising and I expected H5 to differ in fecundity effect in 218 aphids and md10 aphids. *Hamiltonella* has been found to increase aphid success on *M. sativa* but not *T. pratense* so potentially md10 H5 aphids should see reduced fecundity (Henry et al., 2013). Not all strains of *Hamiltonella* were found to increase *medicago* aphids ability to feed on *M. sativa* however so perhaps H5 just does not contain this trait. Again a factorial study involving more symbionts in a complete set of aphid genotypes would offer a better insight into this.

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4.4 Conclusion

In conclusion, I have found evidence for intraspecific and interspecific variation across three trophic levels affecting both aphid fecundity and resistance to the parasitoid wasp *A. ervi* , and so therefore causing wide reaching community level effects. Novel findings included a host plant species level effect on both symbiont and aphid mediated parasitoid resistance as well as host plant and *Hamiltonella* genetic interactions significantly affecting aphid fecundity. I also found evidence for a variation in *Hamiltonella* mediated parasitoid protection based on the aphid genotype.

Thus, I have added to the growing body of evidence which suggests that community traits are driven by complex multitrophic interactions between species and genotypes and cannot be explained by simple species x species interactions. This new way of considering community structure may be helpful in the fight to preserve ecosystem integrity despite the anthropogenic onslaught against the natural world.

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