DOCTOR OF PHILOSOPHY

Genotypic and endosymbiont-mediated variation in parasitoid susceptibility and other fitness traits of the potato aphid, Macrosiphum euphorbiae

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Hannah Victoria Clarke

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List of Abbreviations

AIC/AICc	Akaike information criterion/Corrected Akaike information criterion
ANOVA	Analysis of variance
APSE	*Acyrthosiphon pisum* secondary endosymbiont (bacteriophage)
bp	Base pairs
C_T	Cycle threshold
d. f.	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ETI	Effector-triggered immunity
6-FAM	6-Carboxyfluorescein
FRET	Fluorescence resonance energy transfer
GC	Guanine-cytosine
GLM	General/generalised linear model
HSD	(Tukey's) Honest significant difference
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISSR	Inter-sequence simple repeats
JHI	The James Hutton Institute
kbp	Kilo base pairs
LB	Lysogeny broth (also called Luria-Bertani medium)
LLDPE	Linear low density polyethylene
LR	Likelihood ratio
MACE	Modified acetylcholinesterase
NCBI	National Centre for Biotechnology Information
NTC	No template control
OS	Ordinance Survey
PAXS	Pea aphid X-type symbiont
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH	(Cox) proportional hazard
PTI	Plant pathogen-triggered immunity
PVY	Potato virus Y
qPCR	Quantitative polymerase chain reaction
(T)-RFLP	(Terminal)-restriction fragment length polymorphism
rcf	Relative centrifugal force
R_m	Intrinsic rate of population increase
RNA	Ribonucleic acid
ROX	Carboxy-X-rhodamine
RT-PCR	Reverse-transcription polymerase chain reaction
RTX	Repeats in toxin
s. d.	Standard deviation
SOC	Super optimal broth with catabolite repression
SMLS	*Sitobion miscanthi* L-type symbiont
SSR	Simple sequence repeats
T3SS	Type 3 secretion system
TBE	Tris/Borate/EDTA (ethylenediaminetetraacetic acid) buffer
TEMED	Tetramethylethylenediamine
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y-D	Tyrosine-aspartic acid
YSMS	*Yamatocallis* secondary mycetocyte symbiont

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Declaration

Student Declaration

I hereby declare that the following thesis is based on the results of investigation conducted by myself, and that this thesis is my own composition. This thesis has not in whole or in any part been previously presented for a higher degree. Work other than my own is clearly stated in the text with reference to the relevant researchers or their publications.

Hannah V. Clarke

Supervisor Statement

The relevant Ordinance and Regulations have been fulfilled by the candidate.

Professor Stephen F. Hubbard

Abstract

Almost all aphid species harbour the primary bacterial endosymbiont *Buchnera aphidicola*, which plays a vital role in essential nutrient provisioning. In the last two decades several additional 'secondary' bacterial endosymbionts have been detected across different aphid species, although not across all aphid populations. Recent research has revealed that secondary bacterial endosymbionts can affect several aspects of the morphology and ecology of their aphid host. However, many of these studies have been conducted using the pea aphid (*Acyrthosiphon pisum*), and it is not clear whether facultative bacteria confer the same fitness traits in other aphid species.

The potato aphid, *Macrosiphum euphorbiae*, is closely related to *A. pisum* and is also an agriculturally important pest, utilising several crops including potato, tomato and bean throughout the summer months and transmitting a range of plant viruses. More than half of the 19 clonal lines of *M. euphorbiae* established in culture for this study were found to be singly or doubly-infected with two secondary endosymbiont bacteria, *Hamiltonella defensa* and *Regiella insecticola*, previously characterised from *A. pisum*. However, only the *H. defensa* infections persisted in culture, and these were associated exclusively with two of the seven *M. euphorbiae* genotypes represented in the clonal lines. Under controlled experimental conditions, no inherent fitness costs to the aphid were identified for the two aphid genotypes in which *H. defensa* infections occurred naturally. Neither aphid reproduction and survival nor densities of *B. aphidicola* were detrimentally affected by the presence of the secondary endosymbiont, although this may not be true for other genotypes of *M. euphorbiae*. Despite an established role in conferring parasitoid resistance to *A. pisum* hosts, this study found very little evidence that *H. defensa* protects *M. euphorbiae* against one of its principal natural enemies, the parasitoid wasp *Aphidius ervi*. Instead the innate immunity of one specific genotype of *M. euphorbiae* dramatically reduced parasitoid susceptibility, regardless of secondary endosymbiont presence.

Which aphid genotypes will dominate in a population depends on the relative ability of aphid clones to locate and feed on suitable host plants, withstand abiotic stresses and escape natural enemies, and such knowledge is essential for the successful management of aphid infestations in agricultural systems. The research presented here has contributed to this knowledge by characterising *M. euphorbiae* genotypes and their associated

facultative endosymbionts, and by quantifying genotypic differences in aphid intrinsic fitness and susceptibility to parasitoid wasps. Whilst secondary endosymbionts are recognized as a potential source of heritable traits that could influence the population dynamics of their aphid hosts, the selection pressures acting to favour persistence of the endosymbiotic association between *M. euphorbiae* and *H. defensa* have yet to be determined.

Chapter 1: General Introduction

Aphids are small, soft-bodied, hemipteran insects that feed on plant phloem sap. Despite their size, they are of great economic importance, with many species feeding on crop plants for all or part of their life cycle (Blackman & Eastop, 2000). In addition to diverting nutrients away from the plant, aphids are efficient vectors of a wide range of plant viruses (Ng & Perry, 2004). Furthermore, the complex and varied life cycles of different aphid species and the rapidity with which they can increase their population size has limited the efficacy of current control strategies designed to reduce the damage to both crop yields and to the quality of the produce. In order to manage successfully infestations on crops it is therefore important to have an understanding of the factors influencing aphid fitness and population dynamics (Kindlemann & Dixon, 2010).

There are approximately 4400 known species of aphid within the family Aphididae, and both between and within these species a range of life cycles are exhibited (Blackman & Eastop, 1994). The annual life cycle is typically holocyclic, with one or more generations of asexual reproduction alternated with a single generation of sexual reproduction. These two phases serve different functions; in addition to producing cold-resistant eggs the single sexually reproducing oviparous generation allows genetic recombination and hence the production of novel genotypes, whilst repeated asexual generations allow individuals to achieve maximum reproductive output of viviparous offspring when conditions are favourable (Moran, 1992, Simon *et al*., 2002). This division of function has both enabled separate adaptations to evolve in the sexual and parthenogenetic females, and led to the alternation of host plants arising independently in several different groups of aphids (Moran, 1988). In such heteroecious species sexual reproduction occurs on the primary host, usually a perennial, woody plant, from which offspring disperse to colonise annual, herbaceous secondary hosts. Despite this selective advantage, some species now appear to have abandoned sexual reproduction altogether and instead are anholocyclic, reproducing by continuous parthenogenesis throughout the year on secondary hosts. In other species, however, both types of life cycle are shown, with some populations reproducing continuously by apomictic parthenogenesis whilst other populations produce both sexual and asexual generations (Simon *et al*., 1996a, 1996b, 2002).

1.1 Determinants of aphid fitness

Variation in aphid fitness, that is the relative ability of an aphid clone to survive and reproduce, is well documented (Ferrari *et al*., 2001; Vorburger, 2005; Gwynn *et al*., 2005). Even within asexual summer morphs, the availability and quality of suitable host plants and the abundance and virulence of natural enemies and pathogens, as well as abiotic factors such as temperature and humidity, influence aphid survival and reproduction (Gonzáles *et al*., 2002; Karley *et al*., 2003). Genetic differences between aphid clonal lines within a given species influence the extent to which these factors affect aphid fitness, with maternally-inherited endosymbiont bacteria providing further sources of heritable variation (Vorburger, 2006; Ferrari *et al*., 2006; Dunbar *et al*., 2007; Bieri *et al*., 2009). Whilst the function of the primary bacterial endosymbiont to provide the aphid host with nutrients is well understood, secondary, facultative endosymbionts found with varying prevalence within and between aphid species are increasingly implicated in conferring beneficial heritable traits to their aphid hosts in certain environmental contexts (Oliver *et al*., 2010, and references therein). Moreover, it has only recently been recognised that aphid fitness can be determined by genotypic interactions between aphids, their endosymbionts and both higher and lower trophic levels of organisms with which the aphids interact.

1.1.1 Effects of host plant quality on aphid fitness

Aphids feed exclusively from plant vascular tissues, ingesting the sap from within the phloem sieve tube elements. The resources necessary to aphids for growth and reproduction therefore all come either directly from the host plants on which they feed, or indirectly, converted from available precursors by the obligate primary endosymbiont, *Buchnera aphidicola* (Sandström & Moran, 1999; Gündüz & Douglas, 2009). As a consequence, the quality of the nutrients available from host plants can dramatically influence aphid fitness (Karley *et al*., 2002). Various physical and chemical plant defences, either constitutively expressed or induced upon herbivore attack, may also reduce the accessibility and palatability of the phloem sap (Awmack & Leather, 2002).

1.1.1.1 Plant nutritional quality

As the major conduit for transporting nutrients from source to sink organs and for circulating signalling hormones, secondary metabolites and other macromolecules, the composition of phloem sap is not fixed but is instead responsive to numerous intrinsic and environmental factors (Dinant *et al*., 2010). Temporal variation in phloem sap composition occurs over the course of plant development (Weibull, 1987; Boggio *et al*., 2000; Karley *et al*., 2002) and on shorter timescales, with concentrations of certain amino acids and sugars fluctuating diurnally (Winter *et al*., 1992; Gattolin *et al*., 2008). Phloem composition also varies spatially within a plant, both between older and younger elements (Jongebloed *et al*., 2004), and even between sieve tube elements from the same region of the plant (Gattolin *et al*., 2008). Abiotic factors such as temperature, water availability and nitrogen availability further influence the composition of phloem sap (Mitchell & Madore, 1992; Girousse *et al*., 1996; Ponder *et al*., 2000).

Sugars derived from photosynthesis form the dominant components of phloem sap, providing a plentiful supply of carbon. Concentrations of utilisable nitrogenous compounds such as free amino acids are relatively low in comparison, and dominated by non-essential amino acids. Aphids must therefore ingest large volumes of phloem sap to acquire sufficient amounts of phloem nutrients that are present at low concentrations, and must simultaneously contend with the high osmotic pressure of the imbibed sap (Douglas, 2006). Total amino acid and sucrose concentrations, the quantities of specific essential and non-essential amino acids and the ratio of sugars to amino acids have therefore all been implicated as characteristics of plant phloem that affect the nutritional quality of host plants, and hence the fitness of the aphids that feed on them.

Despite the metabolic capabilities of *B. aphidicola*, shortfalls in essential amino acids present in the phloem sap are not always fully met by the primary endosymbiont, with consequent detrimental effects on aphid growth and reproduction (Douglas *et al*., 2001; Wilkinson & Douglas, 2003; Gündüz & Douglas, 2009). Variation in the essential amino acid requirements of different aphid clones, attributable to genetic differences in either the aphid or the primary endosymbiont, will therefore affect aphid fitness on certain host plants (Srivastava *et al*., 1985; Sandström & Pettersson, 1994; Birkle *et al*., 2002, 2004; Wilkinson & Douglas, 2003). A reduction in the total concentration of amino acids also affects aphid fitness negatively, even when essential amino acid concentrations remain

constant, due to limited nitrogen availability for conversion to other forms (Ponder *et al*., 2000; Bernays & Klein, 2002). Other aspects of phloem sap composition can restrict nitrogen intake by restricting or inhibiting feeding rates, as can occur when certain non-essential amino acids are present in high concentrations, or when concentrations of sugars (a phagostimulant) are low (Srivastava *et al*., 1983; Douglas *et al*., 2006b). Excessively high concentrations of phloem sugars are also detrimental to aphid fitness, as the physiological mechanisms that have evolved in aphids to reduce phloem sap osmotic pressure in the aphid gut are overwhelmed (Karley *et al*., 2005; Douglas *et al*., 2006b).

Some aphid species are able to partially compensate for the nutritionally imbalanced nature of their diet by inducing changes in the phloem composition of their host plants. Feeding by the Russian wheat aphid *Diuraphis noxia* on susceptible cultivars of wheat cause both visible damage (leaf rolling, discolouration) and a concomitant change in the amino acid composition of the plant phloem, which in turn is correlated with an increase in aphid performance; such effects are not observed when the aphids feed on resistant wheat cultivars (Telang *et al*., 1999). Similar systematic changes in amino acid composition within a leaf damaged through feeding are documented for the greenbug, *Schizaphis graminum*, but not for the bird cherry–oat aphid *Rhopalosiphum padi*, which does not induce visible leaf damage (Sandström *et al*., 2000).

1.1.1.2 The primary endosymbiont, Buchnera aphidicola

Aphids and other animals are able to create many of the 20 amino acids that make up proteins, either from precursors or through the conversion of other amino acids. However, several of these amino acids cannot be synthesised, and instead must be obtained from the diet. For most animals, this would preclude an exclusive diet of phloem sap, in which some or all of the essential amino acids may be present in very low concentrations or absent altogether. Analyses of phloem sap from the severed stylets of various aphid species feeding on different host plants have shown that the supply of at least one amino acid is often inadequate for the protein requirements of the aphid (Sandström & Moran, 1999; Gündüz & Douglas, 2009).

Almost all aphids contain a primary bacterial endosymbiont, the γ-Proteobacterium *Buchnera aphidicola*, with which they have an intimate obligate mutualistic relationship.

Only certain aphid genera from the tribe Cerataphidini lack these bacteria, and instead have been found to have a similar symbiotic relationship with extracellular yeast–like microbes (Fukatsu & Ishikawa, 1996). As well as sugars, other non-essential amino acids such as glutamic acid are often present in abundance in the phloem sap diet or can be readily synthesised by the aphid; it is these compounds that the primary endosymbiont *B. aphidicola* utilises to provide the aphid with the essential nutrients lacking from the phloem sap that they are unable to produce themselves.

This ability of *B. aphidicola* to provide essential amino acids to the aphid host has been demonstrated empirically through laboratory studies using aphids cured of their primary endosymbiont. When fed on artificial diets containing sufficient levels of all 20 amino acids, the growth rates of cured aphids are similar to those of controls still bearing their endosymbionts. In contrast, when fed on artificial diets lacking each of the essential amino acids aposymbiotic aphids show a reduction in net protein growth rates (Douglas *et al*., 2001; Gündüz & Douglas, 2009). Radio-labelling of the non-essential amino acid glutamic acid with ^{14}C has also shown that the synthesis of at least three essential amino acids incorporates carbon from glutamate in symbiotic aphids, but not in aphids in which *B. aphidicola* had been eradicated. Other radio-labelled non-essential amino acids and sugars such as sucrose have also been shown to be precursors of essential amino acids synthesised by *B. aphidicola*, if they are first converted into glutamate (Febvay *et al*., 1995, 1999; Douglas *et al*., 2001).

The endosymbiotic association between *B. aphidicola* and aphids is thought to be an ancient one, having become established in a common aphid ancestor between 160 and 280 million years ago. As a result the genome of *B. aphidicola* has become reduced to roughly one-seventh of the size of the genome of *E. coli*, and whilst it includes genes for the biosynthesis of amino acids essential for their aphid host, gene loss means *B. aphidicola* lacks the capability to synthesise non-essential amino acids commonly found in the phloem sap diet. Furthermore, the bacterium has also lost the genes required for the production of certain cell surface components, many regulator genes and genes involved in cell defence, restricting it to a symbiotic existence within bacteriocyte cells (Shigenobu *et al*., 2000). That the relationship between the aphid and the primary endosymbiont is both obligate and mutualistic to both partners is confirmed through the complementarity of the two genomes and the transcriptomic evidence from the pertinent genes (Wilson *et al*., 2010).

It is the aphid, not *B. aphidicola* that induces the formation of the polyploid bacteriocyte cells within which the bacteria reside, and controls the recruitment of further bacteriocytes after the maternal transfer of *Buchnera* to the aphid embryos (Braendle *et al*., 2003). Within the bacteriocytes various aphid genes are up-regulated, including those involved in amino acid production to enable the aphid host to synthesise excess amounts of the non-essential amino acids that *Buchnera* can no longer produce, and genes related to the transport of metabolites and substrates between the two symbiotic partners (Nakabachi *et al*., 2005). Two further genes in the aphid genome that are over-expressed in the bacteriocytes, *rlpA* and *ldcA*, appear to have been acquired by lateral gene transfer from a past symbiotic interaction with an α-Proteobacterium, but to have been lost from *B. aphidicola*. The LdcA enzyme is required for the recycling of a peptidoglycan within the bacterial cell wall, and the acquisition of functional promoters for these two genes and their expression in the bacteriocytes suggests that their expression is necessary for maintaining the *Buchnera* symbionts (Nikoh & Nakabachi, 2009).

1.1.1.3 Host plant preference and acceptance

The vast majority of aphid species are monophagous, restricting their feeding to a genus or single species of plant. Even holocyclic species that alternate between host plants tend to do so between two specific groups of plants. Some, however, are more polyphagous, and can feed on a range of plants within a family, or even plants from many different families. As cultivated varieties of crop plants may provide vacant ecological niches into which aphid species traditionally found on other plants can expand, those that colonise agricultural crops tend to be relatively polyphagous (Blackman & Eastop, 2000).

A series of behaviours characterises host plant selection in aphids, with visual, olfactory and gustatory cues used to select potential host plants on which to alight, and to assess the plant based on surface chemicals and structures (Powell *et al*., 2006). The nutritional composition of phloem sap does not appear to contribute to the range of host plants utilized by an aphid species (Wilkinson & Douglas, 2003). Instead other intracellular compounds within the plant tissues, encountered by probes of the stylet prior to reaching the phloem, act either as deterrents or as signals that cause the aphid to settle, feed and reproduce on the plant (Tosh *et al*., 2002; Powell *et al*., 2006).

Plants exhibit a wide array of physical and chemical defences against herbivory, including the production of constituent and inducible secondary metabolites and other defensive proteins. Plant secondary metabolites in particular are integral to many aphid–plant interactions, and by providing positive or negative stimuli to probing aphids may in part explain the host specificity exhibited by monophagous and oligophagous aphids (Schoonhoven *et al*., 1998). Volatile cyanogenic glucosides and alkanes in the epicuticular wax layer, for example, act as attractants to certain aphid species whilst volatile (*E*)-β-farnesene, diketones present on the leaf surface and 2-tridecanones in glandular trichromes have repellent properties (Niemeyer, 1990). Furthermore, within an aphid species and even within a single genotype, specialist and generalist forms may show differential responses to the metabolites present (Del Campo *et al*., 2003; Tosh *et al*., 2003). As the aphids probe the leaf tissue the molecules encountered within the mesophyll, such as some phenolics, act as gustatory cues that cause the aphid to accept the plant. The Brassicaceae on which the cabbage aphid *Brevicoryne brassicae* feeds, for example, all contain mustard oil glucosinolates that deter feeding by many other aphid species. By introducing the glucosinolate sinigrin into the tissues of a plant normally rejected as a host, cabbage aphids that probe the treated leaves settle, feed and reproduce (Wensler, 1962).

As aphids assess potential host plants they secrete gel-like saliva that protects the stylet as it penetrates between the cells of the plant, whilst watery saliva is secreted into punctured epithelial and mesodermal cells and eventually the sieve tube elements of the phloem (Tjallingii, 2006). The wounding thus caused, and elicitors in the saliva, induce both localised and systemic plant defences. These include the occlusion of the sieve plates between phloem tube elements and the activation of phytohormone pathways, which in turn cause the accumulation of further antixenotic and antibiotic secondary metabolites and defensive proteins that deter aphids from feeding or reduce their fitness (de Vos *et al*., 2007; Giordanengo *et al*., 2010). However, aphids are able to suppress the defensive responses of their host plants through effectors in their saliva. Some of these effectors prevent phloem proteins from changing in conformation and blocking the sieve plate pores, enabling the aphids to continuously feed from a single sieve tube element (Will *et al*., 2007, 2009). Others suppress certain signalling pathways of the plant pathogen-triggered immunity (PTI), although the introduction of effectors may in turn trigger the expression of the plant resistance (R) genes that form the effector-triggered immunity (ETI) (Rossi *et al*., 1998; Walling, 2008; Bos *et al*., 2010; Hogenhout & Bos,

2011). Not all induced plant defences are directly detrimental to the aphids feeding from them; the production of secondary metabolites such as terpenes and other volatiles may indirectly protect the plant by recruiting the natural enemies of the attacking herbivores (Hatano *et al*., 2008; Liu *et al*., 2009).

1.1.2 Natural enemy pressures: predators, parasitoids and pathogens

Natural enemies are a significant cause of mortality in aphid populations, curtailing aphid survival and reproduction (Snyder & Ives, 2003). Specialist aphidophagous predators include the larvae of hoverflies, gall midges, lacewings and both larval and adult ladybirds, and spiders and rove beetles may also consume aphids as part of more generalist diets. In addition, aphids are vulnerable to numerous pathogens, from bacteria such as *Erwinia aphidicola* and *Dickeya dadantii*, taken up when the aphid penetrates the leaf surface to feed (Harada & Ishikawa, 1997; Grenier *et al*., 2006; Santos *et al*., 2009), viruses like *Rhopalosiphum padi virus* (RhPV) that are transmitted horizontally through plant tissues (Ban *et al*., 2007), and generalist and specialist entomopathogenic fungi such as *Beauveria bassiana* and *Verticillum lecanii*, *Neozygites fresenii* and *Pandora* (*Erynia*) *neoaphidis*, the last of which is known to cause severe epizootics outbreaks in natural aphid populations (Pickering *et al*., 1989). Variation in the direct and indirect ability of aphids to avoid and/or defend themselves from predators, parasitoids and pathogens therefore considerably affects aphid fitness, with consequent effects on aphid population dynamics (Hufbauer, 2002).

1.1.2.1 Behavioural defences

Encounters with parasitoids and other natural enemies elicit a number of defensive behaviours seen across aphid species such as dropping from the plant, body raising, kicking, body rotation and walking away (Figure 1.1). The frequency and efficacy of these behaviours tends to increase as the aphid develops (Wyckhuys *et al*., 2008). However, varying as they do in their energetic costs and with the potential to result in lost feeding opportunities or an increased risk of mortality through other means, the defensive behaviours are influenced by factors such as the physiological state of the aphid, climatic conditions and host plant quality (Dill *et al*., 1990; Villagra *et al*., 2002).

The dominant behavioural responses correlate with differences in parasitism rates, although these findings are complicated by other associated traits (Bilodeau *et al*., 2013a, 2013b).

Figure 1.1: *M. euphorbiae* kicking (left) and body rearing (right) in response to an attempted attack by *A. ervi*. Photographs courtesy of David Riley.

As another defence against both parasitism and predation, aphids may produce a waxy secretion from their cornicles that hardens upon exudation (Strong, 1967). As it is most commonly produced in response to an initial attack, such a defence mechanism may not directly benefit the aphid. Indeed, the production of cornicle secretions diverts lipids away from growth and reproduction, decreasing individual fitness by delaying offspring production (Mondor & Roitberg, 2003). Nevertheless, the presence of these dried triglycerides on the aphid body can deter oviposition, with parasitoids potentially using the presence of the secretion as an indication of an already parasitized host (Outreman *et al*., 2001), and if smeared upon a parasitoid the attack rate on the aphid colony is reduced as the parasitoid grooms itself (Wu *et al*., 2010). The greater the number of clone-mates in the colony that benefit from the decrease in host-encounter and oviposition rates resulting from such smearing, the greater the potential indirect fitness benefits to the aphid.

The cornicle secretions of most aphid species also contain the volatile compound (*E*)-β-farnesene, an alarm pheromone that alerts conspecifics and elicits mechanical defence responses such as walking away and dropping (Vandermoten *et al*., 2012). When smeared on to natural enemies, cornicle secretions can also alert aphids to the arrival of a potential threat (Mondor & Roitberg, 2004). Cornicle droplet production is greatest in pre-reproductive aphids, which are most likely to be aggregated and thus a large number of kin will benefit from the warning (Mondor *et al*., 2000). Whilst direct contact with predators or parasitoids can result in the production of a greater proportion of alate

offspring, frequent releases of (*E*)-β-farnesene by conspecific aphids in combination with tactile stimuli also increases the expression of the winged phenotype, increasing dispersal from the perceived threat (Sloggett & Weisser, 2002; Kunert *et al*., 2005; Kunert & Weisser, 2005). The exact defensive behaviours exhibited in response to (*E*)-β-farnesene differ within and between aphid species; the behaviours elicited in the pea aphid, for example, vary in different host-adapted races, potentially as a result of dissimilar natural enemy pressures (Kunert *et al*., 2010).

Aphids may also form associations with ants, which provide additional defence from natural enemies. The ants benefit from the relationship by collecting and feeding on aphid honeydew, which is produced in larger quantities and differs in composition from that produced by unattended aphids. However, the physiological adaptations of aphids to ant tending are costly, and so the relationship is not always a mutualistic one (Stadler & Dixon, 2005).

1.1.2.2 Physiological defences

Aphidophagous predators such as ladybirds and hoverfly larvae kill and consume their aphid prey within minutes, with little opportunity for aphids to mount physiological defences. In contrast, both braconid and *Aphelinus* wasps and pathogens such as the entomopathogenic fungus *Pandora neoaphidis* are fatal to their aphid hosts only after a matter of days, enabling immune responses to be initiated in response to attack.

The innate immune responses of insects are typically composed of humoral defences such as the production of antimicrobial peptides and effector molecules that induce clotting, and cellular defences such as phagocytosis and encapsulation mediated by various haemocyte cells, though the two types of defence are interconnected (Strand, 2008). The repertoire of immune responses in aphids appears much reduced relative to other insects, with many of the genes associated with recognition, signalling and response to bacteria and other pathogens absent from the pea aphid genome (Gerardo *et al*., 2010). The reduced capacity to mount humoral defences is also apparent in *A. pisum* following wounding or bacterial challenges, with functional assays indicating that lysozyme–like activity is induced but with no anti-microbial peptide production, and only

modest upregulation of inducible immunity genes (Altincicek *et al*., 2008; Gerardo *et al*., 2010; Laughton *et al*., 2011).

Cellular immune defences in aphids are more apparent, although the identification of haemocytes based on morphological, histochemical and/or functional attributes has led to conflicting characterisations of cell types (Boiteau & Perron, 1976; Behera *et al*., 1999; Laughton *et al*., 2011; Schmitz *et al*., 2012). Granulocytes, plasmatocytes, oenocytoids and spherulocytes have all been tentatively identified, along with progenitor prohaemocytes. Aphid granulocytes and plasmatocytes actively phagocytose bacteria, and can also adhere to foreign bodies, forming an incomplete cellular layer around the object (Laughton *et al*., 2011; Schmitz *et al*., 2012). Complete cellular encapsulation of invasive bodies such as parasitoid eggs by plasmatocytes is a common immune response of many other insect groups, but has not been reported in aphids (Oliver *et al*., 2005; Bensadia *et al*., 2006). Instead, melanotic capsules have been observed, the formation of which is initiated by phenoloxidase enzymes produced by granulocytes and oenocytoids (Laughton *et al*., 2011; Schmitz *et al*., 2012). The final group of haemocyte cells, spherulocytes, are implicated in the coagulation process following wounding (Schmitz *et al*., 2012). Despite these functional responses observed in immune-challenged aphids, the contribution of haemocytes to aphid defence is limited. Aphids have been shown to exhibit high susceptibility to pathogenic bacteria and endoparasitoids, and only exhibit a weak coagulation response to wounding (Grenier *et al*., 2006; Altincicek *et al*., 2008, 2011).

In addition to innate immune responses, the accumulation and sequestration of plant secondary metabolites, obtained during aphid feeding, can provide aphids with a degree of protection from predators and parasitoids. The cabbage aphid *Brevicoryne brassicae* feeds solely on crucifers, from which they sequester glucosinolates. These plant metabolites are hydrolysed by myrosinase enzymes within the muscles of the aphid to produce toxic isothiocyanates (Bridges *et al*., 2002; Kazana *et al*., 2007). The increased toxicity of the aphids has detrimental, often fatal effects on the fitness of ladybird, hoverfly and lacewing predators (Francis *et al*., 2001; Pratt *et al*., 2008; Kos *et al*., 2011). Generalist parasitoids like *A. colemani* are also unable to use *B. brevicoryne* as hosts, whilst the fitness of specialised parasitoids such as *D. rapae* is reduced with increasing glucosinolate concentrations in aphid tissues (Sampaio *et al*., 2008; Le Guigo *et al*., 2011). Similarly, aphids such as *Aphis nerii* and *A. asclepiadis* sequester toxic

cardenolides from milkweed plants, and again these secondary compounds deter predators and reduce the suitability of the aphids as hosts for parasitoids (Malcolm, 1990; Helms *et al*., 2004; Mooney *et al*., 2008; Desneux *et al*., 2009).

1.1.2.3 Defensive traits of secondary endosymbiont bacteria

In addition to *Buchnera aphidicola*, aphids can harbour other bacterial types. These 'accessory' bacteria or secondary symbionts have been most studied in the pea aphid *Acyrthosiphon pisum*, where at least seven different bacterial taxa occur in natural aphid populations; *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, Pea aphid X-type symbiont (PAXS), and bacteria from the *Rickettsia*, *Rickettsiella* and *Spiroplasma* genera (Chen *et al*., 1996; Fukatsu *et al*., 2000; Darby *et al*., 2001; Russell *et al*., 2003; Sakurai *et al*., 2005; Moran *et al*., 2005c; Guay *et al*., 2009; Tsuchida *et al*., 2005, 2010; Lamelas *et al*., 2008). Two further types of secondary endosymbiont bacteria have been characterised from other aphid species; *Yamatocallis* secondary mycetocyte symbiont (YSMS) and the *Rickettsia*-like *Sitobion miscanthi* L-type symbiont (SMLS) (Fukatsu, 2001; Wille & Hartman, 2009; Li *et al*., 2011a, 2011b). In contrast to *B. aphidicola*, however, the presence of these secondary symbionts is generally not essential for aphid survival and their occurrence in natural populations is highly variable both within and between aphid species (Chen *et al*., 1996; Chen & Purcell, 1997; Fukatsu *et al*., 2000, 2001; Darby *et al*., 2001, 2003; Sandström *et al*., 2001; Tsuchida *et al*., 2002, 2005, 2006; Simon *et al*., 2003; Russell *et al*., 2003; Haynes *et al*., 2003).

The presence of certain secondary endosymbionts can augment the physiological defences of aphids against parasitoids and pathogens. *Pandora* (*Erynia*) *neoaphidis* is one of the most common entomopathogenic fungi to infect aphids in temperate climates (Pell *et al*., 2001). Initial correlations between pea aphid resistance to *Pandora* and the presence of the secondary endosymbiont *Regiella insecticola* were complicated by the concomitant prevalence of *Regiella*-infected aphids on *Trifolium* host plants (see section *1.1.4.3*). However, fungal resistance bestowed on otherwise susceptible pea aphid clonal lines following the establishment of heritable endosymbiont infections has since confirmed *R. insecticola* as the cause of the resistant phenotype (Ferrari *et al*., 2004; Scarborough *et al*., 2005). More recently, strains of four unrelated secondary endosymbiotic bacteria (*R. insecticola*, *Rickettsia*, *Rickettsiella* and *Spiroplasma*) have all

been shown to reduce aphid mortality from *P. neoaphidis*, and to decrease fungal fitness through the reduction and delay of sporulation in those aphids that had succumbed to the pathogen (Łukasik *et al*., 2013a). Although this does not change the fate of the individual, the inclusive fitness of the infected aphid may be increased as surrounding kin (with which the infected aphid shares genes) are less likely to become infected with the fungus (Scarborough *et al*., 2005).

The presence of the endosymbiont *Hamiltonella defensa* and the associated lysogenic lamdoid APSE (*Acyrthosiphon pisum* secondary endosymbiont) bacteriophage can strengthen the immune defences of *A. pisum*, *A. fabae* and other aphid species in response to parasitism (Oliver *et al*., 2003; Desneux *et al*., 2009; Schmid *et al*., 2012). This may manifest as a reduction in parasitism rates, but also in increased fitness following a resisted parasitoid attack relative to aphids lacking secondary endosymbionts (Vorburger *et al*., 2013). Several variants of APSE have now been identified, broadly divisible into three categories based on the gene homologues of protein toxins they contain; Shiga-like toxins, cytolethal distending toxins (*cdtB*), and tyrosine-aspartic acid (Y-D) repeat proteins (Degnan & Moran, 2008a). It is these constitutively-expressed APSE toxins, the delivery of which is potentially mediated by the type 3 secretion systems of *H. defensa*, which are thought to halt the growth of the parasitoids before or during early larval development through targeted cell destruction (Oliver *et al*., 2003; Moran *et al*., 2005a; Degnan & Moran, 2008a). Other *H. defensa* genes such as those coding for RTX toxins may also contribute more directly to the resistant phenotype of the aphid host, though many of these genes are no longer functional (Degnan *et al*., 2009b). Co-infections of *H. defensa* and APSE with another secondary endosymbiont, PAXS, appear to further increase parasitoid resistance in pea aphids, with the parasitoid egg aborted within 24 hours of attack (Guay *et al*., 2009). Transfection of these endosymbionts from pea aphids into a non-resistant clone of *S. avenae* conveys similar levels of protection to the new host (Łukasik *et al*., 2013b).

S. symbiotica, R. insecticola and co-infections with *Spiroplasma* have also been associated with reduced parasitoid susceptibility in *A. pisum* (Oliver *et al*., 2003; Ferrari *et al*., 2004; Nyabuga *et al*., 2010), although the mechanisms by which resistant phenotypes are conferred are unclear. One strain of *R. insecticola* isolated from an Australian clone of *M. persicae*, however, renders its aphid host almost completely resistant to *Aphidius colemani* and *D. rapae* parasitoid wasps (von Burg *et al*., 2008;

Vorburger *et al*., 2010). Again the resistant phenotype has been attributed to the endosymbiont through transfection experiments, with *Aphis fabae* and *A. pisum* clones acquiring almost complete resistance to *A. colemani* and *A. ervi* parasitoids, respectively, upon inoculation with this specific *R. insecticola* strain (Vorburger *et al*., 2010; Hansen *et al*., 2012). No phages have been found in this or any other strain of *R. insecticola* to date, but genes for RTX toxins, secretion systems and other pathogenicity factors identified only in the genome of the resistance-conferring *R. insecticola* strain suggest a putative means by which parasitoid development could be halted (Hansen *et al*., 2012).

In contrast to *B. aphidicola*, which is found only in the specialist bacteriocyte cells of the aphid host, secondary endosymbionts are found in additional secondary bacteriocyte cells, within sheath cells on the periphery of the bacteriocytes and extra-cellularly in the haemolymph within the body cavity (Fukatsu *et al*., 2000; Sandström *et al*., 2001; Darby *et al*., 2001; Sakurai *et al*., 2005; Tsuchida *et al*., 2005; Moran *et al*., 2005c). They are therefore exposed to the limited cellular defences of the aphid immune system, and potentially subject to active phagocytosis by plasmatocytes and granulocytes. However, within a single *A. pisum* line the presence of *H. defensa* or *R. insecticola*, but not *S. symbiotica*, demonstrably reduces the absolute and relative numbers of haemocyte cells present in the aphid haemolymph, indicating an endosymbiont-specific effect on aphid immunity that may reflect the protective roles of the bacteria (Schmitz *et al*., 2012).

1.1.3 The aphid holobiont: genetic variation in aphids and their microbial endosymbionts

Asexual lineages of aphids reproduce by apomictic parthenogenesis, essentially producing true clones. However, in addition to the immigration of novel clones from cyclically parthenogenetic lineages, clonal variation within a population can arise through mutations, chromosomal rearrangements such as gene amplification, and possibly mitotic recombination (Hales *et al*., 1997). Additionally, the genes that affect aphid performance are not restricted to the aphid genome, but are also located on the genomes of the primary and, where present, secondary endosymbionts. The faithful vertical transmission of *Buchnera* and the predominantly vertical transmission of secondary endosymbionts mean both contribute to the heritable genetic variation in fitness of aphid clones, which in turn affects the clonal composition of aphid populations.

As such, the unit of replication upon which selection can act can be thought of not as the aphid genome but rather the aphid holobiont, comprising both the aphid and its symbiotic microbiota (Mandrioli & Manicardi, 2013).

1.1.3.1 Aphid genotypic variation

Variations in numerous aspects of aphid fitness have been attributed at least in part to nuclear genomic differences between aphid clones. The relative fitness of pea aphid clones when in direct competition with another aphid species, for example, is genetically determined, as is the propensity of different clones to produce winged morphs capable of dispersal in response to environmental cues (Hazell *et al*., 2005; Braendle *et al*., 2005). Clones of *M. persicae* demonstrably differ in their thermal tolerances, both of low and high temperature extremes (Vorburger, 2004; Alford *et al*., 2012a, 2012b, 2012c), whilst the susceptibility of *A. fabae* and *M. persicae* aphids to their respective common parasitoids also differs as a result of genetic differences between aphid clones (von Burg *et al*., 2008; Vorburger *et al*., 2009), and is intimated for some *A. pisum* genotypes (Hufbauer & Via, 1999; Ferrari *et al*., 2001; Li *et al*., 2002; Oliver *et al*., 2005, 2003; Ferrari *et al*., 2004). Recent agricultural practices have also resulted in the evolution of insecticide resistance within certain *M. persicae* clones, which have obvious fitness advantages when insecticide usage is high, but which through negative pleotropic effects on aphid behaviour can reduce the fitness of these genotypes relative to insecticide-sensitive clones (Foster *et al*., 2003, 2005; Kasprowicz *et al*., 2008; Fenton *et al*., 2010). In addition, the *E4* insecticide-resistance gene appears to be under epigenetic control, adding further complexity to the genetic factors that determine clonal variation in fitness within *M. persicae* populations (Field & Blackman, 2003).

Clonal variation in aphid fitness on different host plants is particularly well documented for several polyphagous aphid species. The consequent selection pressure for aphids to settle preferentially on plant species on which their fitness is maximised has led to host-adapted races. In *A. pisum*, for example, races have been identified in North American, European and Asian pea aphid populations that are genetically adapted to specific host plants such as pea/bean, vetch, alfalfa and clover (Simon *et al*., 2003; Ferrari *et al*., 2012). Translocation of host adapted pea aphids from their native to alternative host plants shows a reduction in fitness, resulting in strong host plant choices exhibited by *A.*

pisum races in both laboratory tests and field studies (Via, 1999; Caillaud & Via, 2000). Furthermore, hybrids of two host-adapted races perform less well than the parental races on either host plant, further selecting against migration and sexual reproduction on alternative hosts (Via *et al*., 2000). These two traits, host specialisation and host preference are thought to be genetically linked (Hawthorne & Via, 2001), and as a consequence sympatric host adapted populations of *A. pisum* are reproductively isolated, producing genetically divergent populations with low levels of gene flow (Simon *et al*., 2003; Frantz *et al*., 2006; Peccoud *et al*., 2009).

Host adapted races, that is genetically distinct populations whose performance is reduced on alternative host plants, have been identified in several other aphid species including *Aphis gossypii* (Guldemond *et al*., 1994; Charaabi *et al*., 2008; Carletto *et al*., 2009) and *Myzus persicae* (Nikolakakis *et al*., 2003), and are indicated in other species. Genetically distinct populations of the cyclically parthenogenetic lettuce root aphid, *Pemphigus bursarius*, have been identified from different secondary host plants despite sharing a common primary host, although the concomitant reductions in performance of aphid genotypes on alternative plants characteristic of host adaptation have not been demonstrated (Miller *et al*., 2003, 2005). Likewise, genetically and morphologically distinct populations of *Therioaphis trifolii* have been characterised from lucerne and clover (Sunnucks *et al*., 1997), and of *Schizaphis graminum* on sorghum, wheat and non-cultivated grasses (Shufran *et al*., 2000; Anstead *et al*., 2002).

The strength of association between aphid host races and the plants on which they are adapted also varies between genotypes. In the grain aphid *Sitobion avenae*, for example, genetically distinct host races are found on both wheat and cocksfoot (De Barro *et al*., 1995b; Sunnucks *et al*., 1997). Translocation of these aphids to their alternative hosts for several generations shows that cocksfoot-adapted races can utilise wheat better than vice versa (De Barro *et al*., 1995a). Genotypic variation in the preference and performance of host adapted races to alternative host plants have also been described for *A. pisum*, *A. gossypii*, *M. persicae* and *A. fabae* (Nikolakakis *et al*., 2003; Ferrari *et al*., 2006; Gorur *et al*., 2007; Liu *et al*., 2008; Wu *et al*., 2013).

1.1.3.2 Genotypic variation in the primary endosymbiont

The genomes of several *Buchnera aphidicola* strains sequenced from different aphid species show remarkable stability, exhibiting a conserved gene order with no evidence of chromosome rearrangements or gene acquisitions (van Ham *et al*., 2003). Such genomic stability is in part due to the loss or inactivation of genes such as *recA* and *recF* that in other bacteria are involved in homologous recombination (Tamas *et al*., 2002). Furthermore, the parallel divergence seen in the phylogenetic trees of aphid hosts and their associated strains of *B. aphidicola* is consistent with the transmission of the symbionts from parent to offspring only (Munson *et al*., 1991a). Even within closely related aphid species, there is no evidence for the transfer of *B. aphidicola* between different aphid hosts despite overlapping geographical ranges, comparable habitats, shared host plants and mutual natural enemies that could provide opportunities for horizontal transmission of the bacteria (Clark *et al*., 2000; Jousselin *et al*., 2009). Degradation and lack of gene acquisition has diminished the size of the *B. aphidicola* genome, with the main chromosome comprising between 615 and 640kbp in most of the strains sequenced so far. Furthermore, the loss of genes such as *seqA* and *datA* that are normally involved in co-ordinating cell cycle replication appears to have resulted in extreme polyploidy, with an average of 50 to 200 copies of the genome present in each *B. aphidicola* cell (Komaki & Ishikawa, 1999, 2000; Tamas *et al*., 2002). Such gene degradation may limit the functionality of *B. aphidicola*. The degeneration of the genes necessary for the incorporation of inorganic sulphur and the synthesis of cysteine in the primary endosymbiont of *Schizaphis graminum*, for example, a consequence of the naturally high levels of organic, sulphur-containing compounds in the grasses on which the grain aphid feeds, is likely to restrict the diversification of the aphid host to novel plant groups (Tamas *et al*., 2002).

The location of some or all of the genes in the tryptophan and leucine biosynthetic pathways on extra-chromosomal plasmids in *B. aphidicola* and subsequent plasmid amplification to levels above those of the main chromosome may facilitate provision of tryptophan and leucine to the aphid host (Lai *et al*., 1994; Birkle *et al*., 2004). In some strains of *Buchnera*, however, the plasmid copy numbers are lower than that of the main chromosome, and may serve to functionally limit the production of these metabolically expensive amino acids for aphid hosts in which requirements for tryptophan or leucine are relatively low (Lai *et al*., 1996; Thao *et al*., 1998; Plague *et al*., 2003). Intriguingly,

the incongruent phylogenies of the *Buchnera* chromosomal and plasmid genes intimate that the plasmids may have undergone horizontal transfer between the primary endosymbionts of different aphid species (van Ham *et al*., 2000).

Whilst the lack of homologous recombination and gene acquisition limits the genetic variation of *B. aphidicola*, the fitness of the aphid host can still be affected by mutations that occur within the genome of the primary endosymbiont. For instance, exposure to brief periods of heat during development is damaging to the aphid host as a result of depletion of *Buchnera* cells and the consequent loss of function (Ohtaka & Ishikawa, 1991; Montllor *et al*., 2002). A single nucleotide deletion in the *ibpA* gene of *B. aphidicola*, a transcriptional promoter of a heat-shock protein, dramatically reduces the fecundity of the pea aphid host following exposure to high temperatures, but under cooler rearing conditions the aphids reproduce earlier and produce a greater number of offspring than those with the full *ibpA* gene sequence. The occurrence of this mutation has repeatedly been observed in laboratory-reared clonal lines as well as being found in varying frequencies in pea aphid populations in the field, suggesting that the relative fitness of aphids with either form of the gene will in part depend on the prevailing environmental conditions (Dunbar *et al*., 2007; Burke *et al*., 2010).

1.1.3.3 The presence and genetic variation of secondary endosymbiont bacteria can contribute to aphid fitness

In order to spread and persist throughout the population of their host, maternally inherited endosymbionts must enhance the production of infected daughters. Some, such as the *Wolbachia* bacteria that commonly infect arthropod hosts, achieve this through reproductive manipulation, using diverse means to alter the sex ratio of the offspring produced (Werren *et al*., 2008). Other endosymbionts proliferate by providing a net benefit in fitness to their host, resulting in infected individuals surviving and producing more offspring than their uninfected counterparts (Jaenike *et al*., 2010). Within asexually-reproducing clonal aphid lines there is little scope for reproductive manipulation, but the roles that facultative endosymbionts can play in benefiting the fitness of the aphid host, particularly through protective functions, is becoming increasingly apparent. Potentially, the presence of secondary endosymbiont bacteria could benefit the aphid host by, for example, outcompeting invading organisms for

resources or priming the innate aphid immune system (Haine, 2008). As yet, no such generic benefits have been shown to aphids harbouring facultative bacteria, and indeed, some secondary endosymbionts appear to reduce the quantity of circulating haemocytes (Schmitz *et al*., 2012). Rather, the protective phenotypes mediated by endosymbionts stem from the genetic capabilities of the bacteria. Endosymbiotic protective functions, be it against predators, pathogens or abiotic extremes occur repeatedly, though with sometimes dramatic variation evident between isolates of a given endosymbiont in the benefits bestowed on the aphid host. Coupled with genomic evidence, this variation suggests that protective traits have emerged independently in the different endosymbionts and strains, although mobile elements could also have played a role (Hansen *et al*., 2012; Łukasik *et al*., 2013a).

The genomes of three of the better studied aphid secondary bacteria, *S. symbiotica, H. defensa* and *R. insecticola*, all display features typical of host-restricted symbionts: gene inactivations and losses, decreased GC content and reductions in their genome size, though not to the extremes seen in the obligate primary endosymbiont (Degnan *et al*., 2009a, 2009b; Burke & Moran, 2011). Based on the metabolic pathways present, all three facultative bacteria now appear dependent on both the aphid host and the primary endosymbiont to provide certain nutrients or their precursors (Degnan *et al*., 2009a, 2009b; Burke & Moran, 2011). However, the genes for pathogenicity factors such as type 3 secretion systems (T3SS) and RTX toxins are often present, and may contribute to conferring protective roles. Furthermore, whilst the extreme gene loss in *B. aphidicola* resulting from the ancient endosymbiotic relationship between aphids and *Buchnera* has obscured the early genomic changes and produced effective genomic stasis, *S. symbiotica*, *H. defensa* and *R. insecticola* show evidence of extensive genome rearrangements and the possession of a multitude of mobile DNA elements such as insertion sequences and plasmid- and phage-associated genes (Moran *et al*., 2005c; Degnan *et al*., 2009a, 2009b).

The often incongruent phylogenies of secondary endosymbionts and their aphid hosts are indicative of both the occasional loss of facultative bacteria from aphid lines and rare acquisitions by horizontal transmission (Sandström *et al*., 2001; Russell *et al*., 2003; Tsuchida *et al*., 2006). Such transmission events occur within aphid species during sexual reproduction, but also occur within and between aphid species, possibly through oral infection or vectored by natural enemies (Darby *et al*., 2001; Darby & Douglas,

2003; Moran & Dunbar, 2006; Gehrer & Vorburger, 2012). As a consequence, co-infections of multiple secondary endosymbionts can and do occur within aphid hosts, although not all are stable (Oliver *et al*., 2006; Ferrari *et al*., 2012; Russell *et al*., 2013). Presumably the occurrence of multiple endosymbiont strains within an aphid host could arise in the same manner. The opportunity is then present for the exchange of beneficial genetic material either through mobile DNA elements or through homologous recombination, the required genes for which are still retained by *S. symbiotica*, *H. defensa* and *R. insecticola* (Degnan *et al*., 2009a, 2009b; Burke & Moran, 2011; Russell *et al*., 2013). For instance, although *H. defensa* and *R. insecticola* are closely related, the divergence of plasmid-associated genes between strains of *H. defensa* and *R. insecticola* is less than between the endosymbiont genomes, indicating that plasmid genes have indeed been transferred horizontally since the bacterial species separated (Degnan *et al*., 2009a). The only complete, active bacteriophage characterised to date from aphid secondary endosymbionts is the APSE phage present in multiple strains of *H. defensa*. The genomes of the several described APSE variants show evidence of homologous and non-homologous recombination and gene acquisition, with the range of acquired toxin genes within the lysis operon thought to underlie much of the defensive function against parasitoids conferred by *H. defensa* to the aphid host. Coupled with the ability to move laterally between bacterial strains, the APSE bacteriophage can increase the genetic capabilities of *H. defensa* to endow protection to the aphid host against parasitoids far more rapidly than through small-scale mutations of the bacterial genome alone (Moran *et al*., 2005a; Degnan & Moran, 2008b; Oliver *et al*., 2009, 2012).

Such genetic variation means that not all endosymbiont strains confer protection to an aphid clone to the same extent, if at all. The diverse levels of protective function afforded to a common aphid clone inoculated with different strains of a given secondary endosymbiont confirm this genetic basis. For instance, Oliver *et al*. (2005) introduced five different strains of *H. defensa* isolated from *A. pisum* and *Aphis craccivora* into clonal pea aphids, with subsequent aphid survival ranging from less than 20% to almost 100% following exposure to the parasitoid *A. ervi*. Conversely, a single isolate of *H. defensa* conferred approximately the same level of parasitoid resistance in five pea aphid clones with different genetic backgrounds, indicating that in this system, the strain of endosymbiont was the determinant of the level of parasitoid resistance rather than the host genotype.

It has since been ascertained that the degree of parasitoid resistance conferred by *H. defensa* is correlated with the strain of APSE that it harbours, and in particular with the pathogenicity genes present in the virulence cassettes. APSE-1, -4 and -5 bacteriophages all contain genes that encode a shiga-like toxin, which acts by disrupting the 28S RNA fragment of the eukaryotic ribosome, whilst homologues of a cytolethal distending toxin gene, *cdtB*, that encode nucleases that interrupt DNA replication during the eukaryotic cell cycle are found in APSE-2, -6 and -7 variants. The mode of action of the tyrosine-aspartic acid repeat-containing protein encoded by the APSE-3 bacteriophage has not yet been elucidated, but similar Y-D repeat proteins have been shown to be toxic to other groups of invertebrates (Degnan & Moran, 2008a). Within a common aphid host background, the frequently encountered phage variants APSE-2 and APSE-3 confer moderate protection (~40%) and high levels of protection (>85%), respectively (Oliver *et al*., 2009). However, whilst these *cdtB* and Y-D repeat toxins are constitutively expressed, other factors encoded on the *H. defensa* chromosome may still be required in order for the protective phenotype to be realised, for example for the transportation of the toxins outside the bacterial cell (Moran *et al*., 2005a; Degnan & Moran, 2008a; Oliver *et al*., 2009). Although relatively small increases in parasitoid resistance have occasionally been reported from strains of *H. defensa* that lack APSE, other studies found approximately equal rates of parasitism between aphids harbouring strains of *H. defensa* from which the phage has been lost and the same clones without secondary endosymbionts (Degnan & Moran, 2008b; Oliver *et al*., 2005, 2009).

No active intact bacteriophages have been found in the genome of the only strain of *R. insecticola* demonstrated to confer almost complete parasitoid resistance to its aphid host. Instead, whilst the core genes of *R. insecticola* str. 5.15 show little divergence from other *R. insecticola* isolates, the genome also contains unique and intact genes for pathogenicity factors and signalling and secretion systems (Hansen *et al*., 2012). In addition to providing a tentative mechanistic basis for the observed parasitoid resistance endowed to the aphid host, this genetically distinct *R. insecticola* strain illustrates the potential for protective traits to arise independently in facultative endosymbionts.

The same genetic variation in the protective function of isolates is true of endosymbionts that confer resistance to the pathogenic fungus *P. neoaphidis*. Transfections of several isolates of *R. insecticola* significantly decreased the fungal susceptibility of their pea aphid hosts, though the use of different recipient aphid clones for each endosymbiont

strain meant the variation in aphid survival could not be attributed solely to the endosymbiont strains used (Scarborough *et al*., 2005). The establishment of different isolates of several facultative endosymbionts into a common host background, however, resulted in reduced susceptibility of aphids to *P. neoaphidis* when harbouring an isolate of *R. insecticola*, but also almost complete protection when harbouring either of two strains of *Rickettsia*, a strain of *Rickettsiella* and one of three strains of *Spiroplasma* (Łukasik *et al*., 2013a). In addition to the disparate results from the three *Spiroplasma* strains illustrating the effect of genotypic variability within an endosymbiont on the aphid host, these findings again demonstrate the repeated occurrence of protective functions amongst aphid endosymbionts.

As abiotic stressors also affect aphid fitness, a further protective function that has arisen in some secondary endosymbionts relates to thermal tolerance. The presence of *S. symbiotica* can positively affect the ability of pea aphid clones to survive and reproduce at higher ambient temperatures or to mitigate the effects of exposure to heat shock in immature stages (Chen *et al*., 2000; Montllor *et al*., 2002). Again, however, the ameliorative effects differ between strains of *S. symbiotica*, as is evident from the infection of different isolates into a common pea aphid host (Russell & Moran, 2006). Heat shocked aphids harbouring *S. symbiotica* contain significantly more bacteriocytes than the same aphid clones free from secondary endosymbionts, with the release of metabolites from the lysed *Serratia* cells tentatively accredited with protecting the *Buchnera* population (Montllor *et al*., 2002; Burke *et al*., 2009a). As other secondary endosymbionts confer their aphid host with little or no thermal tolerance, the mechanism by which aphid fitness is improved may be more complex than a passive protective role; for example, *S. symbiotica* may be able to compensate partially for the loss of *Buchnera* by supplying certain nutrients to the aphid host for growth and reproduction (Russell & Moran, 2006; Koga *et al*., 2003).

In general, however, and in contrast to *B. aphidicola*, there is little evidence to suggest that the aphid secondary endosymbionts characterised to date have a role in providing nutrients to their host. Studies using pea aphids feeding on artificial diets that vary in their nitrogen or amino acid concentration have shown no differences in the performance of those aphids bearing secondary endosymbionts compared to those without (Douglas *et al*., 2006a). Moreover, whereas the densities of *B. aphidicola* cells increase with elevated levels of dietary nitrogen, those of *S. symbiotica* remain constant regardless of nitrogen

availability. This suggests that, unlike the primary symbiont, the density of *S. symbiotica* is not dependent on regulatory controls responding to the metabolic needs of the aphid host (Wilkinson *et al*., 2007).

One exception is the cedar aphid, *Cinara cedri*, in which the genome of the primary endosymbiont has degraded to such an extent that it no longer contains all of the genes necessary for synthesising tryptophan or riboflavin (Gil *et al*., 2002; Perez-Brocal *et al*., 2006). Although the genes for the first enzyme in the tryptophan synthesis pathway are retained on extra-chromosomal plasmids of *B. aphidicola*, the remainder are located on the chromosome of the secondary symbiont *S. symbiotica* (Gosalbes *et al*., 2008). As tryptophan is essential to the aphid host, in European lineages of cedar aphid this strain of *S. symbiotica* is now an obligate rather than a facultative symbiont, although disparities between the symbiont and aphid clades indicate that *S. symbiotica* remains facultative for at least some South American *C. cedri* lineages (Lamelas *et al*., 2008; Burke *et al*., 2009b). Furthermore, the bacteriocytes containing the secondary endosymbionts are as numerous as those housing *B. aphidicola*, and the *S. symbiotica* cells are larger and more rounded than those in other aphid species (Gómez-Valero *et al*., 2004).

Whilst no other facultative endosymbiont has been shown to provide the aphid host with essential amino acids present at low concentrations in the phloem, the presence of *R. insecticola* can enable otherwise maladapted *A. pisum* clones and even a clone of another aphid species, *Megoura crassicauda*, to feed on clover (*Trifolium* spp.) (Tsuchida *et al*., 2004, 2011). This ability has so far only been demonstrated for two strains of a single endosymbiont, with other isolates failing to improve consistently the performance of aphid clones on host plants (Leonardo, 2004; Ferrari *et al*., 2007; McLean *et al*., 2010).

1.1.4 Aphid fitness as a product of multitrophic interactions and endosymbiotic associations

The multitude of biotic and abiotic factors that influence aphid fitness rarely act in isolation, although in a laboratory setting it is often convenient to assess their impact when other variables are controlled. Instead, the relative fitness of aphid clones and hence aphid population structure depends on the outcomes of numerous interactions

within and between trophic levels and with abiotic elements, examples of which are given in the following sections.

The presence of secondary endosymbiotic bacteria further contributes to the observed variation in aphid clonal fitness in response to multitrophic interactions. That these endosymbionts can confer heritable, beneficial traits to their aphid hosts under certain environmental conditions is clear, yet the failure of bacterial infections to reach fixation within aphid populations is indicative of costs attributable to the endosymbiotic association. Differences in intrinsic life history characteristics of aphid clones harbouring various endosymbiont strains when measured under standardised, benign conditions are evidence of genotypic interactions between the two partners of the endosymbiosis. For instance, Vorburger and Gouskov (2011) demonstrated inconsistent effects of different isolates of *H. defensa* on the size, fecundity and longevity of two clones of *A. fabae*. The persistence of a given endosymbiont within an aphid population will therefore depend, amongst other things, on the prevalence of the conditions under which endosymbiont-mediated traits confer a fitness benefit to their aphid host, and the effects on fitness resulting from genomic interactions between the aphid and bacteria (Oliver *et al*., 2008).

1.1.4.1 Aphid–plant interactions

As the sole source of nutrients, the plants from which aphids feed inevitably exert a strong influence on their survival and reproductive rates. As well as spatial and temporal variation in plant availability, nutritional quality and physiological differences resulting from environmental factors, there are genetic differences within and between plants in terms of morphological traits and the nutrient and defensive compounds present. Many aphid species show intraspecific genetic variation in their ability to utilise different plants, and given the vital role of the obligate endosymbiont *B. aphidicola* in providing nutrients present in low concentrations in the phloem, this observed variation in fitness between aphid clones may in part be a result of genetic variation within the primary endosymbiont (Wilkinson & Douglas, 2003). Secondary endosymbiont infections may also expand or alter the range of plants on which the aphid can survive and reproduce, as has been demonstrated for two strains of *R. insecticola* in pea aphids feeding on clover (Ferrari *et al*., 2007; Tsuchida *et al*., 2004, 2011). Other isolates do not alter pea aphid

performance on clover, and in many instances diminish aphid fitness on the alternative host plant *Vicia faba* (Leonardo, 2004; Ferrari *et al*., 2007; McLean *et al*., 2010).

When coupled with a genetic preference for the plants on which their performance is maximised, the genetic variation between aphid clones to utilise different plants can result in host plant adapted races. This has been particularly well studied in pea aphids, in which several such races have been identified (Via, 1999; Caillaud & Via, 2000). Host plant preference is not immutable, however, and within pea aphid populations associated with different plant species there is further genetic variation between clones in the strength of preference for alternative hosts (Ferrari *et al*., 2006). Similar genotypic variation in their preference for and performance on different host plants is seen in *Aphis fabae*, clones of which differ genetically in both their ability to utilise nasturtium as a host plant, and in their capacity to alter their host plant preference and performance over generations (Gorur *et al*., 2005, 2007). Should secondary endosymbionts alter the plant preference of their aphid host as well as aphid performance, there would be the potential for such bacteria to contribute to the formation of host plant specialisation, but this has yet to be demonstrated (Ferrari *et al*., 2007).

Even within a plant species, the fitness of colonising aphids can vary between plant genotypes or cultivars (Aqueel & Leather, 2011). Combined with genetic variation between aphid clones, plant genotype can affect both aphid preference and performance. Different *S. avenae* clones, for example, preferentially colonize different barley genotypes, with some aphid clones rejecting genotypes on which they are equally or more fecund than their preferred host (Zytynska & Preziosi, 2011).

1.1.4.2 Aphid–natural enemy interactions

Several strains of *H. defensa* and a single strain of *R. insecticola* have been shown to bolster the physiological defences of their aphid host, and hence are clearly beneficial when the aphids are subject to parasitoid attack (see sections *1.1.2.3* and *1.1.3.3*). However, the vulnerability of endosymbiotic populations to high temperatures means such protection can be diminished when aphids are reared at high ambient temperatures, or when exposed to fluctuating temperatures (Bensadia *et al*., 2006; Guay *et al*., 2009).

The presence of such defensive bacteria may also affect the interactions between aphids and their natural enemies through less direct means. For example, harbouring *H. defensa* can produce a concomitant reduction in costly behavioural defences of the *A. pisum* host, such as dropping from the plant (Dion *et al*., 2011a). Whilst the higher rate of parasitoid attack on aphids harbouring *H. defensa* compared with aphids free from secondary endosymbionts that displayed more aggressive behavioural defences did not translate into an increase in successful parasitism, the reduction in behavioural defences may render these aphids more prone to predation from other natural enemies.

The effect on parasitoid behaviour of encountering a potential aphid host harbouring *H. defensa* is unclear though is likely to depend, amongst other things, on the current physiological status of the parasitoid and the perceived likelihood of current and future reproductive opportunities (see section *5.1.2.2*). Under controlled laboratory conditions, naïve *A. ervi* parasitoids allowed to attack singly a cohort of pea aphids either with or without *H. defensa* will oviposit in equal numbers of hosts (Oliver *et al*., 2003; Bensadia *et al*., 2006). Presence of the endosymbiont therefore did not reduce the perceived quality of the aphid host to these parasitoids. That the presence and potential defensive role of *H. defensa* is detected and recognised by *A. ervi*, however, is evident from the attack behaviour and egg distribution of the parasitoids when presented with both infected and uninfected aphid hosts simultaneously. *S. avenae* aphids harbouring *H. defensa* were less acceptable to *A. ervi* parasitoids than the same clones uninfected with secondary endosymbionts, despite no evidence of the bacteria contributing to physiological defences (Łukasik *et al*., 2013b). In contrast, another study found that pea aphids harbouring *H. defensa* were more likely to be super-parasitized by *A. ervi*, an adaptive behaviour that in this instance increased the successful parasitism rate of the infected aphids (Oliver *et al*., 2012). Furthermore, the high mortality rates experienced by developing larvae that encounter defensive strains of *H. defensa* can promote a rapid increase in the virulence of emerging parasitoids over a few generations (Dion *et al*., 2011b). Consequently, the benefits of harbouring *H. defensa* may be reduced when the endosymbiotic association is widespread.

Another aphid secondary endosymbiont, *Rickettsiella*, may also indirectly affect parasitism and predation rates by influencing the colour of the adult aphid. Parasitoids will preferentially attack green over red aphid morphs, whilst ladybirds will consume greater numbers of red than green aphids (Losey *et al*., 1997; Libbrecht *et al*., 2007).

These contrasting selection pressures maintain the co-existence of the two colour phenotypes, which in pea aphids result from genetic variation in a carotenoid desaturase enzyme acquired by lateral gene transfer from fungi (Losey *et al*., 1997; Caillaud & Losey, 2010; Moran & Jarvik, 2010). However, the innate preference of parasitoids for green morphs is not fixed, and can be altered by experience such that attack rates by parasitoids tend to be greater towards the more common aphid colour morph (Langley *et al*., 2006). The searching efficiency of ladybirds too is affected by the proportions of red and green colour morphs present, with red morphs safest from predation when in a 1:3 ratio with green morphs (Balog & Schmitz, 2013). By increasing the production of green pigmentation within the aphid, *Rickettsiella* changes the body colour of developing nymphs from red to green (Tsuchida *et al*., 2010). Any benefits associated with the endosymbiont-mediated change in colour are therefore likely to depend not only on the parasitoids and predators present, but also on both the frequency of genetically determined red and green morphs and the frequency of the *Rickettsiella* association.

Although there is genetic variation in the virulence of parasitoid wasps, there is little evidence that different aphid clones are better suited to resisting different parasitoid genotypes (Henter, 1995; Ferrari *et al*., 2001; Vorburger *et al*., 2009). Aphid resistance to parasitoid attack may instead be determined by interactions between the strain of secondary endosymbiont present within the aphid and the parasitoid genotype. Most parasitoid wasps reproduce sexually, resulting in genetic variation in the virulence of the wasps that is difficult to control experimentally (Oliver *et al*., 2003). The parasitoid *Lysiphlebus fabarum*, however, reproduces by thelytokous parthenogenesis and so clonal lines of the wasps can be reared. *Aphis fabae* harbouring *H. defensa* are less susceptible to attack from the parasitoid than clones lacking the secondary symbiont, yet levels of resistance differ depending on the clonal line of *L. fabarum* they are exposed to. Despite genetic variation in both aphid resistance and parasitoid virulence, there is no evidence for genotype-specific interactions in black bean aphids free from *H. defensa* (Vorburger *et al*., 2009; Sandrock *et al*., 2010). The genetic variability in the virulence of *L. fabarum* observed in the study by Vorburger *et al*. (2009) therefore equates to differences in the ability of the parasitoids to overcome the resistance conferred by the secondary symbiont bacteria.

1.1.4.3 Plant–aphid-natural enemy interactions

As well as being a source of nutrients, the plant on which the aphid feeds influences the biotic and abiotic environment of the aphid, including the natural enemies to which the aphid is exposed. Predators, parasitoids and pathogens and abiotic factors can all impact aphid fitness and so nutrient quality alone is not the sole determinant of host plant preference, as is evident from observations of aphid assemblages skewed towards relatively low-quality host plants (Underwood, 2009). Host plants can differ dramatically in their architecture, with increasing plant structural complexity often accompanied by a decrease in the rate at which aphids are found successfully by predators. Plants in which the morphological structure or the chemical composition reduces natural enemy pressures may therefore be perceived more favourably as alternative host plants. The red deadnettle plant *Lamium purpureum*, for instance, supports a lower abundance of predators than other host plants of the black bean aphid *Aphis fabae*, and so may be colonised despite being of inferior nutritional quality (Adams & Douglas, 1997; Raymond *et al*., 2000; Wilkinson & Douglas, 2003). The searching efficiency of *Coccinella* ladybirds for *A. pisum* aphids is reduced on pea plants with the greatest morphological complexity (Legrand & Barbosa, 2003), although this may not be true for other predators (Reynolds & Cuddington, 2012). Other morphological characteristics of plants, such as stem shape, surface waxes or high trichome densities on the leaves can affect aphid mortality rates through their effects on predator mobility (Carter *et al*., 1984; Grevstad & Klepetka, 1992), whilst physical characteristics such as rolled leaves and blade-sheath junctions may provide refuges for potential prey. Parasitism rates and predation by ladybirds and lacewings of the Russian wheat aphid *Diuraphis noxia*, for example, are reduced on varieties of grass with rolled leaves in which the aphids can aggregate (Reed *et al*., 1992; Messina *et al*., 1997; Clark & Messina, 1998a, 1998b; Messina & Hanks, 1998).

Another factor influencing natural enemy success is the within-plant distribution of the aphid colony, with certain plant structures providing refuge from predators and parasitoids. For instance, *Rhopalosiphum maidis* aphids on Johnsongrass are usually found within the whorl of young leaves despite the poor nutritional quality relative to more mature parts of the plant, but encounter lower parasitism rates than exposed aphids (Gonzáles *et al*., 2001). As a further example, the parasitoid *Aphidius rhopalosiphi* will attack both *Metopolophium dirhodum* and *Sitobion avenae* aphids on wheat plants, but

successfully parasitize the latter less often as a result of these aphids preferentially feeding on the ears of the cereal where they are partially protected between the grains (Gardner & Dixon, 1985).

Just as host plant choice can affect aphid interactions with natural enemies, so too can the presence of natural enemies affect host plant choice. In the absence of natural enemies, both *S. avenae* and *R. padi* select the cultivars of wheat on which their performances are maximised. However, chemical cues signifying the past presence of natural enemies on these cultivars caused the plant preference of both species to shift to nutritionally inferior varieties of wheat where predation risks were lower (Wilson & Leather, 2012).

Interestingly, host plant specialisation in *A. pisum* populations feeding on *Trifolium* is strongly correlated with high frequencies of the endosymbiont *R. insecticola* (Tsuchida *et al*., 2002; Simon *et al*., 2003; Leonardo & Muiru, 2003; Ferrari *et al*., 2004), despite the majority of *R. insecticola* isolates apparently conferring no direct benefits to the aphid in terms of host plant utilisation (Leonardo, 2004; Ferrari *et al*., 2007). Several explanations have been proposed to explain the distribution of this endosymbiont in aphids feeding on different plant species. The association may simply have resulted from the founding of host-adapted lineages by aphid clones more tolerant to harbouring *R. insecticola* (Tsuchida *et al*., 2002; Leonardo, 2004), or may reflect an initial role for *R. insecticola* in host-plant adaptation that has since become redundant as the aphids became reproductively isolated (Leonardo, 2004). Alternatively, the environmental conditions associated with clover may select for particular endosymbiotic associations. The higher rates of aphid infection with *R. insecticola* on *Trifolium* could be a consequence of higher rates of horizontal transfer within the clover-adapted populations (Caspi-Fluger *et al*., 2011) or, given the protective function bestowed by multiple *R. insecticola* strains against the fungus *Pandora neoaphidis*, the observed endosymbiont distribution could arise if the risk of pathogenic infection is greater whilst colonising on clover (Ferrari & Vavre, 2011; Brady & White, 2013). Other aphid species are known in which host plant associated lineages display high incidences of endosymbiont infection with no apparent nutritional contribution, to which the same reasoning can be applied; in the cowpea aphid *Aphis craccivora*, populations associated with alfalfa are commonly infected with the *H. defensa*, whilst only populations assembled on black bean locust harbour *Arsenophonus* (Brady & White, 2013).

1.1.5 Conclusions on determinants of aphid fitness

The structure of aphid populations will be shaped by the relative abilities of different genotypes to locate and acquire nutrients from suitable host plants, avoid predators, pathogens and parasitoids and withstand periods of environmental stress. None of these factors influence aphid fitness in isolation, and the temporal and spatial heterogeneity of the environments in which aphids reside mean the relative pressures from these biotic and abiotic factors fluctuate. Despite the phenotypic plasticity that enables aphids to produce winged morphs capable of dispersal when conditions deteriorate, the rapidity with which parthenogenetic aphids can reproduce means dominant genotypes may quickly be replaced as their environment changes.

The genotypic variation that underlies the observed variation in aphid fitness is not solely attributable to the aphid genome, but also to the genomes of the primary endosymbiont *B. aphidicola* and, where present, secondary endosymbiotic bacteria. The strict vertical transmission of *B. aphidicola* coupled with genomic stasis has reduced the capacity of this endosymbiont to impact upon clonal variation. In contrast, the acquisition of stable secondary endosymbiont infections can instantaneously confer ecologically important traits upon the aphid host, yet genetic variation between the bacterial isolates and in their effects on different aphid clones further contributes to disparities in aphid fitness. The frequency and distribution of secondary endosymbionts will in turn depend on multiple factors, including the prevalence of the conditions under which the endosymbionts benefit their aphid host, how the various endosymbiont strains interact with different aphid host genotypes, and the rates of both horizontal transmission within and between aphid species and the stochastic loss of endosymbiont infections from clonal lines.

1.2 The study system

1.2.1 The potato aphid, Macrosiphum euphorbiae

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Hemiptera
Family: Aphididae
Tribe: Macrosiphini
Genus: *Macrosiphum* (Passerini, 1860)
Species: *Macrosiphum euphorbiae* (Thomas, 1878)

M. euphorbiae is a comparatively large spindle-shaped or pear-shaped aphid, with relatively long legs, antennae, siphunculi and cauda. Usually a shade of green in colour, some potato aphid morphs are pink or magenta, and in alate morphs the thoracic lobes are yellowish-brown. The four nymphal instars are often paler than the adults due to a layer of light-coloured wax, but with a darker central stripe (Figure 1.2) (Blackman & Eastop, 2000).

Figure 1.2: Adult apterous *M. euphorbiae* aphid (left) and *M. euphorbiae* nymphs of various instar (right). Images courtesy of S. Malecki.

The majority of *M. euphorbiae* populations in North America and Canada are holocyclic, producing sexual morphs principally in response to shortening day length and overwintering as eggs on primary host plants from the *Rosa* genus (MacGillivray & Anderson, 1964; Landis *et al*., 1972; Lamb & MacKay, 1997). In contrast, European populations are mainly anholocyclic and overwinter as viviparae on secondary host plants: herbaceous weed species such as common groundsel, nettles and shepherd's purse, potato sprouts in stores and chitting houses and in glasshouses on crops such as

lettuce, although sexual males and oviparous females may occasionally be produced (Turl, 1983; Blackman & Eastop, 2000).

Throughout the summer months the viviparous *M. euphorbiae* are highly polyphagous, with more than 200 plant species from over twenty families known to be suitable host plants (Blackman & Eastop, 2000). These include agriculturally and economically important crops such as potato, tomato, tobacco, bean, lettuce, raspberry, peppers and roses, as well as some cereals. Serious infestations of potato aphid can occur in both glasshouse and field-grown crops, causing both direct and indirect damage to the plants. In certain varieties of potato, for example, high densities of *M. euphorbiae* cause false leaf roll in the upper leaves, reducing the transportation of photosynthetic products and significantly reducing tuber yields (Veen, 1985). In tomato plants, as well as reducing yields the presence of *M. euphorbiae* can stunt plant growth, and attract other insect pests that further damage crops (Walgenbach, 1997; Tomescu & Negru, 2002). The secretion of honeydew as the aphids feed and the resulting sooty moulds can also reduce the marketability of crops such as salads and strawberries (Trumble *et al*., 1983). Furthermore, *M euphorbiae* is also capable of transmitting at least 45 plant viruses as it feeds (Blackman & Eastop, 2000); most, such as potato virus Y (PVY) and cucumber and lettuce mosaic viruses are non-persistent but a few, such as potato leafroll virus, pass into the aphid salivary glands and so the aphid remains viruliferous (Woodford *et al*., 1995; Legarrea *et al*., 2012). Although not as efficient a vector as *Myzus persicae* for the transmission of many of these viruses, *M. euphorbiae* has nevertheless been implicated in disease transmission and subsequent loss of yield in agricultural systems across its range (Howell, 1974; Radcliffe & Ragsdale, 2002).

Originating in North America, *M. euphorbiae* now has an almost global distribution, absent only from the Polar regions (Blackman & Eastop, 2000). First recorded in the UK in 1917 from aphid samples collected in Wye in Kent, the spread of the potato aphid to continental Europe followed soon after (Eastop, 1958). Today *M. euphorbiae* is common throughout Britain, and as such is one of the pest species monitored by the suction trap network as part of the Rothamsted Insect Survey, providing information regarding their phenology and abundance from year to year to ecologists and industry sponsors alike (Harrington & Woiwod, 2007).

Numerous generalist and specialist aphidophagous predators will consume *M. euphorbiae*, including adult and larval ladybirds, hoverflies, lacewings, ground and rove beetles, plant bugs, minute pirate bugs, predatory midges and spiders (Walker *et al*., 1984; Nakata, 1995; Dean & Schuster, 1995; Alvarado *et al*., 1997; Lucas *et al*., 1998). Parasitoids are another cause of mortality, although their impact on *M. euphorbiae* populations may be limited by intraguild predation of parasitized aphids and hyperparasitism (Sullivan & van den Bosch, 1971; Walker *et al*., 1984; Brodeur & McNeil, 1992; Snyder *et al*., 2004). Within North America, *Aphidius nigripes* is the predominant parasitoid of potato aphids reported on potato and tomato crops (Shands *et al*., 1965; Sullivan & van den Bosch, 1971; Walker *et al*., 1984; Walgenbach, 1994), with *A. ervi* also contributing to parasitism rates following its introduction into North America in the 1950s (Feng *et al*., 1992). Within the UK, *A. ervi* is the principal parasitoid of *M. euphorbiae*, and is also sold commercially as part of integrated pest management strategies for combatting glasshouse infestations (van Lenteren *et al*., 1997; van Lenteren, 2003). Other species of *Aphidius*, as well as *Aphilinius* species, *Praon volucre* and *Diaeretiella rapae*, are also documented parasitoids of *M. euphorbiae* (Walgenbach, 1994; Nakata, 1995; Takada, 2002; Starý *et al*., 2007; Lins *et al*., 2011).

1.2.2 The parasitoid Aphidius ervi

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Hymenoptera
Superfamily: Ichneumonoidea
Family: Braconidae
Subfamily: Aphidiinae
Genus: Aphidius
Species: *Aphidius ervi* (Halliday, 1834)

Aphidius ervi is a small, slender-bodied cosmopolitan aphid parasitoid. Approximately 3–5 mm in length, the head and thorax are dark, brown-black, whilst the colouration of the abdomen varies from yellow with dark banding to entirely brown-black. The long thin antennae can reach to the middle of the abdomen, and the legs are yellow with darker brown pigmentation at the ends of the coxae and tarsi (Figure 1.3) (Powell, 1982; Kos *et al*., 2009).

Figure 1.3: *A. ervi* female searching for hosts (left), and attacking an *M. euphorbiae* nymph (right). Images courtesy of David Riley.

All aphidiine parasitoids are solitary koinobionts that use aphids as hosts for their developing offspring. Although the hosts continue to feed and grow for a period of time following parasitism, only a single parasitoid can develop within an individual host (Starý, 1989). An adult female *A. ervi* parasitoid contains less than 100 mature eggs within the first few hours of emergence, but eggs continue to develop (pro-synovigenesis) so that more than 300 aphids can be parasitized by a single female in her lifetime (He *et al*., 2006; He & Wang, 2006). The high numbers of eggs present within the ovaries means each individual egg is small, approx. 50–70 µm in size, and is poorly provisioned with yolk or other energetic reserves. Consequently, the parasitoid embryos must rapidly utilize the resources of their aphid host upon oviposition.

Parasitism begins when the female wasp inserts her ovipositor and lays a single egg into the adipose tissue or haemocoel of the aphid host. She also releases venom that can castrate the aphid host and initiate the degradation of germarial cells and developing embryos, resulting in greater nutrient concentrations in the haemolymph for the parasitoid to utilize (Pennacchio *et al*., 1995; Digilio *et al*., 1998, 2000; Falabella *et al*., 2007). The hydropic egg swells and undergoes rapid cellularization and cell differentiation so that within 24 hours of parasitism the chorion ruptures, releasing a cluster of developing embryonic cells separated from the host haemolymph by a monolayer of serosal cells. The embryo continues to develop until the second hatching, at which time a first instar larva breaks free of the serosal membrane into the host haemocoel (Sabri *et al*., 2011).

The remaining stages of parasitoid development are typical of holometabolous insects, with three larval instar stages followed by pupation and the eclosion of the adult parasitoid (O'Donnell, 1987; Pennacchio & Digilio, 1989). The first instar larva bears large, sickle-shaped mandibles, but no clear gut. Nutrient absorption is therefore thought to occur across the body surface (Pennacchio & Digilio, 1989; de Eguileor *et al*., 2001). The mandibles, meanwhile, are implicated along with physiological suppression mechanisms in competing against conspecific or heterospecific larvae (Bai, 1991). By the second larval instar the gut is fully formed, but with oral appendages absent feeding is restricted to the host fluids, with the continuing uptake of nutrients across the epidermis (Pennacchio & Digilio, 1989; de Eguileor *et al*., 2001; Giordana *et al*., 2003). The teratocytes, derived from the cells of the serosal membrane, aid nutrient acquisition of the first and second instars by synthesising and secreting proteins and redirecting the metabolism of *B. aphidicola* to increase the free amino acid content of the haemolymph (Falabella *et al*., 2000). Only in the third, final larval instar does the parasitoid feed directly on the aphid tissues using heavily sclerotized mandibles, eventually killing the host and leaving only the aphid cuticle (Polaszek, 1986; Pennacchio & Digilio, 1989). By this stage spiracles and a tracheal system are evident, and the silk glands present in the second instar larva now contain secretory products (Pennacchio & Digilio, 1989; de Eguileor *et al*., 2001).

The mature third instar larva cuts a hole on the lower side of the aphid cuticle and fixes the remains of its host to the surface of the substrate using secretion from the silk glands, forming the distinctive 'mummy'. A cocoon of silk is then spun in which the developing parasitoid pupates. Once development is complete, the adult parasitoid emerges through a circular hole it cuts in the dorsal abdomen of the aphid mummy (Figure 1.4).

Figure 1.4: An empty aphid mummy showing the circular hole cut by the eclosed *A. ervi* adult parasitoid. Images courtesy of David Riley.

The development time of *A. ervi* is strongly temperature dependent, and indeed, the induction of diapause in final instar larvae that enables developing *A. ervi* parasitoids to overwinter within their aphid host is induced by both day length and temperature (Christiansen-Weniger & Hardie, 1999; Sigsgaard, 2000; Malina & Praslička, 2008). At 20 °C, *A. ervi* reared on *M. euphorbiae* take approximately 12 days from parasitism to emerge. The daily developmental progress of *A. ervi* under these conditions is shown in Figure 1.5.

Palearctic in origin, *A. ervi* has a wide natural geographic range including Europe, North Africa, Middle East, China, Russia, India and Northern Japan. Following introductions of the parasitoid as a means of controlling aphid populations throughout the last century, the established range has now expanded to North and South America, New Zealand and Australia (Marsh, 1977; Milne, 1986; Cameron & Walker, 1989; Carver, 1989). The range of aphid hosts utilized by *A. ervi* is equally broad, with over 20 species of aphid known to be successfully parasitized (Starý, 1974). Whilst the aphid hosts reported for *A. ervi* in Japan, Taiwan, India and Israel are restricted to *A. pisum* and closely related species, elsewhere the list of potential aphid hosts extends to include *A. kondoi*, *Aulacorthum solani*, *Rhopalosiphum padi*, *Myzus persicae*, *M. euphorbiae* and *Sitobion avenae* (Starý, 1974; Marsh, 1977; Powell, 1982; Takada & Tada, 2000). Consequently, this cosmopolitan parasitoid is considered an important biological control agent in the agricultural systems in which these aphids may be found (Carver, 1989).

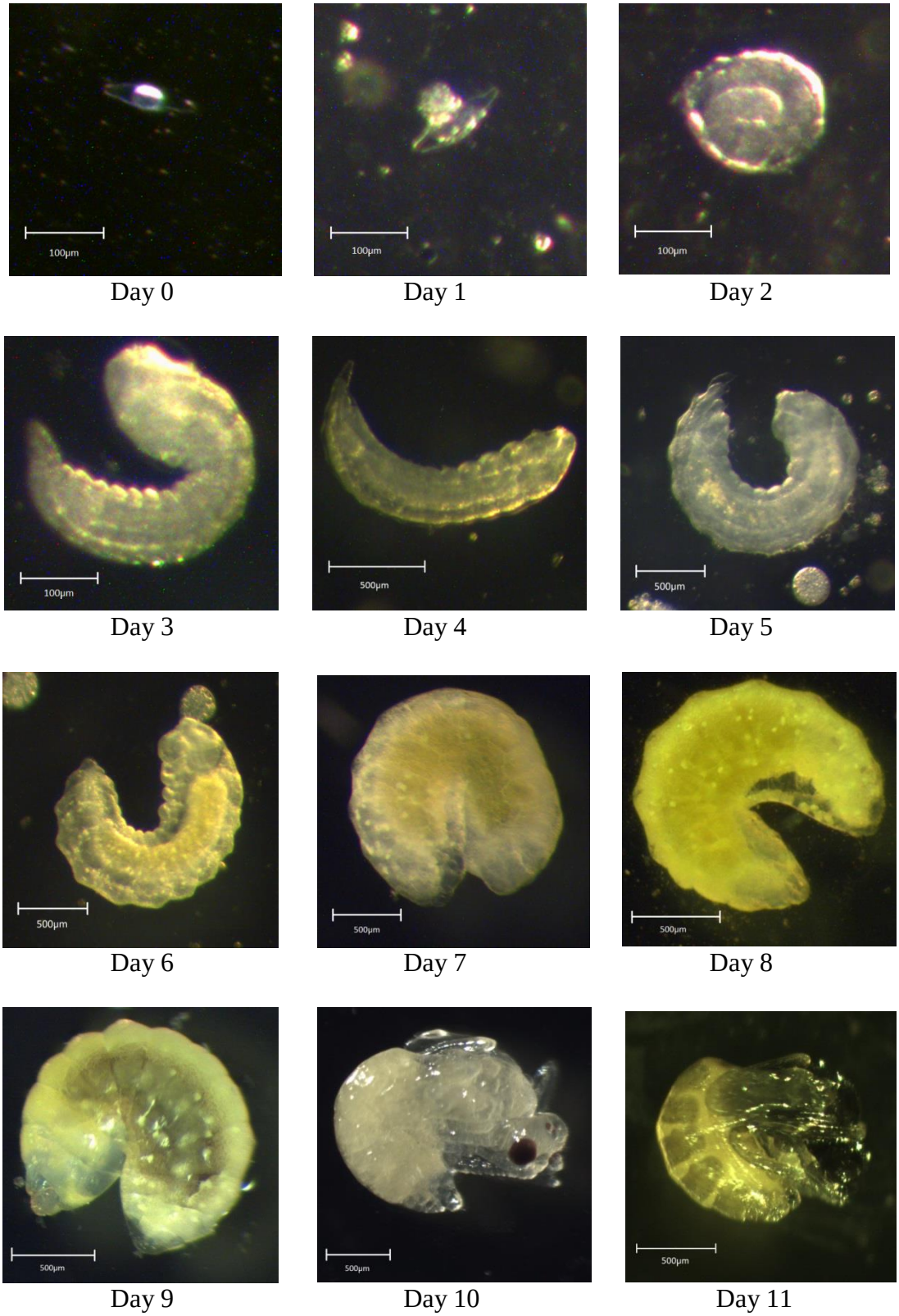

Figure 1.5: Developmental stages of *A. ervi* within a *M. euphorbiae* host, reared at 20 °C. Images taken as part of this study.

1.3 Aims and objectives of the current study

The overarching aim of the work presented in this thesis was to obtain a better understanding of the genetic and endosymbiont-mediated diversity in the aphid crop pest, the potato aphid *Macrosiphum euphorbiae*, and how this relates to aspects of aphid fitness. For the purpose of this study 19 clonal lines of *M. euphorbiae* were established and maintained in culture from founding aphids collected from various locations around eastern Scotland over the course of three years; sub-sets of these lines were used in all of the experimental work presented here.

Unlike the holocyclic life cycle of the pea aphid, in which sexual reproduction in the autumn results in new genetic combinations in the fundatrices emerging in the spring from the cold-hardy eggs in which they overwintered, within the UK *M. euphorbiae* is anholocyclic and so only mutation or immigration will introduce novel genotypes into the population (see section *1.2.1*). Consequently the sample of potato aphid lines collected were hypothesised to comprise of relatively few distinct genotypes, as predicted from theoretical models and from the comparison of asexual and sexual populations of other aphid species (Simon *et al*., 1999; Balloux *et al*., 2003; Kanbe & Akimoto, 2009). Thus, the aim of the research presented in Chapter 3 was to assess the genotypic diversity of these isofemale lineages based on the analysis of microsatellite markers and to establish the presence, identity and titre of known secondary endosymbiont bacteria across the aphid lines using diagnostic and quantitative PCR. Secondary endosymbionts are potential souces of heritable traits to their aphid host, and infections of *H. defensa*, *R. insecticola* and *Rickettsia* have been identified previously from single *M. euphorbiae* clones (Russell *et al*., 2003; Russell & Moran, 2005; Francis *et al*., 2010). By screening the aphid lines on a regular basis, the stability of the endosymbiotic infections was elucidated.

The parthenogenetic nature by which aphids reproduce make them amenable organisms in which to manipulate endosymbiont infections; differences in fitness between genetically identical sub-lines of *M. euphorbiae* varying only in their endosymbiont complement can therefore be attributed to the bacteria. The aim of the research presented in Chapter 4 was to assess the impact on aphid fitness of the secondary endosymbiont *Hamiltonella defensa* using aphid lines that were naturally infected with *H. defensa* and lines artificially cured of secondary endosymbionts using antibiotics. Various fitness

parameters of the different *M. euphorbiae* holobionts (genotype + microbiotia combinations) were evaluated under benign glasshouse conditions to test the hypothesis that innate fitness costs prevent *H. defensa* from reaching fixation within natural potato aphid populations.

Finally, in Chapter 5, I aimed to test whether the susceptibility of the various *M. euphorbiae* holobionts to a common natural enemy, the parasitoid *Aphidius ervi*, varied with the presence of *H. defensa*. In several aphid systems, the presence of the endosymbiont *H. defensa* and the associated APSE phage as the principal determinant of parasitoid susceptibility is well documented (see section *1.1.2.3*). With stable *H. defensa* infections identified in several potato aphid lines, this allowed the hypothesis that the bacteria conferred protection to its aphid host against developing parasitoids to be tested, which in turn could explain some of the selection pressures that may underlie the maintenance of the endosymbiotic association with *M. euphorbiae*. As parasitoids and other natural enemies are a significant cause of mortality in field populations of *M. euphorbiae* (Karley *et al*., 2003), these results will also enable more informed speculation as to how selection pressures from parasitoids may affect the composition of *M. euphorbiae* populations, as well as illustrating potential complications of using *A. ervi* for integrated pest management strategies.

In summary, the objectives of this study were:

1. To quantify the genotypic diversity of isofemale lineages of *Macrosiphum euphorbiae* collectd from sites across Tayside and Fife (Chapter 3);
2. To establish the presence, identity and titre of known secondary endosymbiotic bacteria across the aphid lines (Chapter 3);
3. To quantify the impact on aphid fitness of harbouring the secondary endosymbiont *Hamiltonella defensa* (Chapter 4);
4. To determine whether the secondary endosymbiont *H. defensa* reduces the susceptibility of *M. euphorbiae* holobionts to a common natural enemy, the parasitoid *Aphidius ervi* (Chapter 5).

Chapter 2: General Methods

2.1 Insect cultures

2.1.1 Collection and establishment of aphid clonal lines

Individual *Macrosiphum euphorbiae* aphids were collected from fields or garden plots of potato plants (*Solanum tuberosum*) from the Dundee and Fife areas throughout July and August for three consecutive summers between 2009 and 2011. Each collected aphid was transferred to a potato leaf cutting (cultivar Désirée) within a culture cup formed from two stacked clear polystyrene beakers (No. 16 Clear Container from A. W. Gregory & Co., Sevenoaks, UK). A hole approx. 1cm in diameter in the base of the inner cup allowed the stem of the leaf cutting to sit submerged in 2 cm of water in the outer cup. A linear low density polyethylene (LLDPE) plastic lid from which a central portion had been removed and replaced with gauze provided ventilation and prevented the build-up of condensation. Each culture was labelled with the species name, the provisional clonal line and the location and date collected (Figure 2.1).

Aphid cultures were maintained in a controlled environment room at 18°C ± 2°C, 60% humidity and with 16h light: 8h dark. After two weeks, any parasitized or fungal-infected aphids were discarded, whilst those that appeared healthy and that produced offspring were used to set up clonal lineages, each initiated from a single individual (Table 2.1).

2.1.2 Maintenance of aphid clonal lines

Aphids were cultured on *S. tuberosum* cv. Désirée plants grown from whole tubers or from the dissected eyes of chitted tubers (Marshalls Seeds, Huntingdon, UK) in insecticide-free compost (sand–perlite–peat mix containing N:P:K 17:10:15; William Sinclair Horticulture LTD, Lincoln, UK) under glasshouse conditions.

Clonal lineages of *M. euphorbiae* aphids were maintained at low densities in culture cups under constant conditions (see *2.1.1*) and sub-cultured weekly on to fresh plant material. For each aphid culture the inner and outer cups were thoroughly washed and dried and

M. euphorbiae clonal line	Date collected	Collection location	OS Grid reference
AA09/02	16/07/2009	Dron field E, Balruddery, Dundee	NO 30119 32688
AA09/03	16/07/2009	Dron field W, Balruddery, Dundee	NO 29833 32669
AA09/04	16/07/2009	Mini Rotation, JHI, Invergowrie	NO 33858 29993
AA09/06	16/07/2009	Living Field, JHI, Invergowrie	NO 33964 29897
AA09/11	17/07/2009	City Road allotments, Dundee	NO 38539 30626
AA09/12	23/07/2009	Laystone Farm M, Fife	NO 18800 38200
AA09/13	23/07/2009	Laystone Farm W, Fife	NO 18800 38200
AA09/14	30/07/2009	Living Field, JHI, Invergowrie	NO 33964 29897
HC10/02	02/07/2010	Living Field, JHI, Invergowrie	NO 33964 29897
HC10/05	02/07/2010	Brax Farm, Arbroath	NO 59316 43615
HC10/06	02/07/2010	Brax Farm, Arbroath	NO 59316 43615
HC10/07	03/07/2010	Private garden, Broughty Ferry Rd, Dundee	NO 43083 31091
HC10/08	03/07/2010	Private garden, Broughty Ferry Rd, Dundee	NO 43083 31091
HC10/14	23/07/2010	Living Field, JHI, Invergowrie	NO 33964 29897
AK11/01	27/07/2011	Living Field, JHI, Invergowrie	NO 33964 29897
AK11/02	24/07/2011	Living Field, JHI, Invergowrie	NO 33964 29897
HC11/02	09/08/2011	Mini Rotation, JHI, Invergowrie	NO 33858 29993
HC11/03	09/08/2011	Mini Rotation, JHI, Invergowrie	NO 33858 29993
HC11/09	09/08/2011	Mini Rotation, JHI, Invergowrie	NO 33858 29993

Table 2.1: Date and location from which founding aphids for the 19 *M. euphorbiae* clonal lines kept in culture were collected. The host plant of all collected aphids was *Solanum tuberosum*.

the water replaced before a new leaf cutting from a Désirée potato plant was inserted. Approximately five adults and ten nymphs were transferred from the old leaf cutting using a fine paintbrush, and the remaining aphids on the old leaf cutting were discarded.

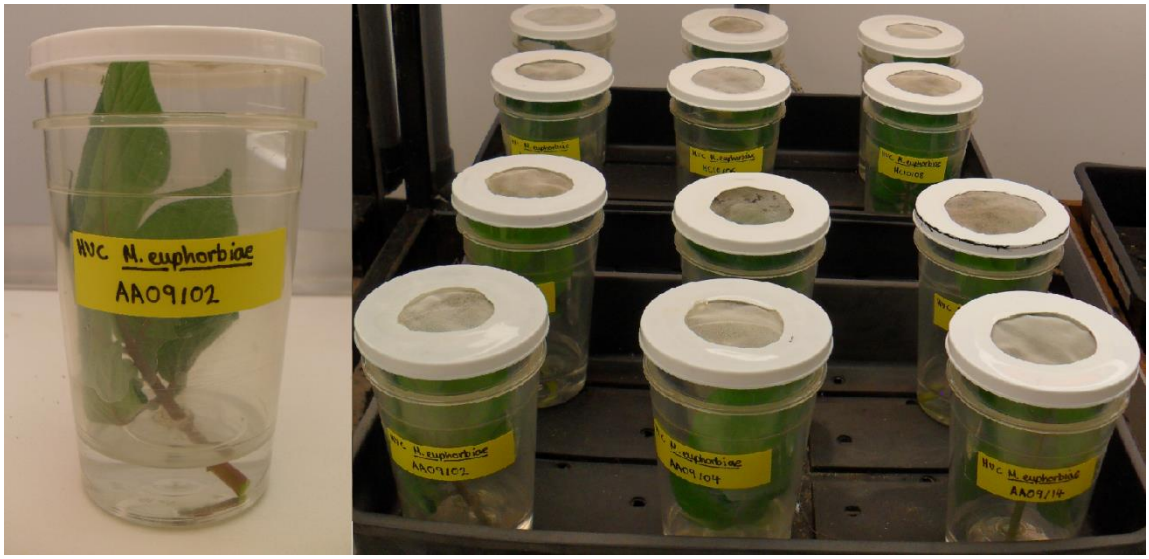

Figure 2.1: Cup cultures of *M. euphorbiae* aphid clonal lines

When greater numbers of *M. euphorbiae* aphids were required, *c*. 15 aphids from a given clonal line were transferred to a young Désirée potato plant and confined using a mesh cage approximately 18cm in diameter and 35cm in height supported by a metal frame and secured at the base around the plant pot with elastic (Insectopia, Austrey, UK). These plant cultures were maintained under constant conditions (see *2.1.1*).

2.1.3 Maintenance of parasitoid cultures

Aphidius ervi parasitoid mummies were purchased from Syngenta-Bioline (Syngenta-Bioline Ltd, Little Clacton, UK). Prior to delivery, the parasitoids had been bred on an *Acyrthosiphon pisum* (pea) aphid culture of mixed clonal genotypes that were periodically refreshed to minimise selection for resistance characteristics within the aphid population, and parasitoids from external stocks were regularly introduced into the *A. ervi* culture in order to prevent inbreeding and maintain the heterozygosity of the population.

The supplied *A. ervi* parasitoid mummies were transferred to a small clear polypropylene container (80 mm × 80 mm × 50 mm; Sainsbury's Supermarkets LTD, London, UK) into which 2 holes (1cm in diameter) had been drilled and covered with gauze to provide ventilation. Emerged individuals were removed daily and transferred to new containers, resulting in cohorts of parasitoids of known age. To prevent parasitoids escaping during the transfer process, the insects were first anaesthetised by directing a stream of CO_2 gas (99.8% purity; BOC, Guildford, UK) into the container through one of the ventilation holes before the parasitoids were moved using a fine paintbrush. Each day cohort was provided with a ball of cotton wool soaked in a 50% (v/v) honey solution on which to feed; this was held within a small plastic lid secured with Blu-Tack (Bostik, Stafford, UK) and was replaced daily to prevent fermentation of the honey.

To ensure a continuous supply of *A. ervi* parasitoids, cultures were reared on *A. pisum* (pea) aphids (clonal line LL01) maintained in culture at JHI under the conditions described above (*2.1.1*). This clonal aphid line harbours no known secondary endosymbionts, and has a high reproductive output. Each generation of parasitoids was produced by infesting 2–3 pots of three broad bean plants (*Vicia faba* cultivar The Sutton; Marshalls Seeds, Huntingdon, UK) with approximately 20 pea aphids before

enclosing the infested plants with mesh cages (see section *2.1.2*). After several days, 5 female *A. ervi* wasps aged at least 2 days and presumed mated were removed from the stock culture and added to each *A. pisum* plant culture. A small ball of cotton wool soaked in 50% (v/v) honey solution was also added to each cage, although unlike for the day cohorts this was not refreshed daily.

Ten days after the parasitoids were first introduced the cages were carefully removed from the bean plants and the mummies that had developed gently prised from the plant material using foil forceps (Figure 2.2). These mummies were transferred to a new ventilated polypropylene container labelled with the species name, the generation and batch number and the date the mummies were collected. If substantial numbers of aphids were still alive on the bean plants the pots were re-covered in the mesh cages for an additional two days to allow further mummies to develop and the removal process repeated, otherwise the plants were discarded. Parasitoids emerging from the collected mummies were transferred to new containers in day cohorts as detailed previously. Parasitoids were bred for a maximum of 12 generations before being discarded to prevent any potential effects of inbreeding depression on parasitoid vigour.

Figure 2.2: *A. pisum* 'mummies' parasitized by *Aphidius ervi* parasitoids

All of the parasitoid cultures and the pea aphid cultures on which they were reared were maintained in growth cabinets or in a growth room (IMAGO F3000 and Reftech custom-made controlled-environment room respectively, Snijders Scientific b.v., Tilburg, The Netherlands) at 20 °C ± 1°C, 60% humidity and with a light regime of 16h light: 8h dark.

2.2 Molecular biology methodology

2.2.1 Preparation of total aphid DNA

2.2.1.1 Extraction of total DNA from M. euphorbiae *aphids*

Total (aphid genomic plus microbiotal) DNA was extracted from whole *M. euphorbiae* aphids using the DNeasy® Blood and Tissue kit (Qiagen, Crawley, UK), using a protocol modified from that provided by the manufacturer for DNA extractions from insects.

DNA was extracted from either fresh aphid material or from aphid samples frozen at -20°C. Each aphid sample was first frozen within a 2 mL Eppendorf tube in liquid nitrogen and the aphid tissue ground using a polypropylene micropestle (Eppendorf, New York, USA) that had been surface sterilised in 100% ethanol. Buffer ATL (180 µL) and 20 µL proteinase K were added, and the sample incubated at 55°C and regularly vortexed to aid lysis of the insect tissues. After an hour, 200 µL buffer AL was added to the lysate and incubated at 70°C for 10 minutes. The sample was then centrifuged for 1 minute at 6000 rcf and, if the sample consisted of more than one aphid, the supernatant was transferred to a fresh Eppendorf tube to prevent the debris material from clogging the spin column membrane in subsequent steps. After the addition of 200 µL of 100% ethanol to the lysate and vortexing, the sample was pipetted into a DNeasy Mini spin column within a 2 mL collection tube and centrifuged for 1 minute at 6000 rcf. The spin column was then transferred to a fresh 2mL collection tube (DNA bound to the central membrane of the column), 500 µL buffer AW1 added and the sample centrifuged for 1 minute at 6000 rcf. The spin column was transferred to another fresh 2 mL collection tube and 500 µL of a second wash buffer, AW2, added and the sample centrifuged at 20240 rcf for 3 minutes; these two wash steps removed remaining contaminants and enzyme inhibitors from the membrane. Finally, the spin column was transferred to a clean, labelled 1.5 mL Eppendorf tube and 200 µL elution buffer (Buffer AE) was added directly to the membrane. Following 1 minute incubation at room temperature the columns were centrifuged at 6000 rcf to elute the aphid DNA. For each aphid sample the eluted DNA was divided into four aliquots (50 µL) to be stored at -20°C.

2.2.1.2 Estimation of DNA concentrations

The concentration of the total DNA extracted from whole aphids was estimated from the absorbance at 260 nm using a NanoDrop ND 1000 Spectrophotometer (ThermoFisher Scientific, Loughborough, UK). The purity of the DNA was assessed from the ratio of sample absorbance at 260 and 280 nm, with ratios considerably lower than 1.8 indicating the presence of protein contamination.

2.2.2 Diagnostic Polymerase Chain Reaction (PCR)

2.2.2.1 Diagnostic PCR reaction contents and conditions

The presence of facultative endosymbionts in each aphid clonal line was determined by PCR reactions using published primers specific to the 16S rRNA gene of *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, PAXS, *Rickettsia*, *Spiroplasma* and *Rickettsiella*; successful amplification of products of the expected size indicated the presence of the bacterial types in a given *M. euphorbiae* template (see Appendix 1, section *A1.1.1* for a list of primer sequences used and the published sources). Reactions using primers for amplifying the 16S rRNA gene and the linked 16S–23S rRNA prokaryotic genes were also conducted. As all bacterial genomes include the conserved 16S rRNA gene, and as the obligate primary endosymbiont *Buchnera aphidicola* is present within every aphid, a positive result in the 16S rRNA screen served to demonstrate successful extraction of bacterial DNA from the aphid samples. The 16S and 23S rRNA genes are no longer linked in the primary endosymbiont, and so this screen was used to indicate the presence of bacteria other than *B. aphidicola*. In addition to screening for facultative endosymbiont bacteria, the presence or absence of the APSE (*Acyrthosiphon pisum* secondary endosymbiont) bacteriophage associated with *H. defensa* was also determined by diagnostic PCR using primers specified in Appendix 1.

Diagnostic PCR products were amplified using GoTaq® DNA polymerase (Promega, Southampton, UK) in 25 µL reactions (see Appendix 1 section *A1.1.2* for full reaction mix contents and volumes). The dNTP stocks (Promega, Southampton, UK) were provided at a concentration of 12.5 µM, giving a final concentration of 0.25 µM in the reaction mix, whilst the primer stocks (Sigma-Aldrich Ltd, Gillingham, UK) were

provided at a concentration of 10 µM, giving a final concentration of 0.4 µM. The restriction enzyme *Hha*1 (Promega, Southampton, UK) was also added (1 unit per 25 µL reaction mix), and after dividing the reaction mix between 0.2 mL PCR tubes (Axygen Inc., California, USA) the mixture was run on a digest programme in the thermocycler (Eppendorf Mastercycler®ep; Eppendorf AG, Hamburg, Germany) for 40 minutes at 37ºC, followed by 10 minutes at 65 ºC. This ensured that DNA from any contaminating bacteria present in the reaction mix was broken down and therefore did not contribute to the PCR results (Vink *et al*., 2014).

After the *Hha*1 digestion step, 1 µL of aphid DNA or other appropriate template was added to each of the PCR tubes. Each PCR screen included a negative control, containing 1 µL of UV-treated filter-sterilised Milli-Q ultrapure water (Merck Millipore, Billerica, USA). Positive controls comprised either DNA extracted from *E. coli* strain O157 for the 16S and 16–23S rRNA screens or DNA extracted from *A. pisum* aphids known to harbour one of the facultative endosymbionts of interest (*S. symbiotica*, *H. defensa* and *R. insecticola*; clonal lines PS01, JF99/04 and JF98/24, respectively). None of the *A. pisum* cultures kept at JHI harboured any other known facultative endosymbionts, and so positive controls were not available for PCR to detect the presence of PAXS, *Rickettsia*, *Spiroplasma* and *Rickettsiella* or the APSE bacteriophage. After adding DNA template, the strips of PCR tubes were centrifuged at 1610 rcf for 1 minute (Sigma 4K15; SciQuip Ltd, Shrewsbury, UK) before being returned to the thermocycler and run under PCR conditions appropriate to each primer pair (see Appendix 1, *A1.1.3*).

2.2.2.2 Visualising diagnostic PCR products on agarose gels

PCR products were run through 1% agarose gels to separate and estimate the size of the amplified DNA. Each gel was made by dissolving UltraPure™ agarose (Invitrogen/ Life Technologies Ltd, Paisley, UK) in 1×TBE buffer with gentle heating, adding SYBR®Safe (Life Technologies Ltd, Paisley, UK) to give a 1× final concentration in the gel, and pouring the solution into a gel casting tray. Once set, the gel was transferred to a gel tank (H1-Set and HU25 horizontal gel units; Scie-Plas Ltd, Cambridge, UK), flooded with 1× TBE buffer and the comb removed. As the 5× Green GoTaq® reaction buffer used in the PCR reaction mix includes a loading dye, 4 µL of PCR product was loaded directly into

each well, and 4 µL of 1 kb DNA ladder (Promega, Southampton, UK) loaded into the outermost wells. The products were separated electrophoretically by running the gel for 1 hour at either 60 V for small 40 mL gels or at 90 V for medium-sized 150 mL gels (Bio-RadPowerPac300 power source; Bio-Rad Laboratories Ltd, Hemel Hempstead, UK and Consort E844 power source; Sigma-Aldrich Ltd, Gillingham, UK). The bands of DNA were viewed by transferring the gel to the UV filter glass stage of the FluorChem® FC2 imaging system light cabinet (Alpha Innotech Corp., San Leandro, USA), exposing the gel to transillumination UV light at 302nm wavelength and then visualising the products through a green filter (537/540nm). Gel images were captured and saved using the AlphaView™ camera and software associated with the imaging system, and the size of DNA fragments scored by comparison with migration of the DNA ladder size markers.

2.2.3 Quantitative PCR

2.2.3.1. Preparation of plasmid standards of known gene copy number

PCR reactions were used to amplify the *EF1-α* and *RpL7* genes of *M. euphorbiae*, the *groEL* gene of *B. aphidicola* and the *gyrB* gene of *H. defensa* using GoTaq® DNA polymerase (Promega, Southampton, UK) from aphid DNA templates. The same components and volumes as were used in the diagnostic PCR reactions were used, along with either published primers or with primers designed from published sequences using the software Primer3 v.4.0 (see Appendix 1 sections *A1.1.2* and *A1.2.1* for reaction mix contents and primer sequences). After the reaction mixtures had been divided between the PCR tubes and run on the digest programme, 1 µL of DNA template from single *M. euphorbiae* aphids were added to each tube and the samples run on the same PCR programme used in the diagnostic PCR screening of potato aphids for the PAXS and *Rickettsiella* symbionts (see Appendix 1, *A1.1.3*).

Once samples of the PCR products had been separated by electrophoresis on a 2% agarose gel to confirm the DNA fragments were of the expected sizes, the four amplified gene fragments were purified using a MinElute® PCR purification kit (Qiagen, Crawley, UK) by following the standard spin protocol given by the manufacturer.

The purified *M. euphorbiae EF1-α* and *RpL7*, *B. aphidicola groEL* and *H. defensa gyrB* PCR products were ligated into plasmid vectors using the pGEM®-T Easy Vector System (Promega, Southampton, UK). Each ligation reaction contained 5 µL 2× Rapid Ligation Buffer, 1 µL pGEM®-T Easy Vector, 1 µL T4 DNA ligase, 2 µL deionised water and 1µL purified PCR product. When the four sets of ligation reaction components had been assembled, they were placed in a fridge at 4°C and left overnight to achieve the maximum number of transformants.

With the purified products ligated into the plasmid vectors, the plasmids were transformed into bacterial cells for propagation. 1 µL of each ligation reaction mixture was added to 20 µL Library Efficiency DH5α Competent Cells (Invitrogen/ Life Technologies Ltd, Paisley, UK), and the cells heat-shocked by first keeping the tubes on ice for thirty minutes, then transferring the tubes to a water bath set to 42°C for 45 seconds before immediately returning them to ice for a further 2 minutes. 180 µL SOC medium (see Appendix 1, section *A1.2.2*) was added to each transformation mixture and the tubes incubated on a shaker (Stuart Scientific Orbital Incubator S150; Bibby Scientific Ltd, Staffordshire, UK) for 90 minutes at 37°C and 150 rpm. 30 µL and 70 µL samples of each of the transformation mixtures were then spread on to plates of agar containing 32 µg mL^{-1} IPTG, 32 µg mL^{-1} X-gal and 100 µg mL^{-1} ampicillin, and the plates incubated overnight at 37°C.

After incubating for approximately 16 hours, the plates were transferred to a fridge for an hour before the bacterial colonies were inspected. Those that had grown from bacteria that had taken up plasmids containing the PCR products were identifiable by their white colour. For each cloned gene 5 white colonies from across the two plates were picked from the agar plate using a small pipette tip and gently mixed with 20 µL sterile distilled water by repeated pipetting.

To verify that the correct inserts had been ligated into the vectors taken up by the competent cells, an end-point PCR was run using the primer sets that had initially amplified the inserts, and the product sizes confirmed through agarose gel electrophoresis (see section *2.2.2*.2). The bacterial colonies could then be grown in liquid culture by adding 10 µL of the bacterial suspension to 5 mL of LB broth (see Appendix 1, section *A1.2.2*) to which 1.25 µL of 200 mg mL^{-1} ampicillin had been added, and the vials incubated on the orbital shaker at 37°C and 110 rpm. After 18 hours on the shaker, 2 mL

of each cell culture was centrifuged at 6000 rcf for 3 minutes to pellet the bacterial cells. The supernatant was discarded, and the cells re-suspended in 250 µL P1 buffer and the plasmids purified using the QIAprep® Spin Miniprep kit (Qiagen, Crawley, UK) by following the standard spin protocol given by the manufacturer.

To confirm further that the correct genes had been amplified and inserted into the vectors and taken up by the competent cells, a sample of each plasmid solution was sequenced in one direction across the insertion site. Plasmid solutions were first diluted using UV and filter-sterilised Milli-Q ultrapure water (Merck Millipore, Billerica, USA) to concentrations of between 20 ng/µL and 150 ng/µL. Inserts were then amplified using the primer T7, which anneals upstream of the cloning site in the pGEM®-T Easy Vector and sequenced using dye terminator chemistry by capillary electrophoresis on the ABI 3730 DNA Analyser (Applied Biosystems/ Life Technologies Ltd, Paisley, UK).

Because circular plasmids can lead to an overestimation of gene copy numbers in qPCR (Hou *et al*., 2010), the solutions of coiled plasmids ligated with the *M. euphorbiae EF1-α* and *RpL7*, *B. aphidicola groEL* and *H. defensa gyrB* genes were linearized using the restriction digest enzyme *Apa1* (Promega, Southampton, UK). For each of the four genes, 4.0 µL 10 × buffer, 0.4 µL bovine serum albumin, 5.0 µL purified plasmid solution and 1.0 µL *Apa1* restriction enzyme were added to 29.6 µL distilled water. The digests were then transferred to a Grant QBTP heat block (Grant Instruments Ltd., Cambridge, UK) set to 37°C for four hours, followed by 20 minutes at 65°C to denature the enzyme. To confirm the digests had been successful, 4 µL of each digested plasmid was combined with 1 µl 5× loading dye (Qiagen, Crawley, UK) and electrophoresed on a 1% agarose gel along with samples of the undigested plasmids (see section *2.2.2.2*). As the linearized plasmids are more mobile than the coiled equivalents, greater migration of the linearized plasmids indicated that the digests had been successful.

The linearized plasmids were again purified using the MinElute® PCR purification kit (Qiagen, Crawley, UK) by following the standard spin protocol given by the manufacturer. The DNA concentration of each plasmid solution was measured three times using a NanoDrop ND 1000 Spectrophotometer (ThermoFisher Scientific, Loughborough, UK), and the values obtained used to calculate the numbers of plasmids per µL. The number of base pairs in each plasmid was determined by adding the length of the relevant insert to 3015bp, the size of the pGEM®-T Easy Vector plasmid, and then

the number of plasmids in 1µl of linearized plasmid solution calculated as given in Equation 1 (Dhanasekaran *et al.*, 2010).

Equation 1) $$\text{No. of copies/µl} = \frac{6.022\times10^{23}\ \text{(molecules/mole)}}{\text{Number of base pairs} \times 660\ \text{daltons}}$$

Where 6.022×10^{23} (molecules/mole) is Avogadro's number and 660 daltons is the average weight of a single base pair.

Finally, for each linearized plasmid solution a ten-fold dilution series ranging from 10^{-1} to 10^{-6} log dilution was made by mixing 10 µL of purified plasmid with 90 µL UV and filter-sterilised Milli-Q ultrapure water (Merck Millipore, Billerica, USA), then by mixing 10 µL of the resulting 10^{-1} dilution with 90 µL water, and so on.

2.2.3.2. Quantitative PCR reaction contents and conditions

Absolute qPCR reactions were conducted using MESA Blue qPCR MasterMix Plus for SYBR® Assay Low ROX (Eurogentec Ltd., Southampton, UK) in 25 µL reactions (see Appendix 1 section *A1.2.3* for full reaction mix contents and volumes). The primers (Sigma-Aldrich Ltd, Gillingham, UK) used for each assay were either published primers or were designed from published sequences using the software Primer3 v.4.0 (see Appendix 1 section *A1.2.4*) and each primer solution used had a concentration of 3 µM, giving a final concentration of 0.3 µM in the reaction mix.

The reaction mixture was distributed into the wells of a MicroAmp® Fast Optical 96-well plate (Applied Biosystems/ Life Technologies Ltd, Paisley, UK), and 1 µl template was added to each well. All DNA templates used in the qPCR assays were extracted from single aphids of known age and clonal line as detailed in section *2.2.1*, and each DNA template and standard curve dilution was run in triplicate.

Plates were loaded into the ABI 7500 Fast Real-Time PCR system (Applied Biosystems/ Life Technologies Ltd, Paisley, UK) after sealing with adhesive film and briefly spun down, and run on the thermocycling programme given in Appendix 1, section *A1.2.5*.

When each run was complete the threshold and baseline were set automatically and the C_T values for each well recorded and analysed.

2.2.4 Genotyping using microsatellites

2.2.4.1 Amplification of microsatellites

The 19 *M. euphorbiae* clonal lines kept in culture were genotyped based on the allele sizes of seven polymorphic microsatellite loci. Primers published by Raboudi *et al*. (2005) were used to amplify the microsatellites from the *M. euphorbiae* templates in 15 µL reactions.

The components of the reaction mix were the same as those used in the diagnostic PCRs and were assembled in the same manner, although the relative volumes differed (see Appendix 1, section *A1.3.2*). However, as the target of the microsatellite primers was eukaryotic DNA, the *Hha*1 restriction digest enzyme and the digest step was omitted. Two different methods of sizing the resulting microsatellite products were used, with the reaction mix contents adjusted accordingly. DNA fragments that were scored on polyacrylamide gels were amplified using non-modified primers and the 5× Green GoTaq® reaction buffer that includes loading dye, whilst those that were scored using capillary electrophoresis were amplified using forward primers modified with the addition of a 6-FAM fluorophore molecule to the 5' end (Sigma-Aldrich Ltd, Gillingham, UK), and 5× Clear GoTaq® reaction buffer. As the aphid lines kept in culture are clonal, 0.5 µL of DNA template extracted from multiple aphids from a given line were added to each well to give a stronger signal.

Once the DNA templates were added and the tubes centrifuged briefly, two different programmes on the thermocycler (Eppendorf Mastercycler®ep; Eppendorf AG, Hamburg, Germany) were used to amplify the microsatellites due to the different annealing temperatures of the primers (see Appendix 1, section *A1.3.3*). The amplification of loci *Me1, Me3, Me7* and *Me9* required an annealing temperature of 54°C, whilst loci *Me10, Me11* and *Me13* were amplified with an annealing temperature of 62°C. When the programmes were complete, the PCR products were stored at -20°C until they could be separated by electrophoresis to determine the sizes of the amplified fragments.

2.2.4.2 Scoring the microsatellite products on polyacrylamide gels

The sizes of the amplified microsatellite products were determined by separating the DNA fragments on a polyacrylamide gel using electrophoresis. To form the 8% polyacrylamide gel, 3.2 mL of 30% acrylamide/bis-acrylamide solution (Sigma-Aldrich Ltd, Gillingham, UK) was combined with 7.6 mL distilled water and 1.2 mL of 10× TBE buffer in a small Duran® bottle and the solution degassed in a sonicator (Branson 2510 Ultrasonic Cleaner; Branson Ultrasonics Corp., Danbury, USA) for 15 minutes. A 200 µL aliquot of 10% ammonium persulfate (Sigma-Aldrich Ltd, Gillingham, UK) and 10 µL TEMED (Sigma-Aldrich Ltd, Gillingham, UK) were then added and, after gentle swirling, approx. 5 mL of the gel solution was pipetted into each of two small gel moulds and a ten-tooth comb inserted into the top of each gel.

Once the gels were solidified the combs were removed and the gels transferred to the inner cooling core of a Bio-Rad Mini-Protean II electrophoresis cell (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). With the inner core in the lower chamber, 1×TBE electrode buffer was poured into the upper and lower chambers and 4 µL of each PCR product was loaded into the wells of the gel. A maximum of eight PCR samples were loaded into each gel, with 4 µl of 25 bp DNA step ladder (Promega, Southampton, UK) loaded into the outermost wells. The gels were run at 200 V for 45 minutes using a Bio-RadPowerPac300 power source (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

To enable the DNA fragments to be visualised, the gels were removed from the glass plates and submerged in a solution of 1×TBE and 1×SYBR®Safe (Invitrogen/ Life Technologies Ltd, Paisley, UK) in a small plastic container. This was then covered with foil and incubated at 30 minutes on an orbital shaker set to 50 rpm (Stuart Scientific Orbital Incubator S150; Bibby Scientific Ltd, Staffordshire, UK). Finally, the gels were transferred to the imaging system cabinet, the images captured and the size of the DNA fragments scored as detailed in section *2.2.2.2*.

2.2.4.3 Scoring the microsatellite products using capillary electrophoresis

To score the size of the microsatellite products using capillary electrophoresis, 1 µL of each PCR product generated using a 5’ fluorescently labelled primer and 0.16 µL of the

internal lane size standard GeneScan™ ROX 500 (Applied Biosystems/ Life Technologies Ltd, Paisley, UK) were suspended in 8.84 µL Hi-Di™ Formamide (Applied Biosystems/ Life Technologies Ltd, Paisley, UK) in the wells of a Thermo-fast® non-skirted 96 well plate (ABGene Ltd, Epsom, UK), and the plate sealed and centrifuged briefly. The fluorescently-labelled DNA fragments were then separated by capillary electrophoresis using an ABI 3730 DNA Analyser (Applied Biosystems/ Life Technologies Ltd, Paisley, UK). The fragment sizes detected in each sample were scored automatically using ABI Prism® GeneMapper™ software v. 4.0 (Applied Biosystems/ Life Technologies Ltd, Paisley, UK), having ensured that internal lane standard peaks had been assigned the correct size labels.

2.3 Experimental methodology

2.3.1 Aphid fitness assays

Aphid fitness assays were conducted in which single aphids, reared on whole potato plants, were monitored and various fitness parameters recorded in order to get a measure of fitness for different *M. euphorbiae* clonal lines. For this, *S. tuberosum* cv. Désirée plants were grown from whole tubers or from the dissected eyes of chitted tubers in insecticide-free compost (see section *2.1.2*) in a glasshouse in which the benches were lined with capillary matting so as to ensure constant water availability. When the plants had been growing for approximately four weeks small clip cages were used to secure the aphids to the leaves. Each clip cage consisted of stacked two rings of acrylic plastic (external diameter 32 mm, internal diameter 26 mm, height 5 mm) to which fine gauze had been fixed across the outer surfaces of the rings and felt used to line the inner surfaces. The two halves of the cage were attached to the ends of the two halves of a small aluminium spring-loaded clip so that the cage could be securely positioned against the leaf surface (Figure 2.3).

Figure 2.3: Clip cage for securing an individual *M. euphorbiae* aphid to a potato leaf

For each *M. euphorbiae* clonal line of interest, adult apterous aphids were removed from the whole plant cultures on which they had been reared and were caged in pairs to the underside of the leaves of the potato plants. After 24 hours, the adult aphids were removed from the clip cages and the nymphs re-caged. If neither aphid had produced any offspring in this time, they were left on the plant for a further 24 hours before being removed and discarded. When the nymphs were five days old, all but one nymph from each clip cage were discarded.

From day 6 onwards, each nymph was monitored daily and records made of the date the aphid underwent final ecdysis to adulthood, the morph (apterous/alate) of the adult aphid and the date offspring were first produced. The nymphs produced by each aphid were counted and removed every 1–2 days for a time period at least equal to the length of the pre-reproductive period, after which time the aphids were collected and frozen so that the genotype and symbiont status of each aphid could be confirmed. Throughout the experiment aphids were transferred to a new leaf on the same plant whenever the original leaf appeared unhealthy or had been visibly damaged by the clip cage. Clip cages were secured to leaves in the lower leaf zone, as the reproductive rate of *M. euphorbiae* has been shown to decrease significantly on older, senescent leaves (Aldamen & Gerowitt, 2009).

Replicates of the performance assays were conducted in temporal blocks in which the positions of the clonal aphid lines were randomly assigned. All replicates were performed within a glasshouse in which supplementary lighting ensured that that light

intensity was at least 200 Wm^{-2} for 16 hours a day, and in which the temperature was maintained at 20°C during the 16 h photoperiod and at least 15°C overnight.

2.3.2 Aphid–parasitoid interactions

2.3.2.1 Experimental arenas for observing parasitoid attacks on aphids

For each experimental arena, 0.2 g UltraPure™ agarose (Invitrogen/ Life Technologies Ltd, Paisley, UK) was dissolved in 20 mL sterile distilled water with gentle heating and the solution poured into the base of a Petri dish 90 mm in diameter (Sterilin Ltd/ ThermoFisher Scientific, Loughborough, UK). As the agarose cooled, a single potato leaf cutting (cultivar Désirée) was embedded in the gel with the abaxial surface uppermost. Once the gel had cooled completely, aphids were transferred to the leaf surface using a fine paintbrush and allowed to settle for at least 10 minutes before a parasitoid was introduced (Figure 2.4).

Figure 2.4: Experimental arena for aphid–parasitoid interactions

2.3.3 Aphid and parasitoid dissections

2.3.3.1 Dissection of M. euphorbiae *aphids*

Each *M. euphorbiae* aphid to be dissected was immersed in 50 µL 1 x PBS buffer (8 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4.12H_2O$ and 0.2 g KCl per litre of sterile distilled water) on a glass slide on the stage of a Leica MZ7$_5$ stereomicroscope (Leica Microsystems Ltd, Milton Keynes, UK). The head of the aphid was pinned down using one pair of dissection forceps whilst another pair of forceps was used to pull gently just above the cauda to remove the gut and reproductive system. The remainder of the contents of the abdomen and the contents of the head were then released into the buffer by running the side of the forceps from the head to the abdomen.

Images of aphid dissections, particularly of developing *A. ervi* larvae from parasitized aphids, were photographed using a GXCAM-5 digital microscope camera and the associated GXCapture software (GTVision, Haverhill, UK).

2.3.3.2 Dissection of A. ervi *parasitoids*

Parasitoids to be dissected were first anaesthetised with CO_2 gas (99.8% purity; BOC, Guildford, UK) and then killed by immersing in 70% ethanol for 2–3 minutes. Each dissection was conducted in 25 µL of sterile distilled water on a glass slide on the stage of a Leica MZ7$_5$ stereomicroscope. As an indicator of parasitoid length, the left hind leg of each parasitoid was removed from the thorax of the wasp using dissection forceps and the tibia measured using a vertical scale graticule within the eyepiece of the microscope. At the highest ×50 magnification, 1 graticule unit equalled 0.02 mm.

To measure egg counts from female *A. ervi* parasitoids, the ovaries were removed by holding the petiole with one pair of fine forceps and pulling just above the ovipositor with another pair of fine forceps. A cover slide was then used to rupture the ovaries and separate the eggs, which were brought into sharper definition using the inclined transmitted light source of the microscope and counted with the aid of the indexed grid graticule in the eyepiece.

Chapter 3: Characterising secondary endosymbiont complements of *M. euphorbiae* clonal lines

3.1 Introduction

3.1.1 Initial discovery and characterisation of aphid primary and secondary endosymbionts

Although the aphid bacteriome was documented as early as the 1850s, the hypothesis that it harboured symbiotic bacteria only developed throughout the twentieth century (Buchner, 1965). A series of histological and ultrastructural studies on the bacteriocytes and surrounding cells first determined that the coccoid primary symbiont, later named *Buchnera aphidicola*, was a Gram-negative bacterium found exclusively within the bacteriocyte cells of the bacteriome along with organelles such as mitochondria and ribosomes. Smaller, rod-shaped symbionts were found occupying the sheath cells associated with the bacteriocytes, and occasionally in bacteriocyte cells separate from those housing the primary symbiont (Buchner, 1965; Hinde, 1971; Griffiths & Beck, 1973; McLean & Houk, 1973). Beyond their morphology however, little was known about these endosymbionts until molecular characterisation was available, beginning with the amplification, cloning and sequencing of ribosomal genes.

3.1.1.1 Amplification and sequencing of bacterial genes from aphid hosts

The prokaryotic 16S ribosomal subunit is functionally constant and is found in all bacterial species, whilst the 16S rRNA gene contains both highly conserved and more varied regions and is easily amplified using universal primers (Weisburg *et al.*, 1991; Moran *et al.*, 1993). As such, comparative analysis of 16S rRNA sequences allows bacterial phylogenies to be assembled. Following the detection of expressed prokaryote 16S rRNA genes within the aphid bacteriome (Ishikawa, 1978), comparisons of 16S rRNA gene sequences amplified from pea aphid primary and secondary endosymbionts with those of other prokaryotes confirmed infections by two distinct γ-Proteobacteria (Unterman *et al.*, 1989). Analysis of the primary symbiont 16S rRNA genes from other aphid species has determined the monophyletic origins of *B. aphidicola* and the subsequent coevolution of the bacteria with their aphid hosts (Munson *et al.*, 1991a,

1991b; Moran *et al.*, 1993). The sequencing of other *Buchnera* genes, culminating in the sequencing of the entire bacterial genome from several aphid genera, has validated different putative roles assigned to the primary endosymbiont (Shigenobu *et al.*, 2000; Tamas *et al.*, 2002; van Ham *et al.*, 2003). For instance, the role of *B. aphidicola* in tryptophan synthesis, suggested by the poor fitness of aposymbiotic aphids fed on tryptophan-deprived diets, was substantiated by the presence of five functioning genes from the tryptophan biosynthetic pathway within the *Buchnera* genome (Douglas & Prosser, 1992; Munson & Baumann, 1993).

Further comparisons of the 16S rRNA sequences amplified and cloned from whole aphid DNA or from isolated aphid tissues have revealed the existence of several other bacteria living in close association with aphid hosts. In addition to the pea aphid secondary endosymbiont identified by Unterman *et al.* (1989), later confirmed as *Serratia symbiotica* (Fukatsu *et al.*, 2000), the presence of two further enterobacteriaceal symbionts, *Hamiltonella defensa* and *Regiella insecticola* (Sandström *et al.*, 2001), as well as a mollicute *Spiroplasma* (Fukatsu *et al.*, 2001) and the first *Rickettsia* endosymbiont identified in a herbivorous insect (Chen *et al.*, 1996) were all determined by 16S rRNA sequence analysis, as was the SMLS endosymbiont of the grain aphid *Sitobion miscanthi* (Li *et al*., 2011a). The same techniques have also identified several groups of aerobic bacteria residing within the aphid gut (Harada *et al.*, 1996).

More recently, cloning and sequencing of other genes has improved the phylogenetic resolution of various secondary endosymbionts, identifying *H. defensa* and *R. insecticola* as sister groups to one another and grouping *S. symbiotica* within a clade that includes other *Serratia* species associated with insects (Moran *et al*., 2005c). The entire genomes of these three bacteria have now been sequenced from various aphid species (Moran *et al*., 2005a; Degnan *et al*., 2009a, 2009b; Burke & Moran, 2011), showing the extent of genome divergence and reduction compared with related free-living bacteria that is characteristic of endosymbionts.

As with the *B. aphidicola* genome, the genes of the secondary bacteria for which functional information is available reflect their endosymbiotic nature. Both *H. defensa* and *R. insecticola*, for example, express type 3 secretion systems (T3SS) that are known to have a role in host cell invasion in other insect symbionts, and both bacteria have lost all but two of the biosynthetic pathways necessary to synthesise essential amino acids,

indicating a metabolic dependence on both their aphid host and the primary symbiont (Haghjoo & Galán, 2004; Dale *et al*., 2005; Moran *et al*., 2005a; Degnan *et al*., 2009a, 2009b). The genes present also allude to the means by which such symbionts may play a mutualistic role; although putative pathogenic RTX toxins have been identified in both *H. defensa* and *R. insecticola*, the type 1 secretion system required to secrete the exotoxins are found in most *H. defensa* strains and in the single strain of *R. insecticola* shown to confer resistance to parasitoid wasps, suggesting a possible mechanism by which this resistance is bestowed (Delepelaire, 2004; Degnan *et al.*, 2009a, 2009b; Hansen *et al*., 2012).

The characterisation of the APSE bacteriophage associated with *H. defensa* followed a similar pattern to that of the bacterial endosymbionts, first with the observation of isometric phage-like particles in electron micrograph images of pea aphid secondary endosymbionts, then through sequencing of the APSE genome (van der Wilk *et al*., 1999). Partial genomic sequencing of several different strains of the APSE bacteriophage have since identified homologues of genes encoding various protein toxins, which may contribute to the resistance phenotype conferred to pea aphids harbouring both the secondary endosymbiont and the phage (Moran *et al*., 2005a; Degnan & Moran, 2008a).

3.1.1.2 Other molecular diagnostic tools for characterising aphid bacterial endosymbionts

In addition to direct sequencing, other molecular methods using amplified 16S rRNA products can be employed to both detect the occurrence of specific secondary endosymbionts and to study the overall microbial diversity. Restriction fragment length polymorphism (RFLP), for example, exploits unique sites within the 16S rRNA gene of the endosymbionts. When 16S rRNA amplicons are subject to restriction digest enzymes that act upon these sites, the number and size of the resulting DNA fragments when electrophoresed on an agarose gel is distinctive for a given type of bacteria (Chen & Purcell, 1997; Sandström *et al.*, 2001; Darby *et al.*, 2001). Terminal restriction fragment length polymorphism (T-RFLP) works in a similar manner, with one of the two primers used to amplify the 16S rRNA gene being fluorescently labelled. Following amplification and digestion, the size of the terminal fragment bearing the fluorescent

marker is determined using capillary electrophoresis. Initially designed as a means by which the diversity of a microbial community could be assessed, through careful selection of digestion enzymes T-RFLP has become another tool by which to identify the bacterial species composition in a given aphid host (Liu *et al.*, 1997; Haynes *et al.*, 2003; Ferrari *et al.*, 2004; Carletto *et al.*, 2008).

An alternative method by which to detect and characterise endosymbionts within aphid hosts is to stain thin tissue sections with labelled probes that hybridise to the bacteria. Such *in situ* hybridisations with probes designed to bind to the 23S rRNA unit of different bacterial phylogenetic divisions revealed the existence of secondary endosymbionts belonging to both the γ- and β-Proteobacteria in various aphid species, although the latter group have not been characterised further (Fukatsu *et al.*, 1998). Immunohistochemical stains have been used in a similar manner, with the primary and secondary endosymbionts differentially stained based on the different heat-shock proteins they predominantly produce (Fukatsu & Ishikawa, 1993, 1998). *In situ* hybridisations using probes designed from unique gene sequences for specific aphid primary and secondary endosymbionts are now commonplace, with the nuclei of the eukaryotic aphid cells visualised with a counterstain; as well as verifying their presence, this technique also reveals the location of the bacteria within the bacteriocyte and sheath cells of the aphid host, confirming their endosymbiotic nature (e.g. see Fukatsu *et al.*, 2000; Darby *et al.*, 2001; Koga *et al.*, 2003, 2007; Sakurai *et al.*, 2005; Moran *et al.*, 2005b; Tsuchida *et al.*, 2005, 2010).

3.1.2 Diagnostic polymerase chain reactions (PCRs)

3.1.2.1 Diagnostic PCR screening for secondary endosymbiont bacteria

The most commonly used technique to screen for known bacterial endosymbionts in both aphids and in other invertebrates is diagnostic PCR, with either one or both primers designed to anneal to regions of a given gene specific to the bacterium. An amplified product is then generated only when the bacteria of interest is present. This technique has allowed wide-scale screening of pea aphid clones for known secondary endosymbionts, revealing their distributions within and between populations (Sandström *et al.*, 2001; Tsuchida *et al.*, 2002; Simon *et al.*, 2003). Furthermore, the limited co-

evolution between secondary endosymbionts and their aphid hosts as a result of horizontal transfer events has enabled other aphid species to be screened for known secondary bacteria using the same sets of primers (Sandström *et al*., 2001; Russell *et al*., 2003; Tsuchida *et al*., 2005, 2006; Li *et al*., 2011b).

Although there may still be secondary endosymbionts as yet uncharacterised, an initial means by which to determine the presence of bacteria other than *Buchnera* is to amplify the 16S-23S rRNA genes using universal primers (Sandström *et al.*, 2001). In most eubacteria the 16S, 23S and 5S rRNA-encoding genes form a single operon, whereas in *B. aphidicola* the 16S rRNA gene is no longer linked to the 23S-5S operon and so primers spanning the two genes will not amplify from the primary endosymbiont (Munson *et al.*, 1993; Rouhbakhsh & Baumann, 1995). While such PCR reactions may generate products from transient bacteria on the surface of the aphids or from bacterial gut fauna, established endosymbionts are likely to be present in greater numbers and therefore generate stronger bands of amplified product when electrophoresed and visualised on an agarose gel.

3.1.2.2 Diagnostic PCR screening for the APSE bacteriophage

With several APSE bacteriophage strains now at least partially sequenced, primers that amplify conserved APSE genes or the integration site of the phage genome with the endosymbiotic bacterial host can be used for diagnostic PCR screening (Moran *et al*., 2005a; Degnan & Moran, 2008a, 2008b; Oliver *et al*., 2009). However, with high rates of homologous and non-homologous recombination typical of bacteriophages, it is possible that such primers may fail to amplify DNA of novel APSE strains (Hendrix, 2002). Southern blot hybridisations may therefore be employed to confirm the presence of the bacteriophage (Degnan & Moran, 2008b; Oliver *et al*., 2009).

3.1.3 Quantifying aphid endosymbiont bacteria

3.1.3.1 Principles of quantitative PCR

As with traditional end-point PCR, quantitative or real-time PCR involves the amplification of a specific nucleotide sequence through repeated cycles of denaturation, primer annealing and elongation. However, whereas the products of end-point PCR are typically separated by gel electrophoresis and visualised using a nucleic acid stain such as ethidium bromide or SYBR®Safe, in quantitative PCR fluorescent reporters are used to measure the amplification of DNA as the reaction progresses, from which the initial quantity of target DNA in the sample can be calculated. The greater the quantity of target DNA, the greater the number of amplicons that will be generated and hence the earlier the reaction cycle at which a given threshold of fluorescence will be reached (Higuchi *et al*., 1993).

A number of fluorescent chemistries are available by which to detect the concentration of PCR product, which can broadly be divided into two categories; non-specific DNA-binding dyes and sequence-specific probes. Dyes such as SYBR®Green emit fluorescence when bound to double-stranded (ds) DNA. However, as such dyes intercalate indiscriminately to all dsDNA, primer-dimers and non-specific amplification products can contribute to the fluorescence emitted and so analysis of the melt curves generated by the PCR products is necessary to ensure that only the intended target sequence has contributed to the fluorescence detected (Ririe *et al.*, 1997). Probe-based reporters are more specific, and so are advantageous when detecting multiple targets within a sample. Hydrolysis probes, typified by TaqMan® probes, are complementary to specific regions within the target sequence and are labelled with both a reporter and a quencher fluorescent dye, the latter of which quenches the emission spectra of the reporter dye through fluorescent resonance energy transfer (FRET) when the probe is intact. As the target sequence is amplified, the exonuclease activity of DNA polymerase degrades the probe and the fluorescence of the reporter can then be detected (Holland *et al.*, 1991; Heid *et al.*, 1996). 'Hairpin' probes such as molecular beacons, scorpions and Sunrise™ primers also rely on FRET reporter quenching, with fluorescence from the reporter dye increasing only when the probe is bound to the target sequence (Valasek & Repa, 2005). Dual hybridisation probes, in contrast, use FRET to amplify a fluorescent signal, with fluorescent emission only detectable when two independent probes, one with

a donor dye and one with an acceptor dye, anneal to the target sequence (Wong & Medrano, 2005).

Regardless of the specific chemistry used, levels of fluorescence increase as the PCR amplicons accumulate to produce an amplification plot in which the magnitude of the fluorescent signal, ΔRn, normalised to a passive reference dye and from which the background fluorescence has been subtracted, is plotted against the cycle number. The amplification curve can be divided into three phases as the PCR progresses; the baseline, the log-linear or exponential phase and finally a plateau phase (Figure 3.1). In the early cycles of the PCR, the fluorescence emitted is weak and cannot be distinguished from the background. Once the background threshold has been exceeded, PCR product amplification and hence fluorescence emission continues to increase exponentially until inhibitors accumulate or one or more reaction components become limiting, at which point the intensity of the fluorescence reaches a plateau (Kubista *et al.*, 2006).

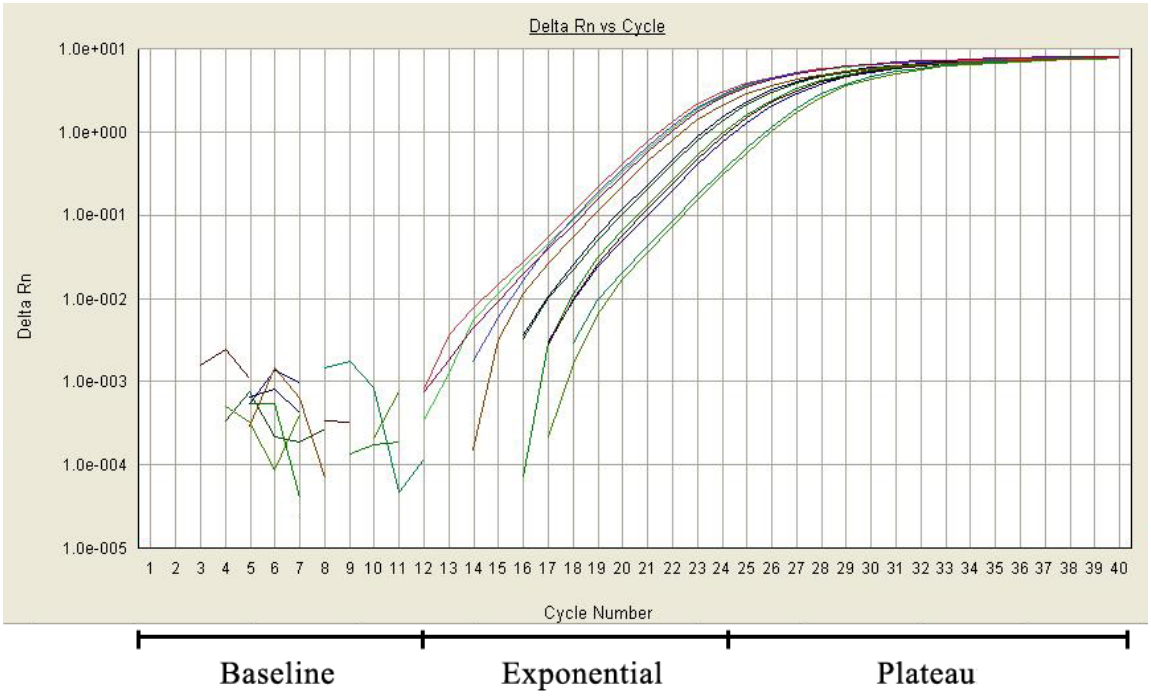

Figure 3.1: Typical quantitative PCR amplification plot illustrating the three phases of the reaction as the PCR progresses.

To quantify the differences in the initial copy numbers of a target sequence from the amplification plots, the cycle numbers (C_T value) at which the fluorescent signal reaches a particular threshold above baseline are compared for each sample of interest. In absolute qPCR, serial dilutions of a standard in which the copy number is known, such as a PCR-amplified target sequence or target sequences ligated and cloned in plasmids, are

used to construct a standard curve. The C_T values generated by the samples are then compared to those of the standard curve to produce absolute copy numbers of the target sequence initially present (Dhanasekaran *et al.*, 2010). In contrast, relative qPCR relates the signal from experimental samples to that of a reference sample and, in conjunction with primers that amplify from reverse-transcribed mRNA, is often used to compare expression levels of one or more target gene to a reference 'housekeeping' gene (Wong & Medrano, 2005). The mean normalized gene expression is then calculated using one of a number of mathematical models, such as the comparative C_T ($2^{-\Delta\Delta Ct}$) method or the Pfaffl model (Livak & Schmittgen, 2001; Pfaffl, 2001).

3.1.3.2 Absolute and relative qPCR applied to aphid endosymbiont bacteria

Titres of primary and secondary aphid endosymbionts can be inferred using absolute qPCR assays, with the number of copies of a given bacterial genome present derived by quantifying the number of copies of one or more bacterial genes that each occur only once within the genome. As *B. aphidicola* exhibits extreme polyploidy, such assays give only an approximation as to the actual number of bacterial cells harboured within the bacteriocytes. However, given that the functional significance of the polyploid genome of *Buchnera* is not known, quantifying genome copy number may give a better indication of the potential productivity of the endosymbiont (Komaki & Ishikawa, 1999, 2000).

For absolute endosymbiont titres to be comparable between aphid samples, the abundance of aphid primary and/or secondary endosymbiont bacteria determined by qPCR assays have been normalised in various published works by dividing each bacterial titre by aphid fresh weight, DNA concentration, or by copy numbers of single-copy genes of the aphid host (e.g. see Oliver *et al.*, 2003; Plague *et al.*, 2003; Wilkinson *et al.*, 2007; Nikoh *et al.*, 2010). The results of such normalised absolute qPCR assays have, for instance, been used to establish how titres of *B. aphidicola* and various secondary endosymbionts change throughout aphid development (Koga *et al*., 2003; Sakurai *et al*., 2005; Douglas *et al*., 2006a; Li *et al*., 2011b; Schmid *et al*., 2012) in response to alternative host plants or differences in diet nutritional quality (Wilkinson *et al*., 2007; Chandler *et al*., 2008), and in response to environmental stimuli such as heat shock (Burke *et al*., 2009a).

Reverse-transcribed relative qPCR, involving the quantification of transcribed mRNA sequences, has been used to understand further the roles of primary and secondary aphid endosymbionts. For example, through RT-qPCR changes in the expression of *B. aphidicola* genes involved in amino acid biosynthesis in response to different dietary inputs have been confirmed (Moran *et al*., 2005b), as have changes in the expression of heat-responsive genes in response to varying temperature regimes (Wilson *et al*., 2006; Dunbar *et al*., 2007), and expression of toxin-encoding genes of the APSE phage relative to highly expressed *B. aphidicola* and *H. defensa* genes (Moran *et al*., 2005a; Oliver *et al*., 2009).

3.1.4 Study objectives and hypotheses

The objectives of the study presented in this chapter are:

1. To screen the *M. euphorbiae* cultures established and maintained in culture at JHI for the presence of seven secondary endosymbiont bacteria known from the pea aphid literature using diagnostic PCR. Given that secondary endosymbiont bacteria are a major source of heritable traits in *A. pisum* and other agriculturally important aphid species, and that *M. euphorbiae* are exposed to many of the same predators, pathogens and environmental factors to which these bacteria serve a mitigating role, some instances of secondary endosymbiont infections were expected.

2. To use microsatellite markers to quantify the range and frequency of *M. euphorbiae* genotypes present in the cultured lines, and to enable associations between secondary endosymbiont infections and host genotypes to be investigated. Neither the exact mechanisms by which secondary bacteria are horizontally transmitted, nor the factors that affect the successful establishment of endosymbionts when introduced to novel hosts are fully understood (Russell & Moran, 2005). In both *A. pisum* and *Aphis craccivora* there is correlative evidence that certain endosymbionts are found in clonal lines associated with certain host plants (Brady & White, 2013; Henry *et al*., 2013), yet the genetic differentiation of host-adapted pea aphid races suggests innate genetic differences between clonal lines may equally be responsible for the observed endosymbiont distribution (Henry *et al*., 2013). All of the potato aphids that founded the cultures were collected from *S. tuberosum*, thus precluding any investigation into host-plant associated endosymbiont infections, yet innate genetic variation between potato aphid lines may directly influence the

endosymbiotic associations capable of becoming established. Thus, I tested the null hypothesis that there would be no association between the secondary endosymbiont presence and *M. euphorbiae* genotype.

3. To quantify titres of *B. aphidicola* and the secondary endosymbiont *H. defensa* within adult *M. euphorbiae* hosts using quantitative PCR, assessing different denominators to determine the most suitable means of normalising the results. As secondary endosymbiotic bacteria are dependent on both the aphid host and the primary endosymbiont for their nutrient provisioning and compete with the primary endosymbiont *B. aphidicola* for space within the bacteriome in which to reside, I hypothesised that infection with secondary endosymbiont bacteria would negatively affect the potato aphid host through suppression of *B. aphidicola* titres.

3.2 Materials and Methods

3.2.1 Screening M. euphorbiae *clonal lines for secondary endosymbionts and an associated bacteriophage using diagnostic PCR*

To determine the presence of known secondary endosymbionts in the 19 *M. euphorbiae* clonal lines kept in culture at JHI, between 5 and 10 adult aphids were removed from each stock culture and frozen at -20 °C. Genomic DNA was extracted as given previously (section *2.2.1*), and formed the template for diagnostic PCR reactions. Universal primers were used to amplify the prokaryotic 16S rRNA and the linked 16S-23S rRNA genes to confirm the successful extraction of DNA from the aphid samples and to screen for bacteria other than *B. aphidicola*, respectively, whilst published primers specific to the 16S rRNA genes of *S. symbiotica*, *H. defensa*, *R. insecticola*, PAXS, *Rickettsia*, *Spiroplasma* and *Rickettsiella* were used to screen for the known pea aphid secondary endosymbionts. Two pairs of primers specific to the P35 and P51 APSE genes were also used to establish the occurrence of the phage. PCR products were then electrophoresed and visualised on an agarose gel (section *2.2.2*). The reproductive endosymbiont *Wolbachia* was not screened for in *M. euphorbiae* owing to the unreliability of the current diagnostic primers available (Augustinos *et al*., 2011).

The stock *M. euphorbiae* cultures were screened for known secondary endosymbionts in this way approximately every six months to determine the stability of the bacterial infections.

3.2.2 Genotyping **M. euphorbiae** *clonal lines using microsatellite markers*

Initially, 10 sets of published primers for polymorphic loci in *M. euphorbiae* were tested using DNA templates from three aphid clonal lines (Raboudi *et al.*, 2005). The allele sizes of the seven polymorphic microsatellite loci that were successfully amplified were used to genotype the 19 *M. euphorbiae* clonal lines kept in culture, using the same aphid DNA templates prepared for diagnostic screening for secondary endosymbionts. This enabled different clonal lines with the same endosymbiont complement to be distinguished and also established which aphid genotypes have a propensity to harbour secondary endosymbionts. Details of the amplification and scoring of the microsatellite loci using both polyacrylamide gels and capillary electrophoresis are given in section *2.2.4*.

The stock *M. euphorbiae* cultures were genotyped using between two and seven molecular markers on an annual basis to ensure the continuing integrity of the clonal lines.

3.2.3 Quantifying titres of endosymbiont bacteria in **M. euphorbiae** *aphids using quantitative PCR*

3.2.3.1 Genes selected to quantify endosymbiont titres in M. euphorbiae *aphids*

The heat-shock protein groEL is encoded by a single-copy gene in *B. aphidicola* and many other bacteria; consequently several studies have quantified the genome copy number of the aphid primary endosymbiont in terms of *groEL* gene copies. Published qPCR primers from Wilkinson *et al*. (2007) and Sakurai *et al*. (2005), designed to amplify sections of the *groEL* gene from *B. aphidicola* harboured by *A. pisum* hosts, successfully yielded detectable amplification from *M. euphorbiae* aphid templates. However, as there is evidence that the *B. aphidicola* bacteria have cospeciated with their

aphid hosts, the *groEL* gene of *B. aphidicola* from *M. euphorbiae* was sequenced to determine whether the complementarity of the primers could be improved (Clark *et al*., 2000; Jousselin *et al*., 2009).

The *B. aphidicola groEL* gene was amplified from two genotypes of *M. euphorbiae* using primers designed using the *groEL* sequence of *B. aphidicola* from *A. pisum* (GenBank: CP001161.1) and with the programme Primer3 (v.4.0), and the products purified and sequenced as given in section *2.2.3.1*. The resulting *B. aphidicola groEL* consensus sequence from *M. euphorbiae* aphids, aligned using the software programme Sequencher (version 4.9), is given in Figure 3.2. The sequences of the forward qPCR primer from Wilkinson *et al*. (2007) and both reverse qPCR primers were modified based on discrepancies between the *B. aphidicola groEL* gene sequences from *A. pisum* and *M. euphorbiae* hosts (Appendix 1, *A1.2.4*), and the two sets of modified primers tested on *M. euphorbiae* aphid DNA templates.

TTGTATCCGTAGCCCGTGAAATTGAATTAGAAGATAAATTCGAAAACATGGGAGCTCAAATGGTAAAAGAAGTTGCAT
CAAAAGCAAATGATGCAGCAGGCGATGGTACTACAACAGCGACATTATTAGCCCAATCTATAGTAAATGAAGGCTTAA
AAGCAGTAGCAGCTGGAATGAATCCAATGGATCTTAAACGTGGAATTGATAAAGCTGTTATTAGTGCTGTAGAAGAGT
TGAAAAATTTATCTGTACCATGTTCTGATTCTAAAGCAATTACGCAAGTTGGAACTATATCTGCAAATGCAGATGAAAA
AGTTGGTTCTTTAATTGCAGAAGCAATGGAAAAAGTTGGTAATGATGGAGTAATTACAGTAGAAGAAGGTACTGGTCT
TCAGGATGAACTTGAAGTCGTTAAAGGTATGCAATTTGATCGAGGTTATTTATCTCCATATTTTATTAACAAGCCAGAA
ACAGGTATTGTTGAATTAGAAAATCCGTATATTTTAATGGCTGATAAAAAAATATCTAATGTTCGTGAAATGTTACCAA
TATTAGAATCTGTTGCAAAATCAGGAAAACCATTATTAATTATTTCAGAAGATCTAGAWGGTGAAGCTTTAGCTACATT
AGTAGTTAATTCTATGAGAGGAATCGTAAAAGTAGCTGCAGTAAAAGCTCCTGGATTTGGTGATCGTCGTAA

Figure 3.2: *groEL* gene sequence of *B. aphidicola* harboured by *M. euphorbiae*; Regions highlighted in blue show the positions of primers used to amplify the sequence whilst regions highlighted in light and dark green show the position of the qPCR primers published by Wilkinson *et al*. (2007) and Sakurai *et al*. (2005), respectively. Gaps within the highlighted regions signify deviations from the published sequences.

Although both sets of primers successfully amplified the *B. aphidicola groEL* gene, the PCR products from the modified Sakurai *et al*. (2005) primers were detected up to two cycles later than the amplification of the same gene from the qPCR primers modified from Wilkinson *et al.* (2007). When tested using serial dilutions of aphid template, the efficiency of the PCR reaction with the modified Sakurai *et al*. (2005) primers exceeded 100% (see section *3.2.3.3*), which in combination with the delayed C_T values indicates the possible presence of inhibitors in the reaction. The primers modified from Wilkinson *et al.* (2007) were therefore selected to quantify genome numbers of *B. aphidicola*. Attempts to extract haemolymph from *M. euphorbiae* aphids harbouring the secondary endosymbiont *H. defensa* were unsuccessful, and so the specificity of the *B. aphidicola groEL* primers could not tested empirically. Instead, the specificity of the primers was assumed based on aligning the *groEL* gene sequences from different aphid

endosymbionts, and confirmed through the single peak generated in the melt curve analysis of the PCR amplicons.

The chaperone protein *dnaK* gene is also represented at a single location within the genome of *B. aphidicola*, and has been used in absolute qPCR assays to approximate the number of primary endosymbiont chromosomes in a given aphid sample (Koga *et al*., 2003; Douglas *et al*., 2006b; Dunbar *et al*., 2007). However, the alignment of available *B. aphidicola dnaK* gene sequences with those from *H. defensa* and *R. insecticola* showed a high degree of similarity, with the sequences of published primers designed to amplify from one bacterium potentially able to amplify from other endosymbionts if present (Figure 3.3). Consequently the *dnaK* gene was not considered further as a suitable target for quantifying absolute genome copy numbers of *B. aphidicola*.

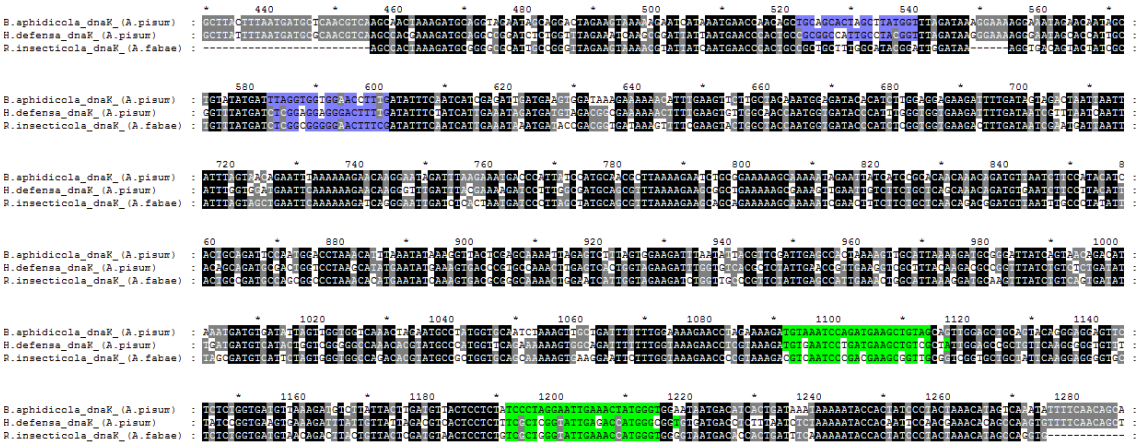

Figure 3.3: Alignment of *dnaK* gene sequences from *B. aphidicola*, *H. defensa* and *R. insecticola*. Regions highlighted in purple and green show the positions of the *B. aphidicola dnaK* primers published by Chandler *et al*. (2008) and Douglas *et al*. (2006b), respectively. Gaps within the highlighted regions signify deviations from the published sequences.

To quantify genome copy number of *H. defensa*, the abundance of the single-copy gene *gyrB*, which encodes a subunit of the DNA gyrase protein, was quantified. Although primers for the amplification of the *gyrB* gene had been successfully used by Oliver *et al.* (2006) to quantify *S. symbiotica* and *H. defensa* densities in pea aphids using absolute qPCR techniques, these primers did not anneal to a region of the gene frequently present in the NCBI database, and hence the likely specificity of the primers to the secondary endosymbiont could not be ascertained. Instead, two sets of qPCR primers were designed based on an available *H. defensa gyrB* sequence from *M. euphorbiae* (GenBank EU021841.1) using the programmes Primer3 (v.4.0) and Primer Express (v. 3.0). Primers published by Degnan and Moran (2008b) were used to amplify the *H. defensa*

gyrB sequence from two infected *M. euphorbiae* clonal lines kept in culture, with the amplicons then purified, sequenced and aligned using Sequencher (version 4.9) (see section *2.2.3.1*). The contig sequence is given below in Figure 3.4; with the exception of one base pair, the *H. defensa* sequence from the *M. euphorbiae* clonal lines kept at JHI matched the published sequence.

CTCATCCCCAGGCGCCTGCGCTTTCTGGTGCCTCGCTCGAAAAATTGGTGCATCAACACTATAGTGTGCAGAAAACGCT
TAATCGAATGGAACGTCGATATCCTCGGGCGCTTTTAAATCAGATGATCTATCAACCAACCTTACAGGAAGGCGACCTC
AAAGATTCCGAAAAACTTCAAGCCTGGATGACCCTGCTGGTGGATAAATTAAACGAAAAAGAAACGCAAGGCAGTCA
TTATATTTTTGTATTGAATAAAAATACAGAGAACGAAATATATGACCCTGTATTGAAGATTCGTACCCATGGTGTTGAC
ACGGACTATCTGCTCGATCTGCACTTTATTCAAAGTAGCGAATATCAAAAAATCTGTCACTGGGGCGATCAATTACGAG
ATCTGTTAGAACCTGGTGCTTTCCTTCAACGAGGAGAAAAAAAGACCTGTATCAACAGTTTTGAAGAGGCCCTGGATT
GGCTGATGAAAGAATCTCGTCGTGGCTTGGCCATTCAGCGCTACAAAGGATTGGGAGAAATGAATCCGGGCCAGCTCT
GGGAGACCACCATGGACCCAGAAACGCGTCGCATGCTACAGGTCAGGATAAAAGACGCGATCGCCGCAGATCAATTG
TTTACAACGTTGATGGGAGA

Figure 3.4: *gyrB* gene sequence of *H. defensa* harboured by *M. euphorbiae*; Regions highlighted in blue show the positions of primers published by Degnan and Moran (2008b) used to amplify the sequence whilst regions highlighted in light and dark green show the positions of the two sets of qPCR primers designed to quantify copy numbers of the *gyrB* gene. Gaps within the highlighted regions signify deviations from the published sequences.

Both sets of *H. defensa gyrB* qPCR primers generated products from the DNA of *M. euphorbiae* aphids that lacked *H. defensa*, albeit with amplification detected at least 10 cycles after that from aphids harbouring the secondary endosymbiont, though no products were generated in the negative (NTC) controls. The equipment and reagents used to extract aphid DNA were ruled out as possible sources of contamination, and surface washing the aphids in 70% ethanol or 1% Tween 20 to remove bacteria present on the cuticle prior to DNA extraction did not reduce the extent of amplification.

Through purification and sequencing, the unknown amplified products were found to match closely a section of the *H. defensa gyrase B* gene from *M. euphorbiae*, as indicated by the similar melt curves of the qPCR products from aphid templates both with and without *H. defensa*. This suggests that the qPCR primers were specific enough not to cross-amplify from other known symbionts such as *B. aphidicola*, but that there is either *H. defensa* or a *Hamiltonella*-like bacterium present in all of the aphids at low titre. Furthermore, amplification from the *H. defensa gyrB* primers was also detected in later cycles from DNA extracted from the peach–potato aphid *Myzus persicae*, which is not known to harbour *H. defensa*, and from DNA samples from the fruit fly *Drosophila melanogaster* (see section *2.2.1.1*). An arbitrary threshold of 30 cycles was therefore set for the *H. defensa gyrB* assay, beyond which detectable amplification was deemed not to represent an established endosymbiotic infection and was hence disregarded.

Other target genes considered for quantifying *H. defensa* titres include the *dnaK* gene that, as with *B. aphidicola*, is present at a single location within the *H. defensa* genome. However, as was described previously, the *dnaK* gene sequences of the various aphid endosymbionts are highly similar. Another potential gene, *recA*, encodes a DNA repair protein and again is a single copy gene within the *H. defensa* genome. The *recA* gene has been lost from *B. aphidicola*, reducing the likelihood of cross-amplification from the primary endosymbiont, and has been used successfully to quantify *S. symbiotica* titres in *A. pisum* (Oliver *et al*., 2003). When available *recA* sequences from various bacterial species including *H. defensa* were aligned, however, the variation within different *H. defensa* strains from *A. pisum* hosts equalled that seen between *H. defensa* and other bacteria such as *R. insecticola* (Figure 3.5). Given that the diversity of *H. defensa* strains within *M. euphorbiae* hosts has not been characterised, it is possible that variations in the gene sequence could result in primers amplifying from the *recA* gene with differing efficiencies, leading to the possible under-estimation of *H. defensa* titres. Neither *dnaK* nor *recA* were therefore used as targets to quantify *H. defensa* genome copy number.

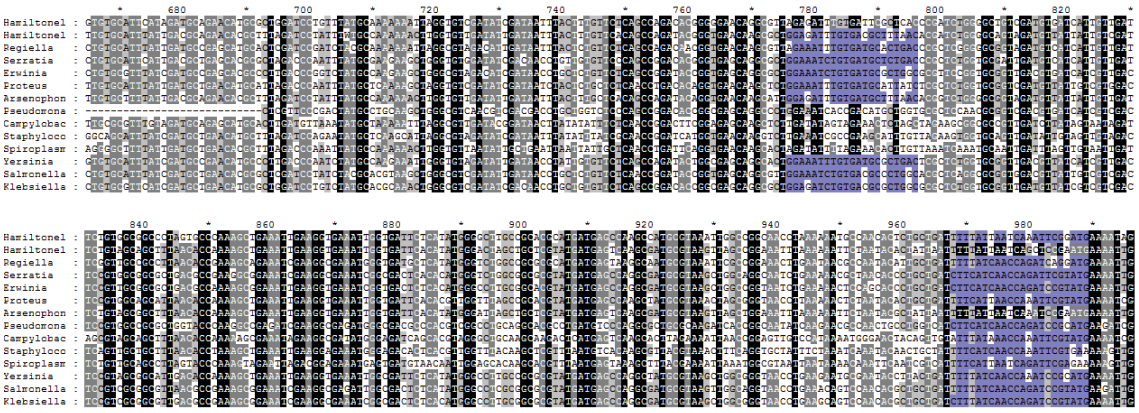

Figure 3.5: Alignment of *dnaK* gene sequences from *H. defensa*, *R. insecticola*, *S. symbiotica* and other bacteria. Regions highlighted in purple show the positions of the *S. symbiotica recA* primers published by Oliver *et al*. (2003). Gaps within the highlighted regions signify deviations from the published sequences.

3.2.3.2 Genes selected to quantify M. euphorbiae *aphid genome copy number*

In absolute qPCR the final result is expressed relative to a defined unit of interest. The number of copies of the *M. euphorbiae* genome present in each sample was therefore quantified so that relative titres of *B. aphidicola* and, where present, *H. defensa* could be reported, allowing comparisons of endosymbiont titres between aphid samples. As any variation in the denominator may obscure differences in the copy numbers of the genes

of interest, the denominator chosen should be absolutely stable across the comparison; two *M. euphorbiae* genes were therefore quantified to enable the most suitable denominator to be selected.

One of the most widely-used aphid normalisation genes, used in both absolute and relative qPCR, is the *elongation factor 1-α* (*EF1-α*) gene, which encodes a protein that, amongst other functions, is important for translation. Although two copies of the *EF1-α* gene are present in the genomes of Hymenoptera and Diptera, most insects, including aphids, have only a single copy of the gene (Danforth & Ji, 1998; Dunbar *et al*., 2007). PCR primers published by Sakurai *et al.* (2005) designed to quantify the genome copy number of *A. pisum* successfully amplified a product of the expected size from *M. euphorbiae* templates, despite possible discrepancies in the complementarity between the target and primer sequences.

To increase the complementarity of the published primers for use with an *M. euphorbiae* template, a section of the *EF1-α* gene spanning the qPCR primer target site was amplified from *M. euphorbiae* DNA. Primers were designed using the *EF1-α* sequence of *Macrosiphum rosae* (GenBank: AY219736.1), the *EF1-α* gene amplified from two genotypes of *M. euphorbia*e aphid, and the products purified and sequenced as before (section *2.2.3.1*). The resulting *M. euphorbiae EF1-α* consensus sequence, aligned using the software programme Sequencher (version 4.9), is given in Figure 3.6. The sequence of the reverse qPCR primer from Sakurai *et al*. (2005) was modified as a result of a single base discrepancy between the *A. pisum* and *M. euphorbiae EF1-α* gene sequences in this region (Appendix 1, *A1.2.4*).

GCTGTGCTTATTGTCGCTGCTGGTACTGGAGAATTCGAAGCTGGTATTTCTAAGAATGGACAAACCCGTGAACACGCTT
TATTGGCTTTCACCTTGGGTGTAAAGCAATTGATCGTTGGTGTGAACAAGATGGACTCTACTGAACCACCATACAGCGA
AGTATGTATTTAAATTCCTTAGTTATGATTATGTATCAAAATTAATAATTGTTTATTTTTAGAACCGTTTCGAAGAAATC
AAGAAGGAAGTCAGCAGTTACATCAAGAAAATCGGTTACAACCCAGCTGCTGTTGCTTTCGTGCCCATCTCTGGATGG
AATGGAGACAACATGTTGGAAGTTTCCGAAAAGATGTCGTGGTTCAAAGGATGGGCCGTTGAACGTAAAGAAGGAAA
GGCTGACGGTAAATGTTTGATTGAAGCTTTAGACGCTATCCTGCCACCCAGTCGCCCAACTGACAAGGCTCTCCGTCTT
CCACTCCAGGTATGAATAAATTTAAATATTTTTTAAYTAATCTTTTTATTTATACACCTTCTAACCTGTGTTTATTTATAG
GATGTCTACAAAATTGGAGGTATTGGAACAGTCCCAGTAGGTCGTGTAGAAACTGGTCTTTTGAAACCTGGTATGGT

Figure 3.6: *M. euphorbiae EF1-α* gene sequence; Regions highlighted in blue show the positions of primers used to amplify the sequence; only partial sequences are given as the chromatogram was ambiguous beyond these points. Regions highlighted in green show the position of the qPCR primers published by Sakurai *et al*. (2005), Gaps within the highlighted regions signify deviations from the published sequences.

Another single-copy aphid gene used in both absolute and relative qPCR to normalise gene copy numbers and expression levels is the ribosomal protein *RpL7* (Nikoh *et al*., 2010; Lu *et al*., 2011). Quantitative PCR primers published by Nakabachi *et al*. (2005)

successfully amplified a product of the expected size from *M. euphorbiae* aphid DNA, and so again the primers were modified to increase their complementarity to an *M. euphorbiae* template.

Primers were designed using an *A. pisum* genomic contig (NW_001920288.1) that contained within it the *A. pisum* mRNA sequence predicted to be the *RpL7* gene (NM_001135898.1) to amplify a region of the *M. euphorbiae RpL7* gene spanning the target site of the published qPCR primers (see Appendix 1, *A2.1.1*). The *M. euphorbiae RpL7* consensus sequence, sequenced and aligned from two genotypes of *M. euphorbi*ae aphid, is given in Figure 3.7. The sequence of the forward qPCR primer from Nakabachi *et al*. (2005) was modified as a result of the single base discrepancy between the *A. pisum* and *M. euphorbiae RpL7* gene sequences in this region (Appendix 1, *A1.2.4*).

GCGCGTGAAAGAAAACTAAGAAGTATTTCAAACGCGCCGAGGCTTATGTTAAGGAATTTAGAATGAAGGAAAGAGAT
GAGATCCGTTTAGCAAGAAATGCAAAGAAATCCGGTGACTTTTATATTCCTCCTGAACCAAAATTAGCATTCATCATGC
GTATTCGTGGGTATGTTTGCTTTCTAAATCTGTTAAATAAGATAATTTCTATTACATATATTATGAACAATATTTTAGTG
TGAACCAAGTAGCTCCTAAAGTGAAGAAAGTATTGCAACTGTTCAGATTGCGTCAGATCAACAATGGAATATTCATCA
AATTAAACAAAGTAAAAATATAATTAAATGTAAATAGTAAGTATTATTTCTTAACTTTTTTTTTATTATTTTTAGGCAAC
ATTAAATATGTTGAGAATTTGTGAACCATATGGGACTTGGGG

Figure 3.7: *M. euphorbiae RpL7* gene sequence; Regions highlighted in blue show the positions of primers used to amplify the sequence, with gaps denoting deviations from the *A. pisum* sequence from which the primers were designed. Regions highlighted in green show the position of the qPCR primers published by Nakabachi *et al*. (2005). Gaps within the highlighted regions signify deviations from the published sequences.

Beyond checking the melting points of the amplicons to ensure that only a single product was being generated in each assay, the specificity of the modified *M. euphorbiae EF1-α* and *RpL7* qPCR primers was not tested further as there should be no eukaryotic DNA other than that of *M. euphorbiae* in each aphid DNA sample.

Other potential aphid normalisation genes considered included *β-tubulin*, which encodes a structural protein and which has been used successfully as a reference gene for the relative quantification of aphid gene expression (Shakesby *et al*., 2009). However, no annotated genomic sequences were available and primers designed based on the mRNA sequences available failed to amplify from *M. euphorbiae* genomic DNA. Furthermore, the *A. pisum* genome is thought to harbour genes encoding four different isoforms of β-tubulin, some of which have undergone duplication events (Nielsen *et al*., 2010), and so the specificity of primers designed to amplify only from one single-copy isoform would be questionable. Another potential aphid normalisation gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), encodes a metabolic protein and has also been used

as an aphid reference gene for reverse-transcriptase qPCR (Shakesby *et al*., 2009). However, the presence of two apparently functioning copies of the gene as well as two copies of the gene that lack the enzyme active site and two *GAPDH*-like genes similar to genes from plants and yeast predicted in the *A. pisum* genome (Tamborindeguy *et al.*, 2010) meant *GAPDH* was also not a suitable gene for quantifying potato aphid genome copy number in absolute qPCR assays.

3.2.3.3 Optimisation of qPCR reactions and conditions

With primers designed to amplify single-copy genes from *B. aphidicola*, *H. defensa* and *M. euphorbiae*, the optimal primer concentrations for each of the four assays were determined by running primer matrices (as given in section *2.2.3.2*). Each combination of forward and reverse primer at final concentrations of 150 nm, 300 nm and 600 nm was tested in duplicate for each assay. No statistically significant difference was found between the resulting C_T values within any of the four assays when compared using ANOVA (*M. euphorbiae EF1-α*, F = 0.390; *M. euphorbiae RpL7*, F = 2.916; *B. aphidicola groEL*, F = 2.283; *H. defensa gyrB*. F = 2.544; d. f. = 8, P > 0.05). A final concentration of 300 nm, within the range recommended in the MESA Blue qPCR MasterMix Plus for SYBR® Assay Low ROX protocol, was therefore used for each primer.

To ascertain that the reproducibility and efficiency of each reaction fell within the recommended limits, qPCR reactions were conducted using ten-fold serial dilutions of both *M. euphorbiae* DNA and linearized plasmids containing cloned sections of the target genes (see section *2.2.3.1*). Linear regression of the resulting C_T values against the log dilutions of the templates produced standard curves from which the reproducibility and the efficiency of each reaction could be determined. The reproducibility of each reaction was given by the correlation co-efficient (R), whilst the efficiency of the qPCR reactions in each plate was calculated using the formula:

Equation 2) $$\text{Efficiency} = (10^{(-1/\text{gradient})} - 1) \times 100$$

Well-optimised qPCR reactions are expected to have a reproducibility > 0.998 and an efficiency of between 90% and 105%, although for absolute qPCR assays it is more

important that the efficiencies of the standards and the targets of a given assay are alike, or that a correction is applied to make them so (Brankatschk *et al*., 2012). Poor linearity of C_T values across a dilution series can be indicative of poor template quality, a sub-optimal quantity of nucleic acid template or too low an annealing temperature, whilst low reaction efficiencies may signify too short an annealing or extension time, too high an annealing temperature, inhibitors within the reaction mix contents or poor primer design. The efficiency and reproducibility of the four assays determined from both aphid DNA and linearized plasmids are given in Table 3.1.

Assay	Efficiency		Reproducibility	
	Aphid DNA	Linearized plasmid	Aphid DNA	Linearized plasmid
M. euphorbiae EF1-α	90.73%	90.70%	0.999	1.000
M. euphorbiae RpL7	96.92%	96.06%	0.999	1.000
B. aphidicola groEL	84.69%	83.71%	0.999	1.000
H. defensa gyrB	88.08%	87.00%	1.000	1.000

Table 3.1: Efficiency and reproducibility of the qPCR reactions to quantify single-copy genes in *M. euphorbiae*, *B. aphidicola* and *H. defensa*, derived from standard curves generated with both aphid DNA and linearized plasmid templates.

The efficiency and reproducibility of both of the assays for quantifying copy numbers of the single-copy *M. euphorbiae* genes *EF1-α* and *RpL7* were within the desired limits, and so neither assay required further optimisation. Although the reproducibility of both the *B. aphidicola groEL* and *H. defensa gyrB* assays were higher than the recommended minimum, the efficiencies were below the desired 90%. Nevertheless, within each assay the efficiencies generated from the aphid DNA template and the linearized plasmid template were very similar, indicating that the kinetics of the reactions were comparable and hence suitable for absolute quantification of gene copy numbers.

3.2.3.4 Collection of M. euphorbiae *aphids for quantifying endosymbiont titres*

Aphids from ten *M. euphorbiae* clonal lines, selected to represent different secondary endosymbiont complements and a range of aphid genotypes (see sections *3.3.1* and *3.3.2*), were used to quantify titres of primary and secondary endosymbionts. To ensure the rearing history, age and developmental stage of the aphids used were standardised, aphids from each of the ten clonal lines were first reared on whole Désirée potato plants for a minimum of 2 generations (see section *2.1.2*). Four adult apterous aphids were then removed from each whole plant culture and individually secured in clip cages to the

underside of a leaf of a fresh Désirée potato plant within a glasshouse (light intensity ≥ 200 Wm^{-2} for 16 hours a day, temperature 18 °C during the 16 h photoperiod and ≥ 15 °C overnight). After 12 hours the adult aphids were removed and the nymphs re-caged, and after five days all but one nymph from each cage were discarded. The remaining nymphs were left to develop for a further 11 days, by which time each aphid was in the early reproductive phase and therefore mitigating any differences in development time between the clonal aphid lines. On day 16, the adult aphids were collected and weighed using a microbalance (Mettler Toledo MX5, Leicester, UK) before being frozen at -20°C.

3.2.3.5 Preparation of qPCR assays

Genomic DNA was extracted from the *M. euphorbiae* aphids as given previously (section *2.2.1*) to form the templates from which copies of the *EF1-α* and *RpL7* genes of *M. euphorbiae*, the *groEL* gene of *B. aphidicola* and the *gyrB* gene of *H. defensa* were quantified. Each of these genes had previously been amplified and cloned to produce linearized plasmid solutions of known gene copy number (section *2.2.3.1*), and serial dilutions used to create standard curves from which the number of gene copies present in each aphid DNA template could be calculated. The qPCR reactions were prepared and the thermocycling conditions set as given in section *2.2.3.2*.

3.2.3.6 Normalisation of quantitative PCR data and analysis of endosymbiont titres

The number of aphid samples from which bacterial titres were to be quantified necessitated two plates for each assay. To reduce the variation between plates, the C_T threshold automatically set by the ABI 7500 Fast Real-Time PCR software for the first plate was set manually for the second plate. For the *H. defensa gyrB* assay, the threshold was manually set for both plates as the high C_T values generated from some of the templates in which *H. defensa* is absent lowered the threshold automatically set.

For each plate run, the log dilution of the linearized plasmid solution was plotted against C_T value to form a standard curve, and linear regression used to determine the gradient of the line. For each assay, the three C_T values generated by the technical replicates of each

aphid DNA template were converted into equivalent dilutions of the standard plasmid solution using the following formula:

Equation 3) $$\text{Equivalent dilution} = \frac{\text{Gradient of the standard curve} - C_T \text{ value}}{\text{Intercept of the standard curve}}$$

The number of copies of the gene present in the sample was established by multiplying the equivalent dilution by the number of plasmids present in 1 µL of undiluted plasmid standard, and an average gene copy number for each aphid biological replicate calculated by taking the mean of the three technical replicates.

The *B. aphidicola groEL* gene copy numbers from the ten *M. euphorbiae* aphid lines were normalised using four different denominators; for each aphid sample, the primary endosymbiont titres were divided by aphid fresh weight, DNA concentration and the copy numbers of the *EF1-α* and *RpL7 M. euphorbiae* genes. The *B. aphidicola* titres of each *M. euphorbiae* line when normalised by the four different denominators were visually compared and linear regression used to determine the relationship between each normalised dataset, and the most appropriate denominator, both statistically and biologically, selected.

Analyses were performed on the normalised bacterial titres using the statistical software SPSS (v. 21, IBM), with graphs plotted in SigmaPlot (v. 12.3, Systat Software). Univariate general linear models (GLMs) were used to compare the *B. aphidicola* endosymbiont titres between the seven *M. euphorbiae* genotypes, with the different qPCR plates included as factors and either the presence or absence of *H. defensa* included as a factor, or the titres of *H. defensa* present within each aphid line incorporated as a covariate. Aphid line was nested within genotype, or within symbiont grouping and within genotype. A univariate GLM was also used to compare titres of *H. defensa* between the two genotypes that naturally harbour the endosymbiont, again with aphid line nested within genotype. The statistical power of each test (the type II error rate (1-β)), was above the accepted threshold of 0.8 unless otherwise stated. Where statistically significant differences in bacterial titres were found, significant differences amongst the means were isolated using Sidak multiple pairwise comparison post-hoc tests, which adjusts for the accumulation of type I errors and thus the potential rejection of a true null hypothesis.

3.3 Results

3.3.1 Presence and stability of secondary endosymbiont infections in M. euphorbiae *clonal lines*

3.3.1.1 Presence of known aphid secondary endosymbionts

The infection statuses of the 19 isofemale clonal lines of *M. euphorbiae* kept in culture, determined through diagnostic PCR, are given below (Figure 3.8, Table 3.2). Secondary endosymbionts that were initially present but subsequently lost from a given clonal line are shown in parentheses.

***M. euphorbiae* clonal line**	**Secondary endosymbiont**						
	Serratia symbiotica	***Hamiltonella defensa***	***Regiella insecticola***	***Rickettsia***	***Spiroplasma***	**PAXS**	***Rickettsiella***
AA09/02	×	×	×	×	×	×	×
AA09/03	×	✓	×	×	×	×	×
AA09/04	×	✓	×	×	×	×	×
AA09/06	×	✓	(✓)	×	×	×	×
AA09/11	×	×	(✓)	×	×	×	×
AA09/12	×	×	×	×	×	×	×
AA09/13	×	×	×	×	×	×	×
AA09/14	×	×	×	×	×	×	×
HC10/02	×	✓	(✓)	×	×	×	×
HC10/05	×	✓	×	×	×	×	×
HC10/06	×	✓	×	×	×	×	×
HC10/07	×	×	×	×	×	×	×
HC10/08	×	×	(✓)	×	×	×	×
HC10/14	×	×	(✓)	×	×	×	×
AK11/01	×	×	×	×	×	×	×
AK11/02	×	×	×	×	×	×	×
HC11/02	×	×	×	×	×	×	×
HC11/03	×	✓	×	×	×	×	×
HC11/09	×	✓	(✓)	×	×	×	×

Table 3.2: Known secondary endosymbiont infections of the 19 clonal lines of *M. euphorbiae* kept in culture at The James Hutton Institute, based on diagnostic PCR screening.

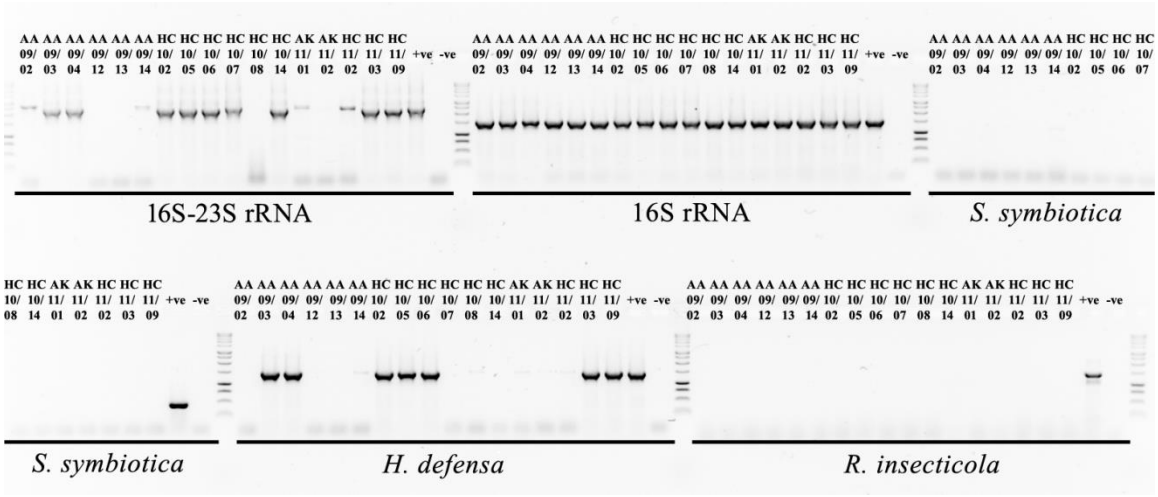

Figure 3.8: Inverted gel electrophoresis image of the diagnostic PCR products generated when stock cultures of the *M. euphorbiae* clonal lines kept in culture at JHI were screened for the endosymbionts *S. symbiotica*, *H. defensa* and *R. insecticola*; only the primers for *H. defensa* generated visible products from the aphid templates. The 16S and 16S-23S rRNA products indicate the presence of bacteria, and of bacteria other than *B. aphidicola*, respectively.

Only two known pea aphid secondary endosymbionts, *H. defensa* and *R. insecticola*, were identified from the *M. euphorbiae* lines, with five clonal lines harbouring only *H. defensa*, three clonal lines harbouring only *R. insecticola* and three clonal lines infected with both secondary endosymbionts. However, whilst the eight *H. defensa* infections were present throughout the period over which the clonal aphid lines were kept in culture, the six *R. insecticola* infections were less stable with each lost within approximately six months of the founding aphids being collected.

None of the nineteen aphid lines produced clear bands of DNA in the *S. symbiotica*, *Rickettsia*, *Spiroplasma*, PAXS or *Rickettsiella* screens, indicating that these endosymbionts were not present in any of the *M. euphorbiae* lines. Although there were no positive controls for the four latter endosymbionts, the lack of substantial product in the 16S-23S rRNA screens in eleven of the nineteen lines confirm the absence of bacteria other than *B. aphidicola* in significant number.

3.3.1.2 Presence of the APSE bacteriophage

Of the eight *M. euphorbiae* clonal lines harbouring *H. defensa*, in all but one the endosymbiont was infected with the APSE phage, as evident from the successful amplification of the P35 and P51 APSE genes. Only the *M. euphorbiae* line AA09/03 harboured *H. defensa* but lacked the APSE bacteriophage.The bacteriophage infection

appeared stable, with lines bearing *H. defensa* also maintaining the phage infection throughout the course of this study.

3.3.2 Genotypic analysis of M. euphorbiae *clonal lines*

3.3.2.1 Genotypic classification of M. euphorbiae *clonal lines*

The allele sizes at three microsatellite loci for a sub-set of the aphid DNA templates were scored by both polyacrylamide gel and capillary electrophoresis, with both producing similar patterns of allele distribution. The resolution of the allele sizes was higher when scored using capillary electrophoresis, however, and so this method alone was used to size the alleles from the remaining four microsatellites.

The allele sizes from each of the seven microsatellite loci from the 19 *M. euphorbiae* clonal lines are shown below (Table 3.3). Allele combinations for a given locus that occur in more than one aphid line are shaded the same colour, whilst alleles that only occur in one given line are marked with an asterisk.

	Genotype 1		**Genotype 2**		**Genotype 3**	
	AA09/03, AA09/04, AA09/12, AA09/14, HC11/02		**AA09/06, HC10/02, HC10/05, HC10/06, HC11/03, HC11/09**		**AA09/11, HC10/14, AK11/01, AK11/02**	
Me1	141	165	159	171	145	165
Me5	102	112	104	112	102	102
Me7	133	139	133	145	149	169
Me9	138	154	148	154	138	142
Me10	154	160	151	154	157	157
Me11	127	139	123	133	129	133
Me13	125	141	149	153	137	143

	Genotype 4		**Genotype 5**		**Genotype 6**		**Genotype 7**	
	AA09/02		**AA09/13**		**HC10/07**		**HC10/08**	
Me1	145	163*	141	151*	145	159	171	171
Me5	112	112	102	112	112	112	102	104
Me7	149	169	133	139	133	133	147*	149
Me9	148	158	138	154	142	154	148	148
Me10	154	157	154	160	154	157	151	154
Me11	129	133	127	139	129	133	123	123
Me13	137	141	125	141	137	149	143	145*

Table 3.3: Allele sizes from seven microsatellite loci amplified from the 19 *M. euphorbiae* clonal lines kept in culture.

Based on these results, the 19 *M. euphorbiae* clonal lines consist of 7 distinct genotypes, 3 of which are represented by multiple lines. Genotype 5, represented by the AA09/13 clonal line, differs from genotype 1 by only one allele at the *Me1* locus; although repeated amplification of the microsatellite loci from multiple aphids confirm the allele sizes, it is not clear as to whether the AA09/13 aphids indeed represent a separate genotype, or whether the clonal line was founded from a genotype 1 aphid in which one of the *Me1* microsatellite alleles had mutated during DNA replication.

3.3.2.2 M. euphorbiae *genotypes and secondary endosymbiont combinations*

The *M. euphorbiae* aphid clonal lines forming each genotype and their secondary endosymbionts are given in Table 3.4.

Genotype	***M. euphorbiae* clonal line**	**Stable secondary endosymbiont infection**
1	AA09/03	*H. defensa*
	AA09/04	*H. defensa* + APSE
	AA09/12	None
	AA09/14	None
	HC11/02	None
2	AA09/06	*H. defensa* + APSE
	HC10/02	*H. defensa* + APSE
	HC10/05	*H. defensa* + APSE
	HC10/06	*H. defensa* + APSE
	HC11/03	*H. defensa* + APSE
	HC11/09	*H. defensa* + APSE
3	AA09/11	None
	HC10/14	None
	AK11/01	None
	AK11/02	None
4	AA09/02	None
5	AA09/13	None
6	HC10/07	None
7	HC10/08	None

Table 3.4: Distribution of *H. defensa* and associated APSE phage across *M. euphorbiae* clonal lines and genotypes

Only a small number of clonal *M. euphorbiae* lines have been both genotyped and screened for stable secondary endosymbiont infections as part of this study. Nevertheless, the confinement of *H. defensa* infection to only two potato aphid genotypes, in all six clonal lines of genotype 2 and two clonal lines of genotype 1,

suggests some genotypes may be more predisposed than others to harbouring the endosymbiont.

Statistically, the observed distribution of *H. defensa* infections does not differ significantly from what would be expected if each aphid genotype was equally likely to harbour the endosymbiont (G-test for goodness of fit with Williams correction; G = 8.721; P = 0.19), but this result should be interpreted with caution as the expected infection rates for the aphid genotypes represented by only one clonal line were very low.

3.3.3 Quantifying primary and secondary endosymbiont infections

3.3.3.1 Comparison of the four normalisation denominators

Although both the *EF1-α* and *RpL7* genes are reported to occur only once within the aphid genome, the absolute copy number of the *EF1-α* gene from each aphid DNA template was on average approximately 2.5 times greater than the absolute copy number of the *RpL7* gene (Figure 3.9).

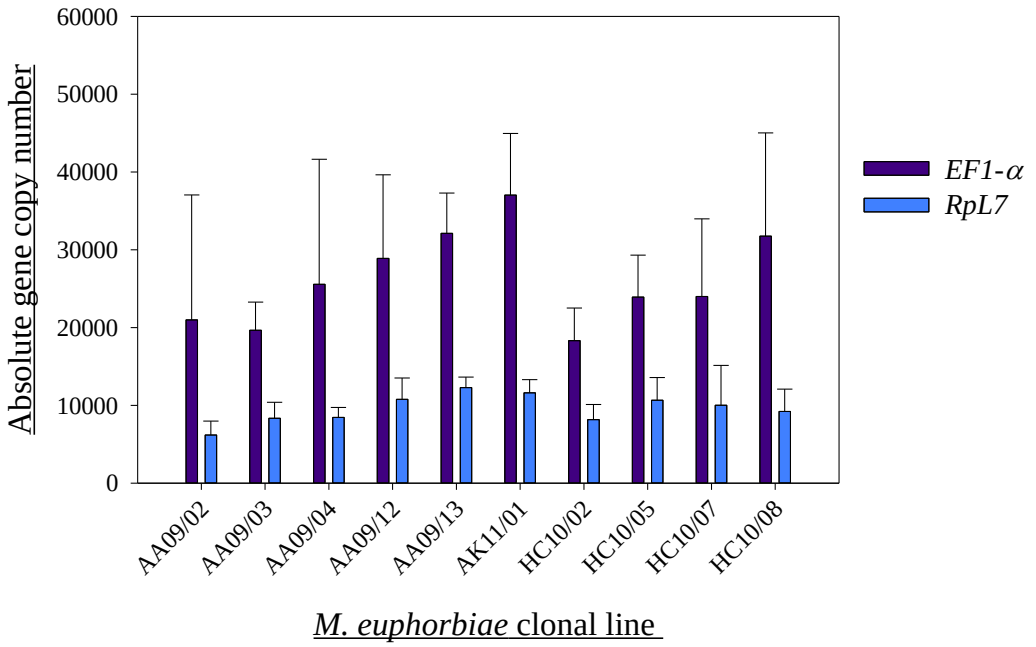

Figure 3.9: Absolute copy numbers of *EF1-α* and *RpL7* genes from individual *M. euphorbiae* aphids across 10 clonal lines. Error bars show +1 s. d.

While this could have been due to poor reaction efficiencies or from non-specific amplification from either set of the qPCR primers used, it is also possible that the aphid genome may contain one or more degenerate duplicates of the *EF1-α* gene from which the primers were partially amplifying. The *M. euphorbiae EF1-α* gene was therefore not used to normalise the bacterial titres.

A linear regression between the fresh weights of the aphid samples and the concentrations of the DNA extracted showed that 67% of the variation in the DNA concentration was explainable by the variation in the aphid fresh weight ($R^2 = 0.669$, Figure 3.10). As aphid material can be lost as the samples are processed for DNA extraction, some of the remaining variation in DNA concentration is likely to be explained by the variation in the efficiency of the DNA extraction method. As there was no means of determining the efficiency of each DNA extraction, aphid fresh weight was also rejected as a standard by which to normalise the titres of primary and secondary bacteria in each aphid sample.

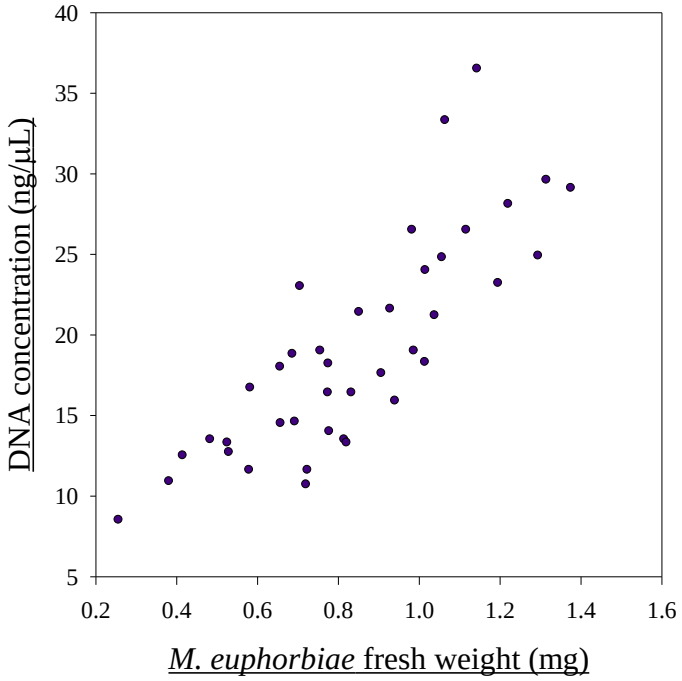

Figure 3.10: Fresh weight of each *M. euphorbiae* aphid plotted against concentration of DNA extracted

The graphs of the mean *B. aphidicola groEL* gene copy number for each aphid line appear very similar when normalised by DNA concentration and by the *M. euphorbiae RpL7* gene copy numbers, and plotting the DNA concentrations against the *RpL7* gene copy number shows a close correlation between the two (R = 0.909; Figures 3.11 and

3.12). A linear regression between the DNA concentrations of the aphid samples and absolute copy numbers of the *M. euphorbiae RpL7* gene shows that nearly 83% of the variation in the *RpL7* copy number is explainable by the variation in concentration of DNA ($R^2 = 0.826$). In contrast, only 54% of the variation in the *M. euphorbiae EF1-α* gene copy number is explainable by the variation in concentration of DNA ($R^2 = 0.542$), again suggesting that the *EF1-α* gene is a less reliable denominator than the *RpL7* gene. As the DNA concentration of each aphid sample was only measured once, the reliability of the NanoDrop ND 1000 spectrophotometer has not been quantified. The *M. euphorbiae RpL7* gene was therefore selected to standardise the *B. aphidicola* and *H. defensa* numbers quantified from each aphid sample, enabling the bacterial titres to be compared between individual aphids and between aphid lines and genotypes.

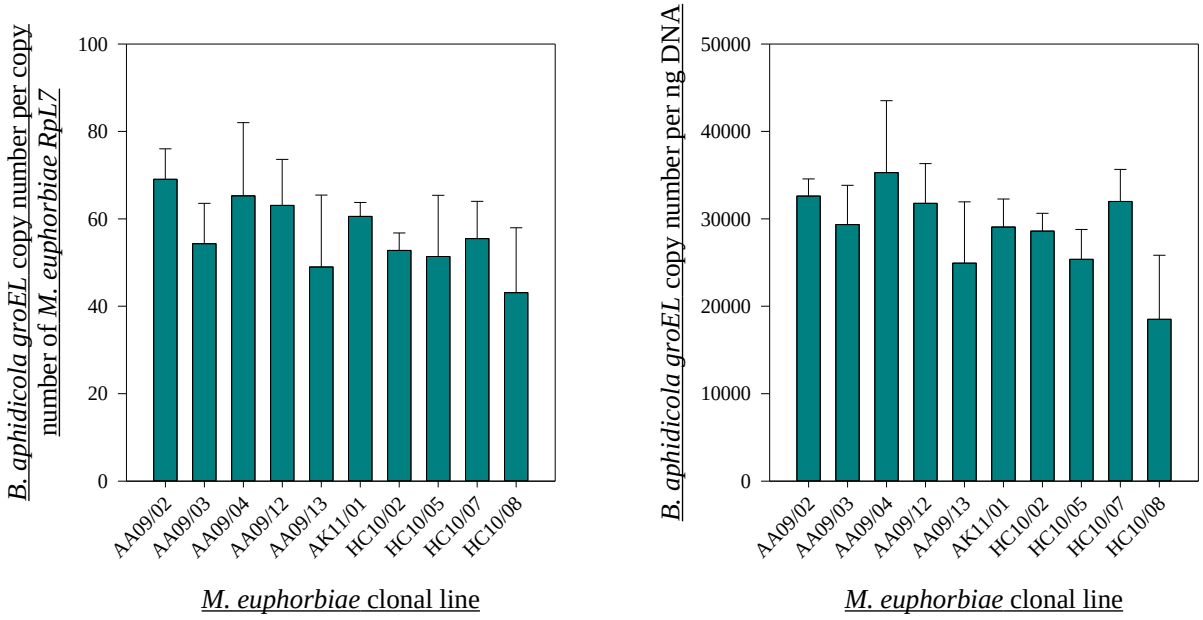

Figure 3.11: Mean *B. aphidicola groEL* gene copy numbers normalised to *M. euphorbiae RpL7* gene copy number (left) and to DNA concentration (right) from individual *M. euphorbiae* aphids across 10 clonal lines. Error bars show +1 s. d.

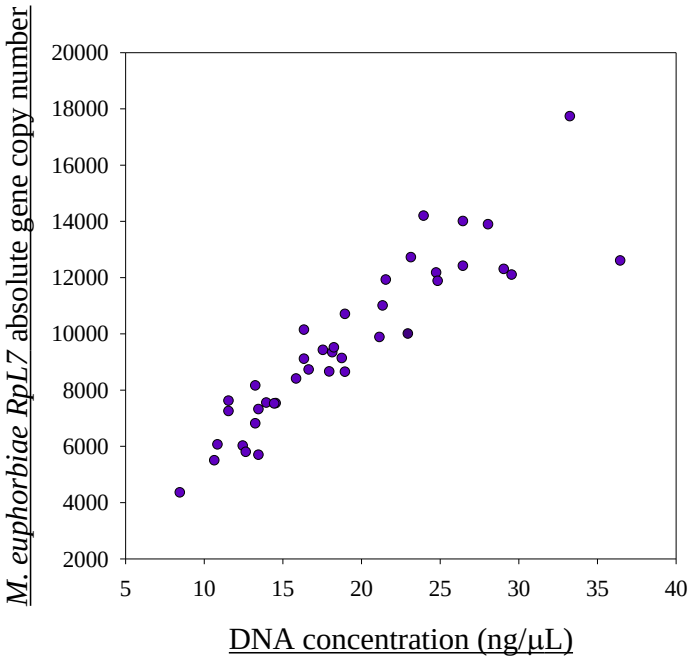

Figure 3.12: Concentration of DNA extracted from each *M. euphorbiae* aphid plotted against the number of copies of the *RpL7* gene present in each sample.

3.3.3.2 Variation in B. aphidicola *titres between* M. euphorbiae *clonal lines and genotypes, and effects of* H. defensa *on primary endosymbiont titres*

A univariate GLM determined that the effect of genotype on the titres of *B. aphidicola* was not significant ($F_{6, 2} = 2.817$, P = 0.285), nor was the effect of symbiont ($F_{1, 2} = 0.234$, P = 0.676), of plate ($F_{1, 29} = 1.078$, P = 0.308) or of aphid line within genotype and symbiont group ($F_{2, 29} = 0.930$, P = 0.406). The levels of significance did not alter with the *H. defensa* titres added as a covariate; the effect of genotype on the titres of *B. aphidicola* was not significant ($F_{6, 1.99} = 3.492$, P = 0.240), nor was the titres of *H. defensa* ($F_{1, 28} = 0.402$, P = 0.531), the plate ($F_{1, 28} = 0.937$, P = 0.341) or of aphid line within genotype ($F_{3, 28} = 0.755$, P = 0.529).

The mean normalised *B. aphidicola* and *H. defensa* titres grouped by aphid line and genotype are displayed in Figures 3.13 and 3.14, respectively.

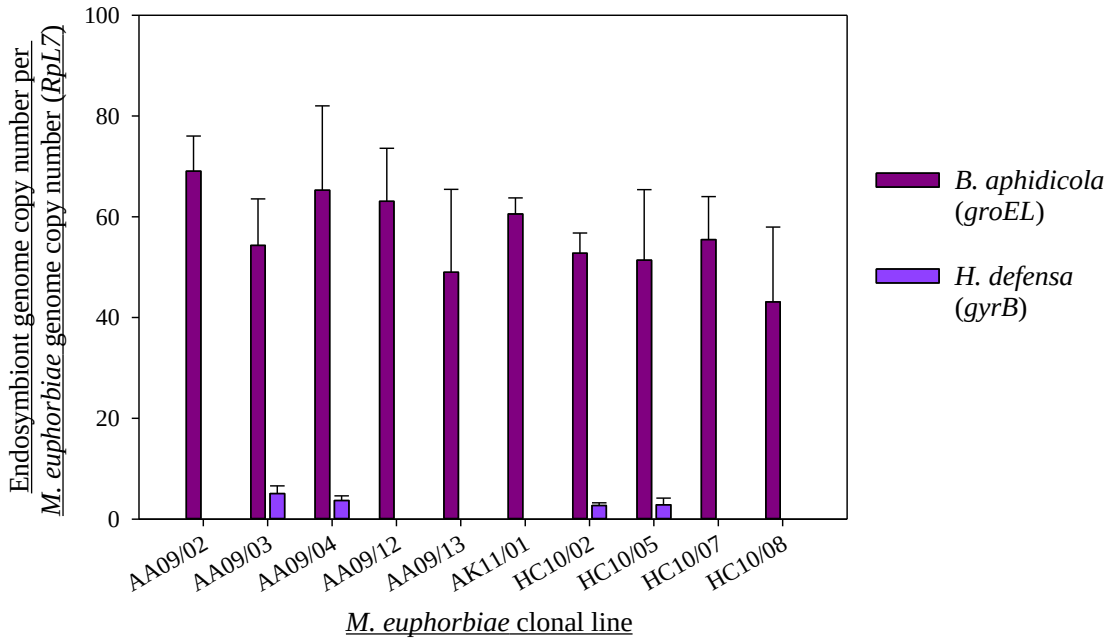

Figure 3.13: Normalised *B. aphidicola* and *H. defensa* titres from 10 clonal *M. euphorbiae* aphid lines, approximated through single copy genes. Error bars show +1 s. d.

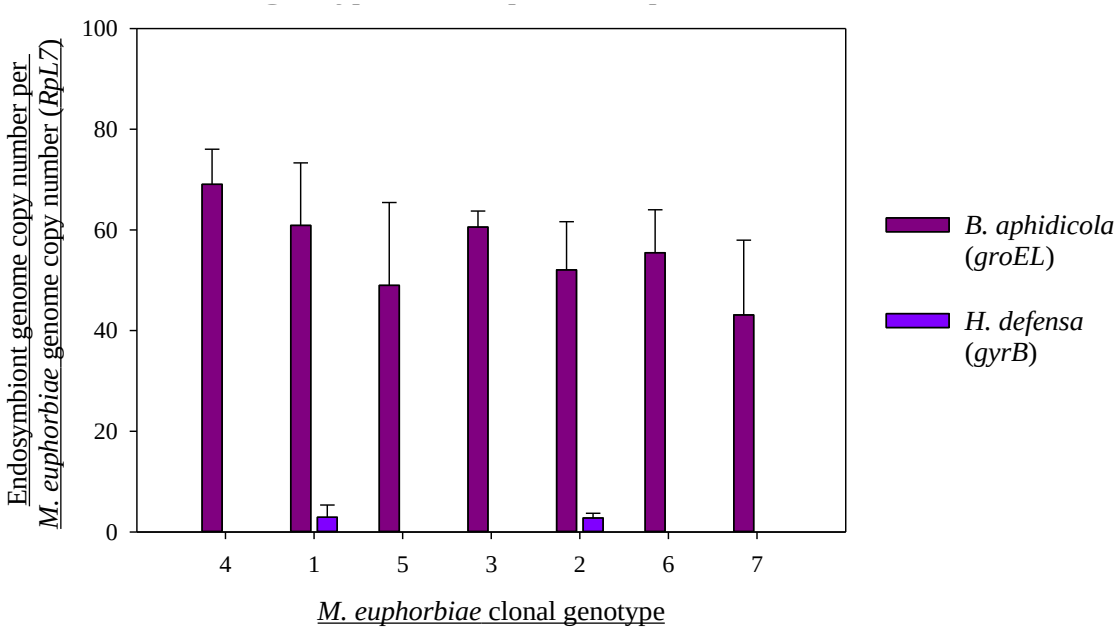

Figure 3.14: Normalised *B. aphidicola* and *H. defensa* titres from 7 genotypes of *M. euphorbiae* aphid, approximated through single copy genes. Error bars show +1 s. d.

In summary, no differences in titres of the primary endosymbiont were found between the *M. euphorbiae* lines, regardless of genotype or secondary endosymbiont status. It should be noted, however, that the powers of these analyses were very low ($1-\beta < 0.8$), increasing the probability of a type II error and reducing the likelihood of detecting a difference when one actually exists. Within the genotypes represented by more than one aphid line, there were no statistically significant differences in the primary endosymbiont titres of the isofemale clones. The mean titre of *H. defensa* in four of the aphid lines was

smaller than the variation (given by the standard deviation) in the mean titre of *B. aphidicola* in both the aphid lines infected with both primary and secondary endosymbionts and in the aphid lines naturally free of *H. defensa* (Figure 3.15). It is therefore perhaps not surprising that the titres of *H. defensa* did not affect the titres of *B. aphidicola*.

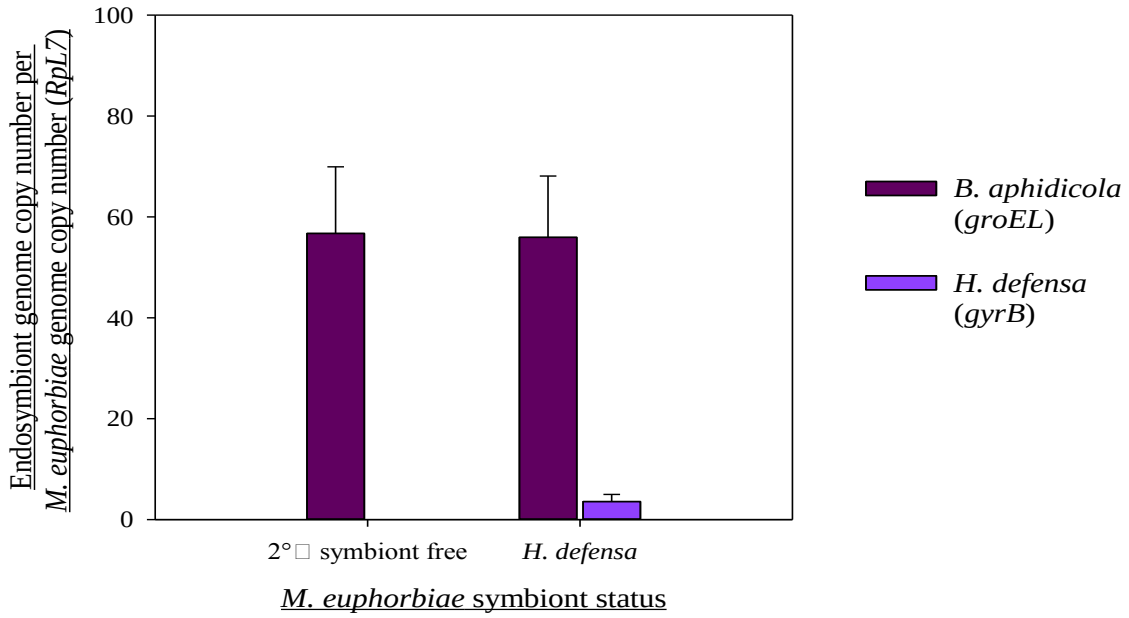

Figure 3.15: Normalised *B. aphidicola* and *H. defensa* titres from *M. euphorbiae* aphids differing in their endosymbiont complement, approximated through single copy genes. Error bars show +1 s. d.

3.3.3.3 Variation in titres of H. defensa *between naturally infected* M. euphorbiae *clonal lines and genotypes*

Figure 3.16 displays the mean normalised *H. defensa* titres from 4 naturally infected *M. euphorbiae* clonal lines, both individually and when grouped by genotype.

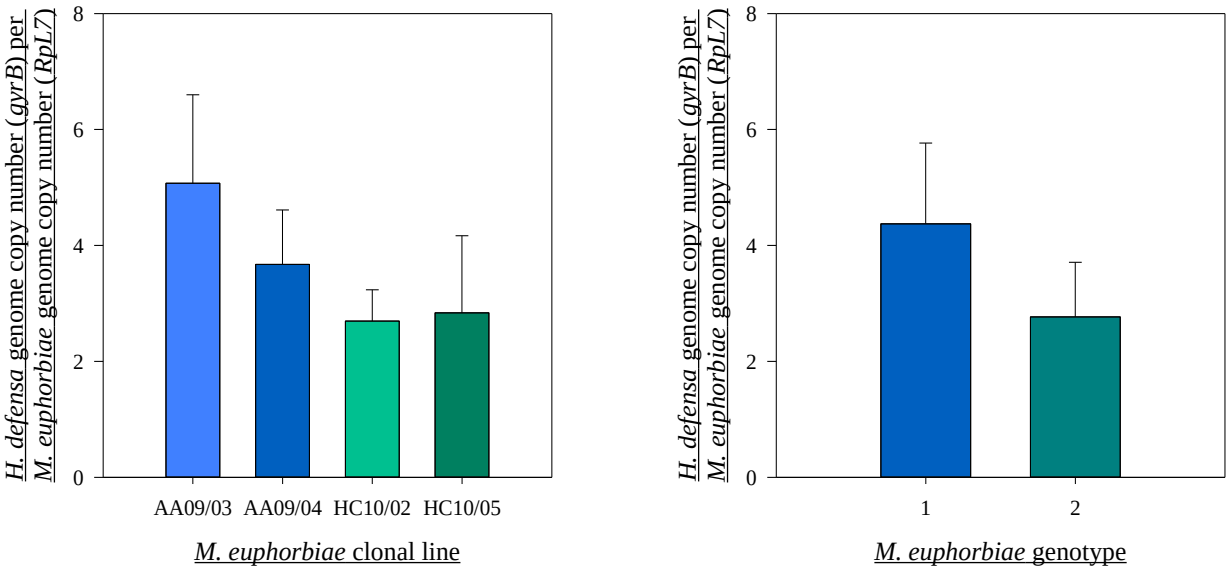

Figure 3.16: Normalised *H. defensa* titres from 4 naturally infected *M. euphorbiae* clonal lines (left), and grouped by genotype (right). Error bars show +1 s.d.

A univariate analysis of variance revealed the titres of *H. defensa* to differ significantly between *M. euphorbiae* genotypes ($F_{1, 13} = 6.886$, $P = 0.021$). When aphid line was nested within genotype, therefore including the variation between aphid lines within each of the two genotypes, the effects of genotype were no longer significant ($F_{1, 2} = 5.205$, $P = 0.150$), nor was the difference between aphid lines within a given genotype ($F_{2, 12} = 1.406$, $P = 0.286$). In aphids that harbour the *H. defensa* endosymbiont, at this particular aphid age and developmental stage, there was therefore no significant variation in *H. defensa* titres between *M. euphorbiae* aphids from different clonal lines and from different genotypes.

Only one of the *M. euphorbiae* lines kept in culture, AA09/03, harbours *H. defensa* but not an associated APSE bacteriophage. By determining there was no significant variation between potato aphid lines within a given genotype, this also showed that within a common host genetic background (genotype 1) the presence of APSE did not significantly affect the titres of *H. defensa*. Again, however, the powers of these analyses were very low ($1-\beta < 0.8$), increasing the likelihood of a type II error.

3.4 Discussion

3.4.1 Presence and frequency of secondary endosymbionts in M. euphorbiae *clonal lines*

This study identified two types of secondary endosymbiont infections in *M. euphorbiae* that have been recorded previously in potato aphids from North America; *H. defensa* and *R. insecticola* (Russell *et al*., 2003; Russell & Moran, 2005). None of the *M. euphorbiae* lines screened here contained *Rickettsia*, previously identified in a clonal line of potato aphid originating from Germany (Francis *et al*., 2010), and no novel infections of other known pea aphid secondary endosymbionts were discovered. The characterisation of endosymbiont infection was limited to 19 isofemale lines of *M. euphorbiae*, and it is possible that other types of endosymbiont infections exist in wider populations of *M. euphorbiae*.

The infection of *M. euphorbiae* with *R. insecticola* appears to be unstable, with all six infections lost from the stock potato aphid cultures within 6 months of the founding aphids being collected. Three of the *M. euphorbiae* lines were infected with both *H. defensa* and *R. insecticola*; losses of endosymbionts from such superinfections have been recorded previously (Sandström *et al*., 2001), and artificially created double infections adversely affect the fecundity of the pea aphid hosts, either due to the over-proliferation of bacteria or from interactions between the endosymbionts (Oliver *et al*., 2006). As greater numbers of aphids are screened for secondary endosymbiont complements, however, so more incidents of superinfection are detected, and at the frequencies expected given the prevalence of each endosymbiont (Russell *et al*., 2013). Despite a lack of published reports of single *R. insecticola* infections lost from pea aphid or potato aphid clones, we have also observed the loss of this endosymbiont from a culture of *A. pisum* kept at JHI. The loss of *R. insecticola* from aphid lines in which no other secondary endosymbionts are present likely indicates a lower transmission efficiency, which in turn suggests few selection pressures acting to maintain the endosymbiotic association. Such positive selection pressures are likely to be particularly weak in the insect culture environment, as many of the benefits shown to be conferred by endosymbionts to their aphid hosts are context dependent. For example, selection pressures on field populations of aphids are likely to maintain the prevalence of an

endosymbiont that provides protection from parasitoid or pathogenic attack by increasing the proportion of infected aphid clones that survive and reproduce.

Furthermore, cultured aphids are typically constrained to feeding on a single plant species, which might restrict the fitness of clones adapted to alternative host species. For example, the frequency of *R. insecticola* infections is highest amongst *A. pisum* populations on clover, although there is contradictory evidence regarding the fitness benefits provided by the endosymbiont to the aphid on *Trifolium* (Tsuchida *et al*., 2004; McLean *et al*., 2010). The only published example of a *Regiella* infection in *M. euphorbiae* was identified in aphids collected from *Penstemon* species, rather than *Solanum tuberosum* (Russell *et al*., 2003; Russell & Moran, 2005). However, the association of specific *M. euphorbiae* genotypes with either particular plant species or particular endosymbiont types has not been investigated. It is possible that the *R. insecticola* infections initially present in *M. euphorbiae* collected here from potato might have persisted in aphids deposited on alternative host plant species where the infection bestowed a benefit to the aphid host.

In contrast, infections of *H. defensa* were maintained in *M. euphorbiae* cultures throughout the period of this study. It is not clear why the two endosymbionts, *H. defensa* and *R. insecticola*, appear to have different transmission efficiencies, at least in culture. *H. defensa* infection would appear to have positive or neutral effects on *M. euphorbiae* fitness in culture, favouring maintenance of the infection. However, *H. defensa* does not achieve fixation within field populations of *M. euphorbiae*, indicating that either the benefits of harbouring *H. defensa* are aphid-genotype specific, or that alternative selection pressures or infection costs come into effect. A further consideration is that the increased availability of nutrients from the excised leaves on which the aphids are reared might mitigate any detrimental effects of the symbiosis (van Emden & Bashford, 1976).

The disparity between the phylogenies of secondary endosymbionts and their aphid hosts in *A. pisum* populations indicates that the proximate cause of the distribution of *H. defensa* between aphid clones is the result of low failure rates in the vertical transmission of the bacteria, combined with occasional horizontal transmission events (Darby & Douglas, 2003; Russell *et al*., 2003, and see section *1.2.1*). The ultimate cause, however, stems from the costs and benefits to each aphid genotype of harbouring *H. defensa*, and

the pressures that select for or against the maintenance of the endosymbiont infection (Kwiatkowski & Vorburger, 2012). The following chapters therefore investigate some of these potential selection pressures, namely the impact of *H. defensa* infection on basic aphid life history characteristics and on interactions with parasitoid wasps.

All but one of the eight *M. euphorbiae* lines that harboured *H. defensa* also harboured genes associated with the lysogenic APSE bacteriophage, the presence of which was stable throughout the project. It is unclear whether the founding AA09/03 aphid initially harboured the APSE phage as the clonal line was established three months before it was screened for phage presence, but after the initial screening the APSE phage was not gained or lost from any aphid cultures. The occurrence of *H. defensa*-infected pea aphids that lack the APSE bacteriophage and the spontaneous loss of APSE from *H. defensa*-infected pea aphids kept in culture are well documented, accompanied by loss of the protection against parasitoids (Oliver *et al*., 2003, 2009; Degnan & Moran, 2008b). As the phage is not carried on every *H. defensa* chromosome, the loss of APSE is likely to be the results of stochastic events during transovarial transmission to aphid embryos (Sandström *et al*., 2001; Moran *et al*., 2005a; Degnan & Moran, 2008a; Oliver *et al*., 2009). There is also evidence to suggest that without the APSE phage the uncontrolled proliferation of the *H. defensa* endosymbiont is detrimental to the pea aphid host (Weldon *et al*., 2013), which could explain why the loss of the APSE phage is not more frequently observed in aphid cultures.

The lack of stable *H. defensa* infections in all but the two predominant *M. euphorbiae* genotypes implies a possible incompatibility with certain aphid genotypes. The potential fitness costs and benefits to the different potato aphid hosts of harbouring the endosymbiont bacteria form the focus of later chapters. In *A. pisum*, differential effects of endosymbiont infection on aphid fitness depending on aphid genotypes might contribute to the disparity in endosymbiont distributions amongst aphid populations (Ferrari *et al*., 2007; Simon *et al*., 2011).

The uneven frequency of potato aphid genotypes amongst the clones in this study is not unexpected, with studies of population structures revealing a few common genotypes and several rarer genotypes in a number of aphid species (Sunnucks *et al*., 1997; Fuller *et al*., 1999; Haack *et al*., 2000). UK populations of the peach–potato aphid *Myzus persicae*, for example, are dominated by two insecticide-resistant genotypes and several less

common genotypes, the proportions of which fluctuate temporally both within seasons and between years and with host plant availability around the collection sites (Malloch *et al*., 2006). Further genotypes might have been characterised had the collection of founding *M. euphorbiae* aphids been expanded to include alternative secondary host plant species. Aphids such as the pea aphid and the grain aphid, *Sitobion avenae*, show host plant-specialised populations whose degree of genetic divergence has led to distinct host races (Frantz *et al*., 2006; Sunnucks *et al*., 1997; Peccoud *et al*., 2009). Furthermore, in *A. pisum* there is a strong association between aphids that feed on clover and the presence of the secondary endosymbiont *R. insecticola*, although the effect of the endosymbiont on aphid performance when fed on *Trifolium* plants appears to depend in part on aphid genotype (Simon *et al*., 2003; Leonardo & Muiru, 2003; Leonardo, 2004; Tsuchida *et al*., 2004; Ferrari *et al*., 2004, 2007). In *M. euphorbiae*, the only reported *Rickettsia* infection was found within a clone adapted to overcome the *Mi-1.2* resistance gene of certain tomato cultivars, with proteomic analysis suggesting the endosymbiont may contribute to the virulence of the aphid (Francis *et al*., 2010); screening of *M. euphorbiae* aphids from the wide range of alternative secondary host plants that they can infest may therefore reveal other endosymbiont associations.

The uneven distribution of secondary endosymbiont bacteria amongst potato aphid genotypes, although not statistically significant, warrants further study. Screening of larger numbers of *M. euphorbiae* aphids, such as those collected by the network of suction traps run as part of the Rothamsted Insect Survey, would allow the frequencies of different aphid genotypes to be established, increase the likelihood of discovering novel endosymbiont infections and enable consistent endosymbiont–aphid genotype associations to be detected.

3.4.2 Primary and secondary endosymbiont titres in M. euphorbiae

The *B. aphidicola* genome copy numbers quantified in each potato aphid line relative to the number of *M. euphorbiae* genome copies were approximately an order of magnitude greater than the number of genome copies of *H. defensa* present. As *B. aphidicola* exhibits extreme polyploidy, thought to result from repeated genome duplication without subsequent cell division within the spatial confines of the bacteriocytes, the titres quantified here do not relate directly to bacterial cell number (Komaki & Ishikawa,

1999). However, in both *A. pisum* and *Schizaphis graminum* the number of genome copies of *B. aphidicola* increases as the aphid develops, decreasing again only in post-reproductive aphids (Baumann & Baumann, 1994; Komaki & Ishikawa, 2000; Koga *et al*., 2003); as *B. aphidicola* supplies essential nutrients required by the aphid for growth and reproduction, concomitant changes in genome copy number are likely to ensure that developmental changes in aphid requirements are met. The observation that genome copy numbers of the primary endosymbiont vary in response to changes such as dietary nitrogen availability or sucrose concentration further validates the use of genome copy number as an indication of functional capacity (Douglas *et al*., 2006b; Wilkinson *et al*., 2007). Quantifying either the number of *Buchnera* cells within a bacteriocyte or the number of bacteriocytes within the bacteriome would be less representative of the potential contribution of the primary endosymbiont to aphid nutrition, as both the number of genome copies present within a single *Buchnera* cell and the number of *Buchnera* cells present within a given bacteriocyte are highly variable (Komaki & Ishikawa, 1999; Mira & Moran, 2002).

The densities of *B. aphidicola* did not differ significantly across the *M. euphorbiae* aphid lines and genotypes, contrasting with the results of Vogel and Moran (2011) who demonstrated that *B. aphidicola* titres varied across different *A. pisum* genotypes. The loss of genes involved in regulation of the prokaryote cell cycle from the genome of *B. aphidicola* over the course of a long endosymbiotic history means it is likely that the proliferation of the endosymbiont is controlled to some extent by the aphid host; proposed mechanisms include the regulation of bacteriocyte development and the restriction of precursor metabolites to the bacteria (Shigenobu *et al*., 2000; Braendle *et al*., 2003; Nishikori *et al*., 2009; Macdonald *et al*., 2011). Titres of both the primary and secondary endosymbionts have also been shown to vary in response to the availability of various plant nutrients, with *B. aphidicola* levels differing in black bean aphids reared on different host plants, and increasing in pea aphids as dietary nitrogen levels increase (Wilkinson *et al*., 2001, 2007). The potato aphids from which the bacterial densities were quantified were reared to the same age and developmental stage under identical controlled conditions and on a single variety of potato plant. It is therefore possible that different titres might have been observed in at least some of the potato aphid genotypes if reared on alternative host plants or with changes in the availability of certain nutrients.

The presence of *H. defensa* did not significantly affect *B. aphidicola* titres. Published work in which pea aphid clonal lines have been cured of either *S. symbiotica* or *Rickettsia* suggest that titres of *B. aphidicola* are decreased in the presence of secondary endosymbionts (Koga *et al*., 2003; Sakurai *et al*., 2005), but this was not apparent in the potato aphid lines tested here. However, these earlier studies demonstrated that *B. aphidicola* titres tended towards similar densities in pea aphids with and without secondary endosymbionts as aphids approached the latter stages of their reproductive life. It is therefore possible that differences in *B. aphidicola* density might have been detected had teneral aphids of *M. euphorbiae*, rather than mature reproductive adults, been assessed. It is also possible that the mechanisms governing endosymbiont proliferation are more stringent in *M. euphorbiae* than in *A. pisum*, and titres of the primary endosymbiont are maintained throughout the period over which the aphid host is reproductively active, regardless of other bacteria present. Both the aphid host and *B. aphidicola* consume and supply metabolites to a shared metabolic pool, resulting in a mutual dependence. In contrast, analysis of the *H. defensa* genome indicates that it is dependent on both the aphid host and *B. aphidicola* for the supply of several essential amino acids (Degnan *et al*., 2009b), but contributes little to the nutrition of the symbiotic partners in return (Douglas *et al*., 2006a). Although at this stage of the aphid life cycle the presence of *H. defensa* in *M. euphorbiae* does not appear to suppress genome duplication of *B. aphidicola*, other interactions between the bacteria, such as competition for metabolic precursors, may in turn be detrimental to the growth and development of the aphid host. Measuring intrinsic fitness characteristics of potato aphids both with and without *H. defensa* could therefore reveal any negative effects associated with harbouring the secondary endosymbiont.

Within the *M. euphorbiae* aphids that harbour *H. defensa*, there were no significant differences in the normalised secondary endosymbiont titres between different genotypes or between different aphid lines within genotypes, implying that at this stage of aphid development, the proliferation of *H. defensa* is generally restricted. Titres of *S. symbiotica* and *Rickettsia* in pea aphids, and of the SMLS endosymbiont of the grain aphid *Sitobion miscanthi*, have been shown to increase throughout aphid development, reaching the highest titres in late-reproducing adults when titres of the primary endosymbiont tend to decrease (Koga *et al*., 2003; Sakurai *et al*., 2005; Li *et al*., 2011b). The proliferation of secondary endosymbionts as the aphid host ages indicates developmental relaxation of regulation over secondary endosymbiont titres, as might

reasonably be expected for an evolutionarily recent, facultative symbiotic association. Further study to determine the population dynamics of endosymbiont densities throughout *M. euphorbiae* development would elucidate whether titres of *B. aphidicola* are maximal early in the reproductive phase of the host, as might be predicted due to rapid division of *B. aphidicola* in the embryonic bacteriocytes, to provide the adult aphid with the essential nutrients needed for growth and repair and for embryo development (Humphreys & Douglas, 1997; Wilkinson & Ishikawa, 1999). Low titres of *H. defensa* early in aphid development might therefore result from limited metabolic resources or spatial competition.

The aphid line that showed the highest titres of *H. defensa*, line AA09/03, is the only *M. euphorbiae* clone maintained in culture that harbours the *H. defensa* endosymbiont without the associated APSE bacteriophage. With the techniques currently available it is not possible to manipulate phage presence within the aphid host without also affecting the associated *H. defensa* endosymbiont, limiting the capacity to isolate the effects of APSE on the population dynamics of aphid endosymbionts. Such studies must therefore rely upon the natural loss of the phage from aphids maintained in culture, and may be hindered further as some variants of the phage are purportedly transmitted with higher fidelity than others (Weldon *et al*., 2013). Nevertheless, titres of *H. defensa* increased in sub-lines of an *A. pisum* clone that had naturally lost the APSE phage compared to sub-lines that had retained the phage (Weldon *et al*., 2013), suggesting that the lytic capabilities of the APSE phage contributes to the regulation of *H. defensa* densities in the aphid host. Although the mean titre of *H. defensa* in the APSE-free *M. euphorbiae* line AA09/03 was greater than the three lines harbouring *H. defensa* and APSE, it was not statistically so; again small sample sizes and the subsequent low power of the statistical analysis may have limited the ability to detect a difference. The potential for *H. defensa* infection and the presence or absence of the APSE phage to influence aphid fitness will be examined in Chapter 4.

3.4.3 Summary and conclusions

Of the 19 *M. euphorbiae* lines examined for this study, over half were found to harbour one or more secondary endosymbionts in addition to the obligate endosymbiont *B. aphidicola*, a proportion comparable with frequencies of endosymbiont infection in pea aphid populations. However, the size of the sample screened was very small, and so may

not be truly representative of infection rates in natural populations. Only two known secondary endosymbionts, *H. defensa* and *R. insecticola*, were found within these cultures, and only the *H. defensa* infection persisted for more than six months. Collecting the founding aphids from a single species of host plant might have biased the findings towards particular endosymbiont associations, and other endosymbiont types might also exist in *M. euphorbiae* populations.

Using microsatellites to genotype the cultured *M. euphorbiae* revealed 3 genotypes represented by multiple clonal lines and 4 represented by only single clones. Given that there were several potato aphids collected each summer between 2009 and 2011 that did not survive to found clonal lines, it is unclear whether these well-represented genotypes are the most common in wild potato aphid populations, or whether they are simply better suited to being reared in culture. The *H. defensa* infection was only present within aphid clones from two of the more common cultured genotypes, although there were not enough clonal lines harbouring the endosymbiont to determine if this relationship was significant or merely the result of a small sample size.

Larger scale, unbiased screening would therefore be required in order to identify less common potato aphid endosymbiont infections, to determine the dynamics of aphid genotypic distribution across heterogenic landscapes rather than monocultures of potato crops from year to year and to ascertain any real correlations between endosymbiont infections and aphid host genotypes.

The use of quantitative PCR enables not only the presence of specific bacteria but also their densities to be measured, which in turn can provide information on the dynamics both between different endosymbionts and between each endosymbiont and the aphid host. In contrast to published work in *A. pisum*, in which the presence of *H. defensa* has been shown to suppress primary endosymbiont titres, at the aphid development stage assessed here there were no differences in *B. aphidicola* densities between potato aphids with and without the secondary endosymbiont. The titres of *H. defensa* were approximately an order of magnitude smaller than those of *B. aphidicola* owing to the extreme polyploidy in the latter. Furthermore, there were no significant differences in the titres of *H. defensa* between the four aphid clonal lines representing the two different genotypes that naturally harboured the endosymbiont, suggesting there is a limit to the population growth attainable by the secondary endosymbiont in potato aphids. The higher titres of *H. defensa* observed in the single clonal line from which the APSE

bacteriophage has been lost indicates that the phage may have a role in this growth regulation. Whilst the proliferation of *H. defensa* appears restricted in actively reproducing *M. euphorbiae* aphids, their presence could still adversely or beneficially affect the fitness of their aphid host, either through competition with or inhibition of the functional role of *B. aphidicola* or through some other mechanism. Investigations into the performance of the different potato aphid lines, particularly comparisons between clonal lines of the same genotype that differ only in their endosymbiont status, should show the extent of such effects of harbouring *H. defensa*, and may help to explain the observed distribution of the endosymbiont in the wider potato aphid population.

Chapter 4: Manipulation of the endosymbiont community to explore the effects of secondary endosymbionts on aphid fitness

4.1 Introduction

4.1.1 Manipulating the insect endosymbiont community

There are numerous examples of arthropods forming associations of varying intimacy with bacterial symbionts (Moran *et al*., 2008; Wernegreen, 2012), and consequently techniques have been developed to manipulate bacterial complements and investigate the effects on their host. As cyclical parthenogens, aphids are particularly amenable to this approach, using experimental manipulation to produce genetically identical clonal sub-lines differing only in their endosymbionts, enabling changes in aphid life history characteristics to be attributed solely to the bacteria. Such endosymbionts can be eliminated through the use of selective antibiotics, whilst artificial infections can be generated using the haemolymph of naturally infected aphids; both treatments are commonly administered either through artificial diets or through microinjection. Other insect curing methods, such as heat treatment (Gotoh *et al*., 2007) are less suitable for eliminating aphid secondary endosymbionts due to the necessity of maintaining the obligate *B. aphidicola* infection (Ohtaka & Ishikawa, 1991).

4.1.2 Elimination of bacterial endosymbionts

4.1.2.1 Selective antibiotics

The specific antibiotics used to cure aphids depend on whether the target bacterium is the primary or a secondary endosymbiont. Chlortetracycline and rifampicin are most commonly used to eliminate the primary endosymbiont *B. aphidicola*, both of which leave secondary endosymbionts intact (Koga *et al*., 2003, 2007; Hardie & Leckstein, 2007). Although the exact cause of this specificity is unknown, the outer cell membrane of *B. aphidicola* is fragile as a result of dramatic genome reduction and so is unlikely to act as a barrier to certain antibiotics in the same way as that of secondary endosymbionts such as *S. symbiotica*, which has a thick outer membrane (Koga *et al*., 2003, 2007).

Ampicillin, cefotaxime and gentamicin have been used successfully in various combinations to cure aphids selectively of their secondary endosymbionts (Douglas *et al*., 2006a; Chandler *et al*., 2008). The presence of cell walls in secondary symbionts such as *S. symbiotica*, *R. insecticola* and *Rickettsia* renders them targetable by ampicillin and cefotaxime, both beta-lactam antibiotics that inhibit cell wall synthesis (Koga *et al*., 2007). Gentamicin, an aminoglycoside that binds to the bacterial ribosome, may also selectively target secondary endosymbionts as the *Buchnera* genome includes genes encoding acetyl-transferases that, in free-living bacteria, modify the antibiotic (Poole, 2005). Furthermore, the fact that secondary facultative endosymbionts tend to be localised in sheath cells and in the haemolymph may increase their exposure to antibiotics (Koga *et al*., 2007).

4.1.2.2 Elimination of endosymbionts through artificial diets

Artificial diets have been used to study the physiology of aphids from as early as the 1930s; initially based on crude plant extracts (Hamilton, 1935), these were developed further in the 1960s to produce diets entirely composed of laboratory chemicals on which aphids could be raised for several generations (Akey & Beck, 1972). These diets do not exactly reflect the composition of the plant phloem sap on which the aphids normally feed, with attempts to replicate phloem constituents in artificial diets failing to achieve the long-term survival of aphid cultures (e.g. Prosser & Douglas, 1992; Liadouze *et al*., 1995). Such holidic, chemically-defined diets, in which individual components can be omitted or altered in concentration, have enabled researchers to assess the significance of separate amino acids, sugars, vitamins and minerals for aphid growth. By eliminating the primary symbiont *B. aphidicola* with antibiotics added to the diet, the contributions of symbiotic bacteria to the nutritional needs of the aphid host have also been deduced (Akey & Beck, 1972; Liadouze *et al*., 1995; Wilkinson & Douglas, 1995; Douglas *et al*., 2001; Wilkinson *et al*., 2001; Douglas *et al*., 2006b; Gündüz & Douglas, 2009).

Numerous formulae for holidic diets have been published, many of which are modifications of the diets published by Akey and Beck in 1971 and 1972, for example to reflect the amino acid profile of aphid carcasses (Febvay *et al*., 1988). The 'Formulation A' diet (Prosser & Douglas, 1992), consisting of a modified amino acid composition of the Akey and Beck diet and the non-amino acid components of 'diet a' (Kunkel, 1976,

originally from Mittler & Koski, 1976), has been used in several recent studies, including to administer antibiotics (e.g. Wilkinson & Douglas, 1995; Adams & Douglas, 1997; Douglas *et al*., 2001; Gündüz & Douglas, 2009).

Different constructs such as refillable glass chambers (Akey & Beck, 1971) and continuous flow systems (Harrewijn, 1973) have been used to administer chemically-defined diets. The most common technique used, however, involves enclosing a small volume of the holidic diet solution between two squares of Parafilm or Nescofilm stretched over a Perspex cylinder, with the aphids piercing the underside of the sachet to feed (Mittler & Dadd, 1964; Cloutier & Mackauer, 1975).

4.1.2.3 Elimination of endosymbionts through treated plant material

When the exact dietary composition does not need to be controlled, an alternative means of administering antibiotics orally is to immerse the roots of a suitable host plant into a solution of the selected antibiotic, and transferring aphids on to the plant to feed. Aposymbiotic aphids have been generated in this way using rifampicin (Miao *et al*., 2003, 2004; Douglas, 1992, 1996; Cheng *et al*., 2011), and *S. symbiotica*, *H. defensa* and *R. insecticola* have all been eliminated from pea aphids using a combination of ampicillin, cefotaxime and gentamicin (McLean *et al*., 2010).

4.1.2.4 Elimination of endosymbionts through microinjection

Endosymbiont bacteria can also be eliminated by injecting antibiotics directly into the aphid host. Aphids are first restrained, typically by being held on to the end of a pipette tip with a vacuum (Oliver *et al*., 2003; Russell & Moran, 2005), and antibiotic solution is then injected into the abdomen at the base of the mid or hind leg using a glass capillary tube pulled into a fine point (e.g. see Chen & Purcell, 1997; Leonardo, 2004; Koga *et al*., 2007). Microinjection of rifampicin has been used successfully to eliminate *B. aphidicola* from various aphid species (Nakabachi *et al*., 2003; Koga *et al*., 2003, 2007), whilst injections of ampicillin, gentamycin and cefotaxime have effectively eliminated secondary endosymbionts from pea aphids (e.g. see Koga *et al*., 2003, 2007; Tsuchida *et al*., 2010; Simon *et al*., 2011).

4.1.2.5 Timing of antibiotic administration and attenuation of treatment effects

As aphids from which the primary symbiont has been eliminated are generally either sterile or produce sterile offspring, aposymbiotic nymphs are commonly generated from antibiotic-treated adults, maximising the time available for treated aphids to be used in experimental work (Wilkinson & Douglas, 1995; Adams & Douglas, 1997; Douglas *et al*., 2001; Wilkinson *et al*., 2001). When secondary endosymbionts are selectively eliminated, the treated aphids continue to produce viable offspring due to the presence of *B. aphidicola*. It is therefore possible to use later generations of aphids in experiments. Exposing young nymphs to the antibiotic treatment increases the likelihood that the developing embryos of their offspring are cured.

To ensure that any differences observed between infected and uninfected aphids result from the presence of secondary bacteria rather than from the antibiotics or the method of administration, cured aphid sub-lines are usually maintained for at least 10 generations before any fitness comparisons are conducted (Koga *et al*., 2003, 2007; Sakurai *et al*., 2005; Douglas *et al*., 2006a; Chandler *et al*., 2008). Furthermore, molecular techniques such as the amplification of polymorphic microsatellites or SSRs (simple sequence repeats) or of inter-sequence simple repeats (ISSRs) are often employed to enable the different aphid clonal lines to be identified and ensure the fidelity of manipulated sub-lines (Leonardo, 2004; Scarborough *et al*., 2005; Oliver *et al*., 2005, 2006; Russell & Moran, 2006).

4.1.3 Establishing secondary endosymbiont infections in uninfected aphid lines

Although not attempted as part of this study, the artificial introduction of heritable secondary endosymbionts enables the effects of these bacteria on novel host genotypes to be determined, which can complement curing experiments. Also referred to as transfection, such endosymbiont infections are achieved by introducing bacteria from infected donor aphids into secondary symbiont-free recipient aphids, again through diet preparations or microinjection. The success of both these methods of transfection in published works gives credence to two current hypotheses regarding possible means of secondary symbiont acquisition by aphids in the field: through feeding, with symbiotic bacteria taken up from the surface of the plants contaminated with infected honeydew, or

through aborted attacks by parasitoid wasps that have previously oviposited in aphids harbouring the bacteria (Darby *et al*., 2001; Darby & Douglas, 2003).

Whilst none of the aphid symbionts identified thus far have been successfully grown freely in culture, Harrison (1989) developed a method for crudely isolating symbiotic bacteria from aphid tissue. Although the symbiont yield is low, it is sufficient to achieve oral transfection when added to an artificial diet (Darby & Douglas, 2003; Chandler *et al*., 2008). Alternatively, haemolymph from donor aphids extracted using a glass needle or by removing a leg and collecting the exudate contains sufficiently high titres of secondary endosymbiont bacteria to alter the symbiont status of a recipient aphid when injected into the abdomen (Chen & Purcell, 1997; Fukatsu *et al*., 2000, 2001; Oliver *et al*., 2003; Tsuchida *et al*., 2005; Vorburger *et al*., 2009).

The potential costs of harbouring secondary endosymbiont bacteria are generally not severe enough to prevent infected aphid lines from being maintained in culture. Again, for treatment effects to attenuate and to allow the endosymbionts to reach densities and levels of organisation seen in natural infections, aphid lines are usually maintained for several generations before being used in experiments (Chen *et al*., 2000; Koga *et al*., 2003; Oliver *et al*., 2003; Tsuchida *et al*., 2004; Russell & Moran, 2006; Chandler *et al*., 2008). Furthermore, in addition to diagnostic PCR to confirm the presence or absence of a given secondary symbiont, techniques such as haemocytometer counts, *in situ* hybridisation and quantitative PCR have been used to ensure the inoculated bacterial symbionts are present within the aphids in similar tissue locations and at comparable densities as naturally infected aphid hosts (Oliver *et al*., 2003; Scarborough *et al*., 2005; Ferrari *et al*., 2007; Chandler *et al*., 2008).

4.1.4 Effects of secondary endosymbionts on aphid fitness

4.1.4.1 Measuring aphid fitness

Natural selection favours those genotypes that survive and produce the greatest number of surviving offspring, and so measures of fitness quantify those traits that directly or indirectly affect survival and fecundity. Fitness, defined as the contribution made by an *average* individual of a specified genotype to the gene pool of future generations, thus

incorporates the variations that result from the assorted phenotypes expressed as a consequence of extrinsic factors. In asexually reproducing organisms such as obligatory parthenogenetic aphids, there is very little genetic variation between clonal individuals (although see Wilson *et al*., 2003; Monti *et al*., 2012). Instead of acting on allelic variants of genes that affect an organism's fitness, natural selection therefore acts on the entire clonal genotype. As a result, aphid population structure often largely consists of a small number of clonal lineages best suited to the current environment, but which may be replaced rapidly as conditions change and rare clones come to dominate (Vorburger *et al*., 2003).

Aphids show considerable phenotypic plasticity in their reproductive and wing morphology, both of which affect individual fitness. In holocyclic aphid populations sexual morphs are produced in response to shortening day length (Trionnaire *et al*., 2008), and so the fitness of cyclically parthenogenetic clones, in terms of fecundity, relates to the number of overwintering eggs produced. In contrast, fecundity of obligatory asexually reproducing populations is measured in terms of nymph production. Within asexually reproducing aphid lines, alate morphs are frequently produced in response to high aphid density, induced by tactile stimulation, or by poor host plant quality (Müller *et al*., 2001; Braendle *et al*., 2006), although the maternal and grand-maternal phenotypes may also affect the propensity to produce winged forms (MacKay & Wellington, 1976). Alate aphids that disperse to new feeding sites can fail to find suitable host plants, and tend to have lower fecundity or delayed reproduction as a result of a trade-off between reproductive output and wing muscle production (Zera & Denno, 1997; Ishikawa & Muira, 2009). Consequently, the fitness of an aphid clonal line in part depends on the relative investment in alate and apterous forms in response to appropriate cues (Weisser & Stadler, 1994). Ideally therefore, to understand the prevalence of clonal genotypes it is aphid clonal fitness that should be measured, taking into account winged morph production and dispersal and the reproductive contribution of every member of the clone from a single foundress (Hodgson, 2001). In reality, clonal fitness is extrapolated from the fitness of individual aphids within a single generation, based on parameters such as relative growth rate, development time, age at first reproduction, total offspring production, longevity and intrinsic rate of increase.

4.1.4.2 Known effects on aphid fitness of harbouring secondary endosymbionts

The effects of secondary endosymbionts may further add to phenotypic differences exhibited by aphids with the same genotypic background. As a consequence of the fidelity with which they are transmitted from one generation to the next, endosymbiont genomes contribute to the heritable genetic variation of their host. Therefore, in order to persist and spread, facultative endosymbionts must affect their aphid hosts in a manner that increases their frequency, either by improving their fitness or through reproductive manipulation. Given that the costs to aphids of harbouring endosymbiont bacteria are likely to be greater than would be the case for carrying extra nuclear genes conferring the same benefits, one possible reason why secondary endosymbionts have not reached fixation in many aphid populations is that such costs may have led to only the most tolerant of aphid genotypes supporting stable infections (Ferrari *et al*., 2007; Koga *et al*., 2007; McLean *et al*., 2010).

Various studies have investigated the impact of harbouring one or more secondary endosymbionts on aphid fitness, in part by comparison of the performance of clonal sub-lines in which the endosymbiont complements have been manipulated through curing and transfection techniques. Experiments with pea aphid sub-lines have shown that, under standard rearing conditions, *S. symbiotica*, *H. defensa*, *R. insecticola*, *Rickettsia* and *Spiroplasma* can have negative effects on aspects of aphid fitness such as fecundity, longevity and adult body weight (Fukatsu *et al*., 2001; Sakurai *et al*., 2005; Simon *et al*., 2007, 2011; Koga *et al*., 2003, 2007). In other studies, however, the same endosymbionts do not appear to influence pea aphid growth rate or development time, and in some aphid genotypes the presence of *H. defensa* has been shown to increase host fecundity and shorten generation time (Russell & Moran, 2005; Oliver *et al*., 2008; Vorburger *et al*., 2009). The endosymbiont strain can also influence the effect on host fitness; different strains of *H. defensa* consistently increases or reduce fecundity in *S. avenae*, regardless of host genotype (Łukasik *et al*., 2013b).

The effects of secondary endosymbionts on aphid fitness under laboratory conditions are therefore not straightforward, and such measurements of life history traits offer only a snapshot perspective of how aphid clones perform over a single generation under a limited set of environmental conditions. Nevertheless, the relative performance of different aphid clones harbouring either natural bacterial complements or artificially

manipulated endosymbiont infections may still give some insight to secondary endosymbiont distributions in aphid populations.

4.1.5 Study objectives and hypotheses

The objectives of the study presented in this chapter are:

1. To generate cured sub-lines of *M. euphorbiae* clones naturally infected with *H. defensa* through the administration of antibiotics selective in their action. Clones of *M. euphorbiae* that were genetically identical but that varied only in their endosymbiont complement could then be used to quantify various aspects of aphid fitness, with any differences attributable to the secondary endosymbiont infection.

2. To quantify titres of *B. aphidicola* in aphid lines with a common genetic background differing only in their secondary endosymbiont complement. This allowed the hypothesis to be tested that the presence of *H. defensa* may inhibit the proliferation of *B. aphidicola* , whilst controlling for possible differences in *B. aphidicola* titres steming from the genetic backgrounds of the aphid hosts.

3. To determine whether innate fitness traits differ between *M. euphorbiae* genotypes and clonal lines varying in their endosymbiont complement. I tested the null hypothesis that development, fecundity and survival of *M. euphorbiae* do not vary with aphid genetic background and *H. defensa* infection under a defined set of conditions.

4.2 Materials and methods

4.2.1 Administration of antibiotics through artificial holidic diets

4.2.1.1 Assembly of artificial diets

A variation of the Prosser and Douglas (1992) 'Formulation A' diet was used to administer antibiotics to selected clonal lines of *M. euphorbiae* kept in culture. The composition of the diet and the volumes and concentrations used are shown in Table 4.1.

Table 4.1: The composition of the standard diet used to administer antibiotics to *M. euphorbiae* nymphs, modified from Prosser & Douglas (1992)

	Amino acid	mol %	Molecular weight	mM in a total of 150 mM	mg in 10 mL	mg required to make 50 mL
Non-essential	Alanine	3.8	89.09	5.70	5.07	50.78
	Asparagine	9.5	132.12	14.25	18.82	188.27
	Aspartic acid	9.5	133.10	14.25	18.96	189.67
	Cysteine	1.8	121.16	2.70	3.27	32.71
	Glutamic acid	5.6	147.13	8.40	12.35	123.59
	Glutamine	11.0	146.15	16.50	24.11	241.15
	Glycine	0.8	75.07	1.20	0.90	9.01
	Proline	3.8	115.13	5.70	6.56	65.62
	Serine	3.8	105.09	5.70	5.99	59.90
	Tyrosine	0.4	181.19	0.60	1.08	10.87
Essential	Arginine	9.5	210.67	14.25	30.02	300.20
	Histidine	5.8	209.63	8.70	18.23	182.38
	Isoleucine	5.8	131.20	8.70	11.41	114.14
	Leucine	5.8	131.18	8.70	11.41	114.13
	Lysine	5.8	182.65	8.70	15.89	158.91
	Methionine	1.9	149.21	2.85	4.25	42.52
	Phenylalanine	1.9	165.19	2.85	4.70	47.08
	Threonine	5.8	119.12	8.70	10.36	103.63
	Tryptophan	1.9	204.23	2.85	5.82	58.21
	Valine	5.8	117.15	2.70	10.19	101.92

Mineral	Molecular weight	mg in 10 mL	mg in 10 mL of 100× stock
$FeCl_3.6H_2O$	270.3	0.11	11.0
$CuCl_2.2H_2O$	170.48	0.01	1.0
$MnSO_4.H_2O$	169.02	0.02	3.0
$ZnSO_4.7H_2O$	287.55	0.17	17.0

Vitamin	Molecular weight	mg in 10 mL	mg in 10 mL of 20× stock
Biotin	244.31	0.01	0.20
Pantothenate-Ca	238.30	0.50	10.00
Folic acid	441.40	0.20	4.00
Nicotinic acid	123.11	1.00	20.00
Pyridoxine hydrochloride	205.64	0.25	5.00
Thiamine hydrochloride	337.27	0.25	5.00
Choline chloride	139.63	5.00	100.00
Myo-Inositol	180.16	5.00	100.00

Sucrose solution	Molecular weight	mg in 10 mL
Ascorbic acid	176.12	10.0
Citric acid	210.10	1.0
$MgSO_4$	120.37	6.0
Sucrose	342.3	1700.0

Other	Molecular weight	mg in 10 mL
$K_2HPO_4.3H_2O$	228.23	150.0

The amino acid stock solution was prepared in 50 mL distilled de-ionised water, and stored at -20°C in 5 mL aliquots. The vitamin and mineral solutions were prepared in 10 mL distilled de-ionised water, and stored at -20°C in 0.1 mL and 0.5 mL aliquots, respectively. The sucrose and potassium phosphate solutions were prepared fresh in 3 mL and 1 mL distilled de-ionised water, respectively. To assemble the dietary components, defrosted aliquots of the mineral and vitamin stock solutions were added to an aliquot of the amino acid solution, followed by the fresh sucrose solution. After mixing the solutions by inversion the phosphate solution was added, the solutions mixed again and the pH confirmed to be between pH 6.8 and 7.5 using pH indicator paper. After the relevant volumes of antibiotics had been added, the completed solution was filter sterilised into a fresh sterile 10 mL tube before being assembled into diet sachets.

In total six antibiotic curing trials by oral administration were conducted using a combination of ampicillin, gentamicin and cefotaxime, the concentrations of which varied based on the results of the previous trial. A further two control trials were conducted using the same diet both with and without antibiotics, fed to *M. euphorbiae* aphids from a clonal line free from secondary endosymbionts (Table 4.2).

4.2.1.2 Administration of antibiotics in artificial diets

Diet sachets were created by dispensing 100 µL of diet solution on to the surface of a square of Nescofilm (3 cm × 3 cm) stretched over a Perspex ring (internal diameter approx. 20 mm), and sealed with a second Nescofilm square. Several feeding chambers were assembled for each curing trial, with two aphids transferred to the underside of each diet sachet. The chambers were placed in a ventilated tissue-lined plastic container along with a small beaker of saturated NaCl solution to maintain the chamber humidity at 70–75%, and kept in a growth cabinet at 20°C ± 1°C, 60% humidity and with a light regime of 16h light: 8h dark.

For each diet trial, between six and twelve nymphs (designated F_0) of known age and produced by single adult apterous *M. euphorbiae* aphids (designated F_{-1}) were fed on the holidic diet for a 12 hour period. The F_{-1} adult aphids were frozen at -20°C for subsequent molecular analysis of their bacterial endosymbiont complement.

When the F_0 nymphs were two or three days old, they were transferred to the feeding chambers and left to feed for between three and eight days (see Figure 4.1, Table 4.2). Diet sachets were regularly checked and were replaced if they showed signs of discoloration (indicating oxidation of diet components) or bacterial growth. At the end of the feeding period the F_0 aphids were transferred to individual culture cups maintained in a controlled environment room at 18°C ± 2°C, 60% humidity and with 16 h light: 8 h dark.

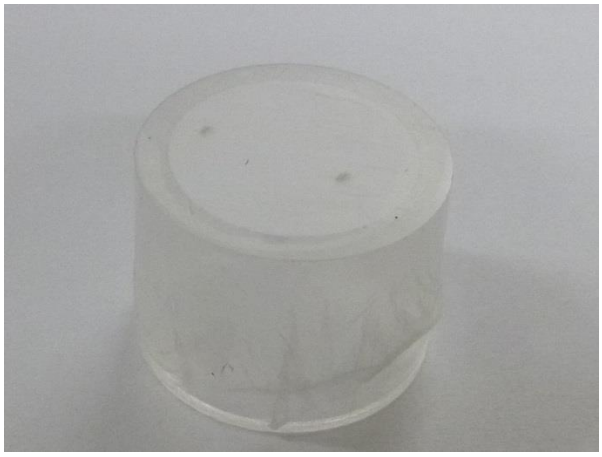

Figure 4.1: Artificial diet sachet and feeding chamber, with two *M. euphorbiae* nymphs feeding from the underside of the diet sachet.

The initial offspring produced in the first 5 days of adulthood by each F_0 aphid were discarded. Subsequently, F_1 offspring were collected in 3-4 successive cohorts by transferring the F_0 aphids to new culture cups every 2–4 days. After collecting the final cohort of F_1 offspring, the F_0 aphids were frozen at -20°C for subsequent molecular analysis of their bacterial endosymbiont complement. Each cohort of first generation offspring was reduced to five nymphs, which were allowed to mature and produce second generation (F_2) offspring prior to being frozen for molecular diagnostic screening.

To explore factors contributing to the success of endosymbiont curing, a number of factors were varied: 1) the concentration of antibiotics in the holidic diet; 2) the age at which the F_0 nymphs were transferred to the diet; 3) the length of time the F_0 nymphs were allowed to feed from the diet sachets; 4) the time from which the F_1 cohorts of offspring were collected from the treated F_0 aphids; and 5) the length of time over which F_1 cohorts of offspring were collected (Table 4.2).

Curing trial	*M. euphorbiae* clonal line	Secondary endosymbionts present	Age of F_0 nymphs transferred to diet	Length of time F_0 nymphs fed on diet	Conc. of each antibiotic in diet	Day of first F_1 cohort collection	Length of each F_1 cohort collection	No. of F_1 cohorts collected
1	AA09/04	*H. defensa* (+ APSE)	3 days	3 days	25 µg mL^{-1}	6 days after adulthood	3 days	4
2	AA09/04	*H. defensa* (+ APSE)	3 days	3 days	50 µg mL^{-1}	6 days after adulthood	3 days	3
3	AA09/04	*H. defensa* (+ APSE)	2 days	6 days	50 µg mL^{-1}	6 days after adulthood	3 days	3
4	AA09/03	*H. defensa* (- APSE)	2 days	8 days	50 µg mL^{-1}	14 days after removal from diet	2 days	3
	AA09/06	*H. defensa* (+ APSE)						
5	HC10/02	*H. defensa* (+ APSE)	2 days	6 days	50 µg mL^{-1}	10 days after removal from diet	3 days	3
	HC10/08	*R. insecticola*						
6	AA09/06	*H. defensa* (+ APSE)	2 days	6 days	100 µg mL^{-1}	7 days after removal from diet	3 – 4 days	4
	HC10/05	*H. defensa* (+ APSE)						
7	AA09/12	None	2 days	6 days	50 µg mL^{-1}	6 days after adulthood	3 days	3
8	AA09/12	None	2 days	6 days	None	6 days after adulthood	3 days	3

Table 4.2: *M. euphorbiae* clonal lines used, antibiotic dosages and the timescales of the eight artificial diet curing trials

4.2.1.3 Screening of treated aphids, attenuation of treatment effects and confirmation of clonal integrity

Genomic DNA was extracted from both the treated aphids (generation F_0) and the cohorts of F_1 offspring they produced (see section *2.2.1*). Diagnostic PCR analysis was then used to determine whether the secondary symbiont had been eliminated and at what stage (section *2.2.2*). Diagnostic PCR was also used to confirm that the endosymbiont infection was present and stable in the stock aphid cultures using DNA extracted from the F_{-1} aphids that generated the nymphs fed on the diet. Successfully cured sub-lines were maintained in culture, with five nymphs from each of the first ten generations following the antibiotic treatment collected and frozen to enable confirmation of the elimination of the endosymbiont.

The aphids that generated the nymphs for the diets (generation F_{-1}), the aphids fed on the diets supplemented with antibiotics (generation F_0), five aphids from each of the cured cohorts of offspring produced by the treated aphids (generation F_1) and five aphids from each of the fifth and tenth generations of offspring (F_5 and F_{10}) from the successful curing trials were genotyped at seven microsatellite loci and the results determined by capillary electrophoresis (see section *2.2.4*). This enabled the integrity of the sub-clonal lines to be verified, ensuring that the cultures had not become contaminated with aphids from other *M. euphorbiae* clonal lines that lacked secondary endosymbionts.

4.2.2 Administration of antibiotics through microinjection

Preliminary trials were first conducted into the microinjection process, as a combination of needle size and shape, the volume of solution being injected and the injection process itself were thought likely to contribute to the survival rate of the treated *M. euphorbiae* aphids.

To create needles, glass capillary tubes (length 89 mm, internal diameter 0.5 mm) were heated and pulled using a needle puller (Narishige PN-3, Narishige International Ltd, London). A conical needle shape that narrowed uniformly and was not too elongated was generated using the settings given in Table 4.3.

Variable	Setting
Heater	5.0
Main magnet	5.0
Sub magnet	9.0
Micro-switch	25mm

Table 4.3: Needle puller settings used to create fine glass needles for microinjection of saline and antibiotic solutions

The Nanoliter 2000 microinjector (World Precision Instruments, Hitchin, UK) used to inject the aphids could dispense set volumes of solution between 2.3 nL and 69.0 nL. A range of volumes of insect saline solution (containing 9 g NaCl, 0.2 g KCl, 0.27 g $CaCl_2 \cdot 2H_20$, 4 g glucose and < 5 g $NaHCO_3$ per litre of solution; see Fox, 2001) were injected into test aphids to determine the appropriate injection volume. An aliquot of 50.6 nL was deemed suitable as the aphid abdomen visibly swelled as the solution was administered. Fluid seeped from the puncture wound when larger volumes of saline were injected, whilst smaller volumes would have limited the amount of antibiotic dispensed.

Ten fourth instar nymphs from the *M. euphorbiae* clonal line AA09/04 harbouring *H. defensa* were injected with ampicillin solution (20 mg mL^{-1}) and eight nymphs were injected with insect saline solution as controls. Each aphid was positioned ventral side uppermost on the apex of a cut pipette tip covered with gauze, and held in place through the suction generated by a low-powered vacuum pump (Figure 4.2). The glass needle was backfilled with inert oil and either the antibiotic or insect saline solution, then positioned by means of a micromanipulator at the base of the second leg of the aphid. When a small amount of haemolymph seeped from the puncture site the needle was deemed to have successfully pierced the abdomen, and the set volume of 50.6 nL of solution expelled using the microinjector controls.

The initial offspring produced in the first three days by each treated aphid were discarded, and then subsequent offspring were collected in cohorts every three days for 12 days. The treated aphids were collected and frozen at -20°C for subsequent molecular analysis of their bacterial endosymbiont complement. The aphids in each cohort of first generation offspring were allowed to mature and produce second generation offspring before they too were collected and frozen, ready for molecular diagnostic screening.

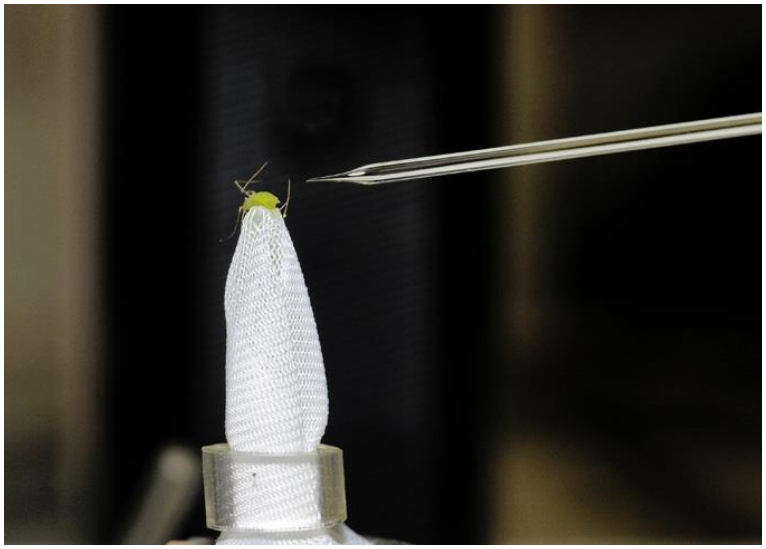

Figure 4.2: *M. euphorbiae* 4th instar nymph prepared for microinjection, held by a low-powered vacuum pump

As with the artificial diet curing trials, DNA was extracted from both the injected aphids and from the cohorts of offspring they produced (see section *2.2.1*). Diagnostic PCR analysis was then used to determine whether the secondary symbiont had been eliminated (section *2.2.2*), with any successfully cured sub-lines maintained in culture.

4.2.3 Quantifying titres of endosymbiont bacteria in artificially cured M. euphorbiae *aphids using quantitative PCR*

Quantitative PCR assays were used to determine whether the process of curing *M. euphorbiae* aphids of *H. defensa* affected titres of *B. aphidicola*, despite rearing the aphids for several generations to allow the effects of the antibiotics to attenuate. Table 4.4 shows the treated *M. euphorbiae* sub-lines and the parental clonal lines used.

Four aphids from each of the treated sub-lines and the original parent lines were reared and collected aged 16 days (section *3.2.3.3*). Genomic DNA was extracted from the *M. euphorbiae* aphids to form the templates from which to measure copy numbers of the *RpL7* gene of *M. euphorbiae* and the *groEL* gene of *B. aphidicola* (section *2.2.1*). Serial dilutions of linearized plasmids containing these genes were used to create standard curves from which the number of gene copies present in each aphid DNA template could be calculated (section *2.2.3.1*), and the qPCR reactions prepared and the thermocycling conditions set as given in section *2.2.3.2*. The absolute *B. aphidicola* titre from each aphid sample was normalised by dividing by the copy number of the *M. euphorbiae*

RpL7 gene, and the normalised titres from each *M. euphorbiae* clonal line and sub-line and each treatment group were compared using univariate GLMs (section *3.2.3.6*).

***M. euphorbiae* clonal sub-line**	**Endosymbiont status**	**Treatment**
AA09/03	*H. defensa*	Stock culture
AA09/03^{ab}4.1b	Cured of *H. defensa*	Artificial diet + antibiotics
AA09/04	*H. defensa* (+ APSE)	Stock culture
AA09/04^{ab}3.8c	Cured of *H. defensa* (+ APSE)	Artificial diet + antibiotics
AA09/12	None found	Stock culture
AA09/12^{ab}7.1c	None found	Artificial diet + antibiotics
AA09/12^{ab}7.4c	None found	Artificial diet + antibiotics
AA09/12do8.1c	None found	Artificial diet only
AA09/12nd8.0c	None found	Stock culture

Table 4.4: Endosymbiont status and treatment of *M. euphorbiae* clonal and treated sub-lines used in quantitative PCR assays from comparisons of *B. aphidicola* titres.

4.2.4 Aphid fitness assays

4.2.4.1 Measuring M. euphorbiae *clonal and genotypic fitness in a glasshouse environment*

Initial pilot trials determined that controlled environment rooms were not suitable for performance assays, principally because the quality of the plant material was inadequate when grown under artificial light. Performance assays were therefore conducted as detailed in section *2.3.1* in a glasshouse with supplementary lighting, using aphids from ten *M. euphorbiae* clonal lines selected to represent different secondary endosymbiont complements and a range of aphid genotypes, and two of the sub-lines cured of the *H. defensa* endosymbiont (Table 4.5, Figure 4.3).

Aphid line	Genotype	Secondary endosymbionts present
AA09/03	1	*H. defensa*
AA09/04	1	*H. defensa* (+ APSE)
AA09/12	1	None found
AA09/03^{ab}4.1b	1	Cured of *H. defensa*
AA09/04^{ab}3.8c	1	Cured of *H. defensa* (+ APSE)
HC10/02	2	*H. defensa* (+ APSE)
HC10/05	2	*H. defensa* (+ APSE)
AK11/01	3	None found
AA09/02	4	None found
AA09/13	5	None found
HC10/07	6	None found
HC10/08	7	None found

Table 4.5: Genotype and endosymbiont status of the 12 aphid clonal lines and sub-lines used to characterise genotypic differences and the effects of *H. defensa* on *M. euphorbiae* fitness.

4.2.4.2 Analysis of M. euphorbiae *fitness data*

Analyses of the life history characteristics of ten different *M. euphorbiae* clonal lines were performed using SPSS (v. 21, IBM), with graphs plotted in SigmaPlot (v. 12.3, Systat Software). In addition to the time to adulthood and the length of the pre-reproductive period, the intrinsic rate of population increase (R_m) for each aphid was calculated using the formula given by Wyatt and White (1977):

Equation 4) $$R_m = 0.738(\ln M_d) / T$$

Where T is the pre-reproductive period and M_d is the number of offspring produced in the time equivalent to the pre-reproductive period. Aphids that died before the end of the observation period were included in the R_m analysis, whilst those few that escaped from the cages before the end of the observation period were excluded.

Figure 4.3: *M. euphorbiae* performance assay in a controlled glasshouse environment

The effects of aphid genotype and of endosymbiont complement on time to adulthood, length of pre-reproductive period and R_m were tested separately using univariate general linear models (GLMs), followed by Sidak multiple pairwise comparison post-hoc tests to isolate differences amongst the means. The wing morph of each aphid, the temporal trial number and the positional block number were also included as factors, whilst aphid clonal line was nested within genotype or endosymbiont group. The statistical power of each test, scored in terms of the type II error rate (1-β), was above the arbitrarily accepted threshold of 0.8 unless otherwise stated.

Given that only adult aphids can produce offspring, there is likely to be a positive relationship between the time to adulthood and the length of the pre-reproductive period. A multivariate GLM was therefore used to investigate the effects of aphid genotype and endosymbiont status on the three fitness traits, independent of associations between time to adulthood, length of pre-reproductive period and intrinsic rate of population increase. Survivorship plots were constructed for the original ten *M. euphorbiae* clones using the Kaplan-Meier estimate of survivor function (Kaplan & Meier, 1958), which shows the probability of an individual surviving longer than time *t*. This method takes into account the removal of individual aphids from the observations as each reached a time equal to that of their pre-reproductive period, with such right-censored data points marked on the plots using circular symbols. Aphids that were unaccounted for by the end of the

observation period were excluded from the survivorship analysis. Log-rank, Wilcoxon-Breslow and Tarone-Ware non-parametric tests were then conducted to compare survival distributions and determine whether significant differences in survival existed between the ten aphid lines.

Both a parametric model based on a Weibull distribution and a semi-parametric Cox proportional hazards (PH) model were fitted to the survival data in an effort to describe and quantify the effects of genotype, wing morph and secondary endosymbiont presence on the instantaneous hazard rate h(t), i.e. the instantaneous rate of mortality. Both models have been used in the survival analysis of aphids in published literature (Vorburger & Gouskov, 2011; Bernardi *et al*., 2012), but neither was wholly appropriate for these data as the log-log of the cumulative survival function S(t) plotted as a function of log time yielded neither a linear relationship nor parallel gradients, indicating that the observed data did not fully meet the assumptions of either a Weibull distribution or a proportional hazards model. Nevertheless, both models were fitted using the software programme **R** (v. 2.14.0, www.R-project.org), with analyses of variance (ANOVA) used to assess the significance of each factor to the models. Akaike Information Criterion (AIC and AICc) scores and weights were used to assess the relative goodness of fits of models with various combinations of the factors genotype, wing morph and trial included.

The analyses described above were also used to test for differences in time to adulthood, time to first reproduction, intrinsic rate of increase and survival between five of the *M. euphorbiae* lines from genotype 1 that differed in their secondary endosymbiont status; lines AA09/04 and AA09/03 that naturally harboured *H. defensa* with and without APSE, respectively, line AA09/12 that was naturally free of secondary endosymbionts and lines AA09/03^{ab}4.1b and AA09/04^{ab}3.8c, both of which had been cured of their *H. defensa* infection.

4.3 Results

4.3.1 Curing through artificial diets

4.3.1.1 Curing M. euphorbiae *lines of naturally occurring secondary endosymbiont infections*

Of the six artificial diet curing trials in which ampicillin, gentamicin and cefotaxime were administered to *M. euphorbiae* aphids harbouring either *H. defensa* or *R. insecticola*, only the third and fourth trials generated successfully cured sub-lines. As a result of the continuing presence of the *H. defensa* endosymbiont in both the treated potato aphids and in the first generation offspring from the first and second trials, the concentration of each antibiotic within the holidic diet and the length of time the aphids were left to feed on the diets were increased for the second and third curing trials, respectively (Table 4.2).

Diagnostic PCR indicated that bacteria were present within all of the surviving F_1 AA09/04 *M. euphorbiae* aphids from the third artificial diet curing trial, shown by bacterial 16S rRNA gene product (Figure 4.4). However, the aphids from the second cohort of offspring from the twelfth F_0 aphid (3.12b) and from the third cohorts of offspring from the second, eighth, eleventh and twelfth F_0 aphids (3.2c, 3.8c, 3.11c and 3.12c) failed to generate products for either the bacterial 16S-23S rRNA PCR screen or the *H. defensa* screen, indicating that only the *B. aphidicola* bacterial endosymbiont was present in significant numbers and that the secondary endosymbiont had been eliminated. Given that the F_{-1} and F_0 aphids from this curing trial were positive for *H. defensa* when screened, the administration of antibiotics appears to have either sufficiently reduced the endosymbiont titres of the treated aphids to prevent *H. defensa* from being transmitted to the developing embryos, or else eliminated *H. defensa* from the embryos before it became established.

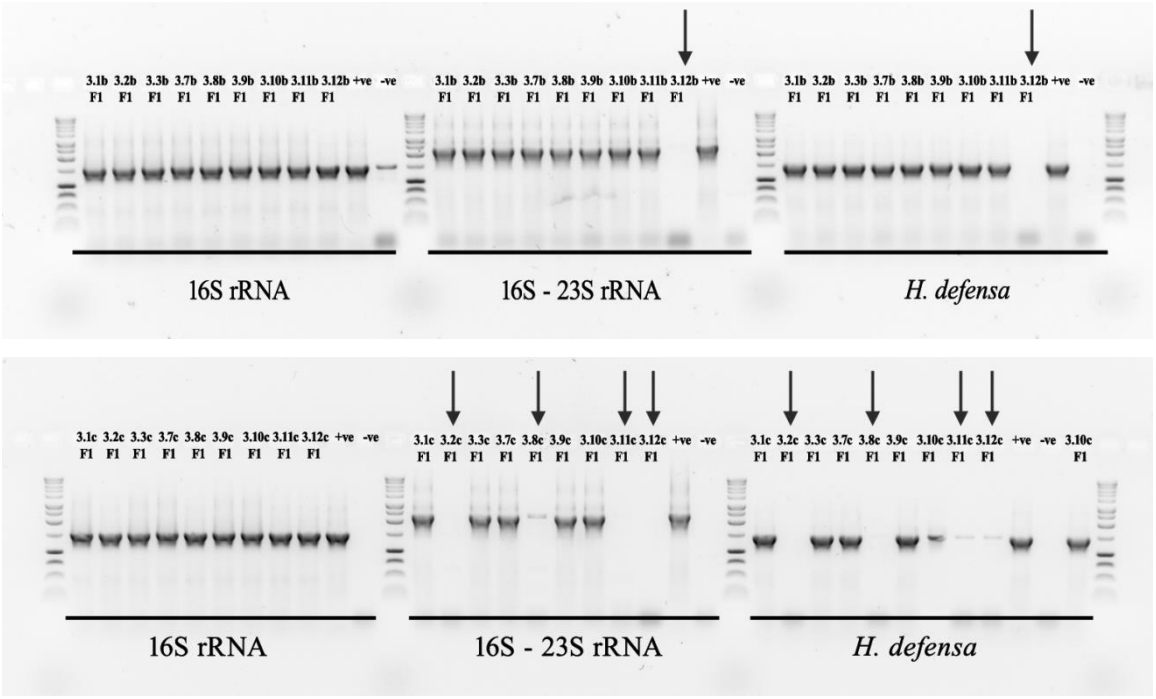

Figure 4.4: Inverted gel electrophoresis images of the diagnostic PCR products generated when the second and third cohorts of F_1 AA09/04 *M. euphorbiae* offspring from the third artificial diet curing trial were screened for the presence of *H. defensa*. Arrows indicate lanes in the *H. defensa* and 16S-23S rRNA screens in which products were not clearly visible, indicating the elimination or suppression of the secondary endosymbiont in these sub-lines.

The diagnostic PCR screening of the F_{-1}, F_0 and F_1 AA09/03 *M. euphorbiae* aphids from the fourth artificial diet curing trial again showed that the *H. defensa* endosymbiont was retained in the treated aphids, but had been eliminated from the second and third cohorts of F_1 offspring from the second F_0 aphid (sub-lines 4.2b and 4.2c), as evident from the lack of products generated in the 16S-23S rRNA and *H. defensa* screens. The cured sub-lines from both the third and fourth sub-lines were maintained in culture, although two (AA09/04^{ab}3.11c and AA09/03^{ab}4.1c) were lost due to the limited availability of good-quality plant material for culture cups. Diagnostic PCR of a sub-set of the aphids from the F_5 and F_{10} or F_{11} generations of surviving aphid sub-lines confirmed that the *H. defensa* endosymbiont had been completely eliminated.

In contrast, the subsequent curing trials were all unsuccessful with the aphids treated with antibiotics either not surviving or else producing offspring that still bore secondary endosymbionts. In the fifth artificial diet curing trial, routine diagnostic PCR screening of the HC10/08 aphid used to generate the F_0 aphids for treatment revealed no secondary endosymbionts, and so the resulting sub-lines were discarded; routine screening of the stock *M. euphorbiae* cultures later confirmed the loss of *R. insecticola* from the HC10/08 clonal line.

The success rate of the artificial diet curing trials was low, with only 29 of a total of 50 treated F0 aphids across the six diet trials surviving to produce offspring and of those, only 5 generating one or more cohorts of offspring from which the secondary symbionts had been eliminated, giving an overall success rate of 10%. Although various antibiotic concentrations and treatment lengths were tried, the very limited number of successfully cured sub-lines from later cohorts of offspring suggests that there is only a small window of time in which the antibiotics administered can affect bacterial titres significantly.

With one exception, the allele sizes at all seven microsatellite loci for the F_{-1}, F_0, F_1, F_5 and $F_{10/11}$ generations of the five surviving cured aphid sub-lines were the same as those from the two parental stock cultures AA09/03 and AA09/04 (Table 3.3). Only one allele at the *Me7* locus for the AA09/04^{ab}3.8 aphid fed on the artificial diet (gen. F_0) was larger than expected at 143bp. Although the cause of this discrepancy is unknown, as both the previous and the subsequent generations of aphids match the parental genotype the AA09/04^{ab}3.8 cured sub-line is likely to have remained true. Aphids from these cured sub-lines were therefore genetically identical to those in the stock cultures of the AA09/03 and AA09/04 lines, but differed in their secondary endosymbiont complement.

4.3.1.2 Administration of artificial diets to M. euphorbiae *lines naturally free of secondary endosymbionts*

Four of the six AA09/12 potato aphids fed on artificial diet containing antibiotic solutions survived to produce cohorts of offspring, which for logistical reasons was culled to two sub-lines, whilst only one of the six AA09/12 potato aphids fed on artificial diet alone did so. Although three cohorts of F_1 offspring were collected from each of the control sub-lines, only the third cohorts of offspring were maintained as these would be most comparable with the successfully cured AA09/03 and AA09/04 sub-lines.

Diagnostic PCR confirmed that all of the F_{-1}, F_0 and F_1 AA09/12 aphid templates were positive in the 16S rRNA screen (Figure 4.5), indicating the presence of bacteria. Whilst the lack of amplified product in the *H. defensa* screen confirmed the absence of the endosymbiont in these aphid sub-lines, bands of varying intensity were also produced in the 16S-23S rRNA screen, signifying the presence of transient gut bacteria or bacteria on the aphid cuticle unaffected by the antibiotic treatment.

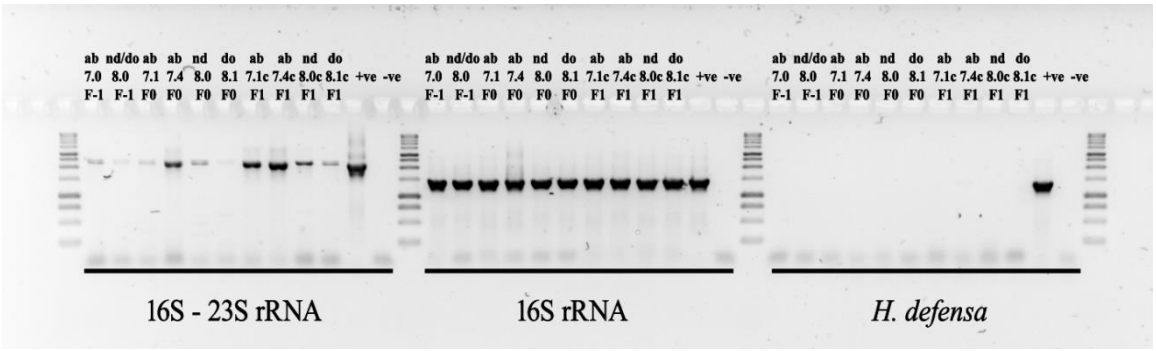

Figure 4.5: Inverted gel electrophoresis images of the diagnostic PCR products generated when the F_{-1}, F_0 and F_1 AA09/12 *M. euphorbiae* aphids from the seventh and eighth artificial diet control trials were screened for *H. defensa*. Like the parental line, none of the AA09/12 sub-lines harboured *H. defensa*, though products in the 16S-23S rRNA screen indicate the presence of other bacteria.

Diagnostic PCR screening of aphids from the F_5 and F_{10} generations of the two sub-lines treated with antibiotics (AA09/12^{ab}7.1c and AA09/12^{ab}7.4c), the sub-line fed on artificial diet only (AA09/12do8.1c) and the control sub-line not fed on artificial diet (AA09/12nd8.0c) again showed that none of the AA09/12 sub-lines harboured either *H. defensa* or any other secondary bacteria, as evident from the lack of significant levels of product in the 16S-23S screen (data not shown).

The allele sizes at all seven microsatellite loci for the F_{-1}, F_0, F_1, F_5 and F_{10} generations of the four aphid sub-lines were the same as those from the AA09/12 stock cultures (Table 3.3). These sub-lines therefore represent genetically identical aphids that differ only in the diet treatment they received over 10 generations previously, and are therefore suitable for testing the effects of oral administration of antibiotics.

4.3.2 Curing through microinjection

Eight of the ten AA09/04 *M. euphorbiae* aphids injected with ampicillin survived to produce one or more cohorts of offspring, whilst only three of the eight injected with insect saline solution did so. However, none of the aphids injected with the antibiotic solution (gen. F_0) or their offspring (gen. F_1) were successfully cured of the *H. defensa* endosymbiont. Furthermore, the first generation offspring of aphids from both treatment groups were rather small and slow-growing, and themselves produced few offspring that were also undersized. Further trials to cure *M. euphorbiae* aphids of the *H. defensa* endosymbiont through the microinjection of antibiotics were therefore not attempted.

4.3.3 Variation in titres of B. aphidicola *between untreated* M. euphorbiae *clonal lines and sub-lines treated with antibiotics*

4.3.3.1 Variation in titres of B. aphidicola *between* M. euphorbiae *clonal sub-lines naturally infected and artificially cured of* H. defensa

A univariate GLM determined that the effect of endosymbiont status on the titres of *B. aphidicola* between the groups of *M. euphorbiae* was highly significant ($F_{2, 16} = 7.680$, P = 0.005), with Sidak post-hoc tests finding significant differences in the titres of *B. aphidicola* between the cured aphids and both the *H. defensa*-infected and naturally symbiont free aphids at the 5% level.

When aphid clonal line was included in the analysis, the effect of endosymbiont status was no longer significant at the 5% level ($F_{2, 2} = 9.566$, P = 0.095), though the power of the analysis was decreased to 0.411, increasing the likelihood of a type II error occurring and hence reducing the likelihood of detecting a difference when one actually exists. The differences between aphid clonal lines with the same endosymbiont status were not significant ($F =_{2, 14} 0.781$, P = 0.477).

Figure 4.6 shows the mean normalised *B. aphidicola* titres from 5 *M. euphorbiae* clonal lines and sub-lines from within genotype 1.

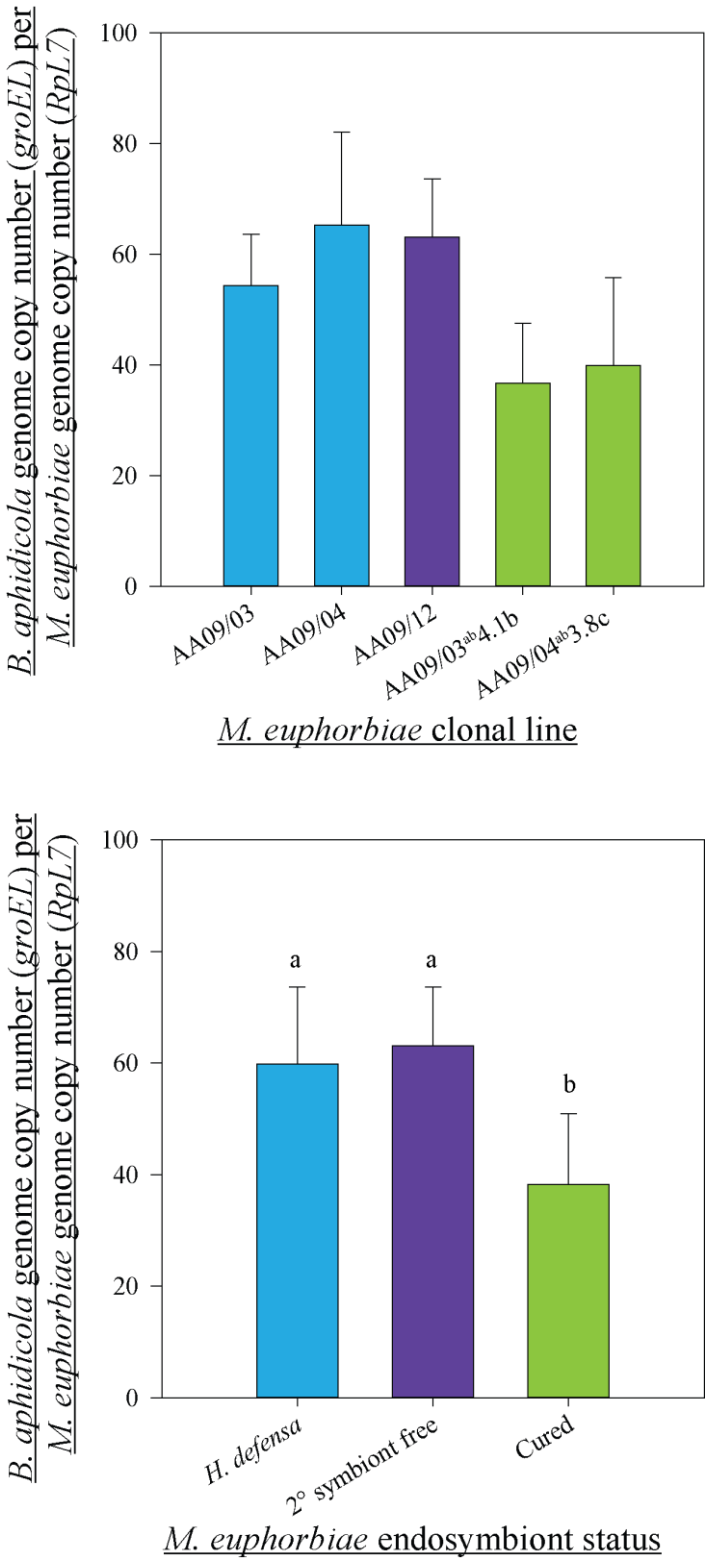

Figure 4.6: Normalised *B. aphidicola* titres from five *M. euphorbiae* clonal lines from a single genotype (top), and grouped by endosymbiont complement (bottom). Error bars show +1 s.d. Columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

4.3.3.2 Effect of antibiotic administration on B. aphidicola *titre*

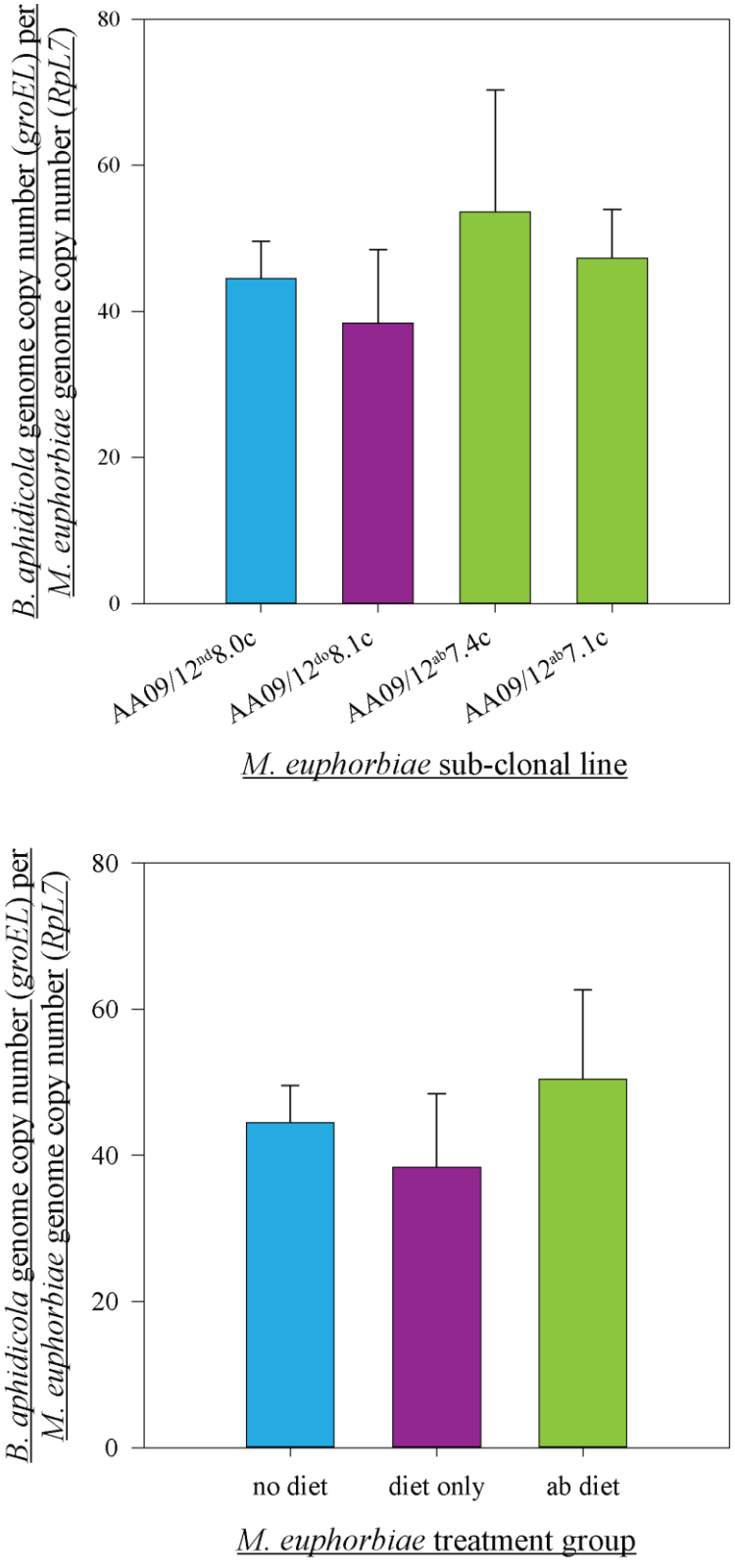

Figure 4.7: Normalised *B. aphidicola* titres from *M. euphorbiae* aphids subject to different diet treatments, grouped by sub-line (top) and by treatment (bottom). Error bars show +1 s.d.

There was no significant effect of artificial diet alone or antibiotic ingestion on *B. aphidicola* titre in the four *M. euphorbiae* AA09/12 sub-lines ($F_{3, 10}$ = 1.134, P = 0.382, 1-β = 0.221; Figure 4.7). Similarly, there were no significant differences in the *B. aphidicola* titres when the aphids were grouped by treatment, pooling the titres of the two sub-lines treated with antibiotics ($F_{2, 11}$ = 1.417, P = 0.283, 1-β = 0.241). The low powers of these analyses reduce the likelihood of detecting a difference when one actually exists, but the higher mean *B. aphidicola* titres of antibiotic-treated AA09/12 sub-lines compared with those either untreated or treated with diet alone suggest it is unlikely that a significant reduction in primary endosymbiont densities would be seen in the 'cured' aphid sub-lines with an increased number of replicates.

4.3.4 Genotypic and endosymbiont–mediated effects on aphid fitness

4.3.4.1 Fitness comparisons between M. euphorbiae *clonal lines and genotypes differing in their natural secondary endosymbiont complement*

Although the data for time to adulthood, time to first reproduction and intrinsic rate of population increase were not normally distributed and could not be normalised through commonly used transformations, frequency histograms of the three datasets showed a bell-shaped probability function, albeit slightly skewed to the right (not shown). Whilst analyses of variance assume that the distribution of the data are normal, small violations of this assumption are known to have little impact on the rate at which differences between means are erroneously scored as significant (Glass *et al*., 1972), especially for reasonable sample sizes, and so were used in these analyses.

Differences between genotypes in time to adulthood were very highly significant ($F_{6, 5.5912}$ = 77.132, P = <0.001; Figure 4.8A), with Sidak post-hoc tests confirming that aphids from genotypes 4, 6 and 7 reached adulthood significantly later than those from genotypes 1, 2, 3 and 5. Aphids harbouring *H. defensa* tended to reach adulthood more quickly than secondary symbiont-free aphids, although this difference was not significant (P = 0.157; Figure 4.8B) and was largely driven by the longer development times of genotypes 4, 6 and 7. The differences in development time between aphid lines from the same genotype and with the same endosymbiont infections, i.e. between lines AA09/03

and AA09/04, and between lines HC10/02 and HC10/05, were also not significant (P = 0.818; Figure 4.8C).

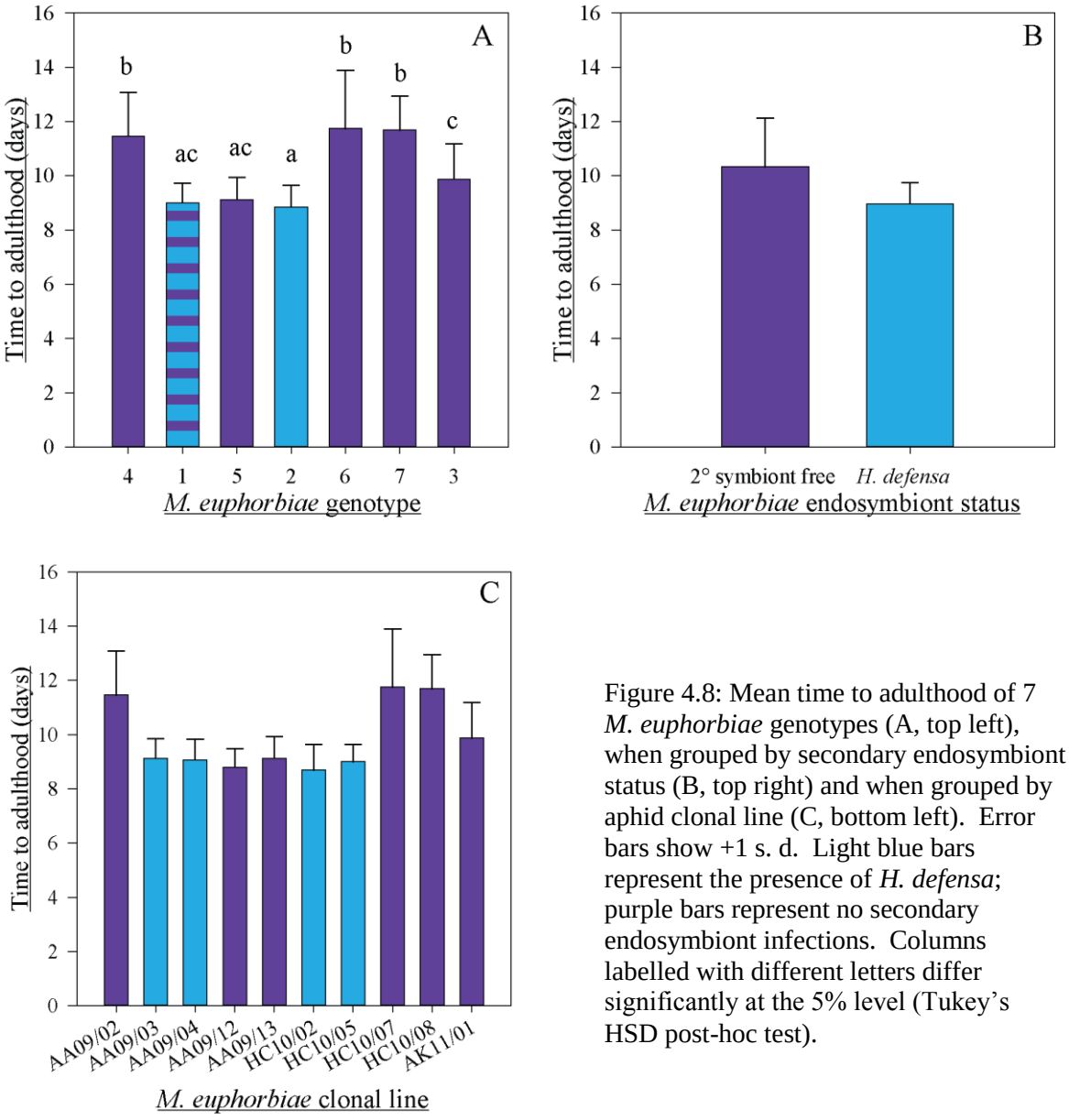

Figure 4.8: Mean time to adulthood of 7 *M. euphorbiae* genotypes (A, top left), when grouped by secondary endosymbiont status (B, top right) and when grouped by aphid clonal line (C, bottom left). Error bars show +1 s. d. Light blue bars represent the presence of *H. defensa*; purple bars represent no secondary endosymbiont infections. Columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

Despite the aphids from the ten aphid lines being reared under optimal conditions at low densities on high-quality plants for a minimum of two generations prior to the performance assay, a number of the aphids generated for the assay were alate. Both the absolute numbers of alate morphs from each line and the proportion of alate morphs differed between the aphid lines (Figure 4.9). There was a very highly significant difference between alate and apterous aphids in development time ($F_{1, 130} = 26.631$, P = <0.001). There was also a significant effect of spatial position in the glasshouse on aphid development time ($F_{7, 130} = 3.039$, P = 0.005), with aphids reared on plants in the seventh block in the glasshouse reaching adulthood significantly later than those in blocks one

and two. No temporal differences between the two sequential trials were detected in aphid development times (P = 0.976).

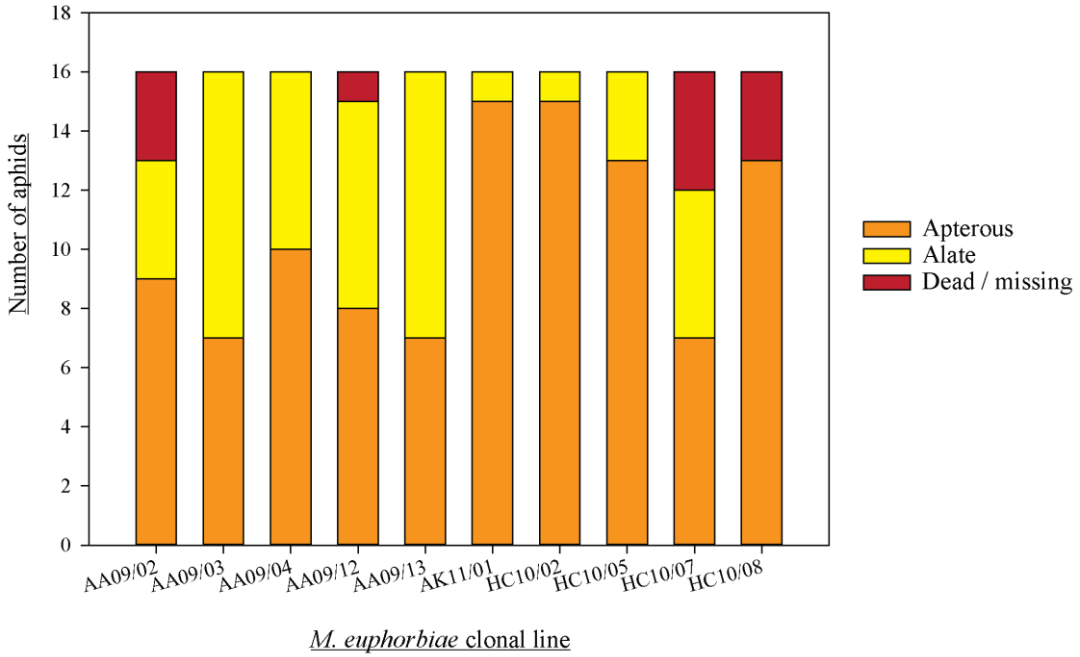

Figure 4.9: Numbers of aphids from the ten original *M. euphorbiae* lines that were alate, apterous or that died or escaped

Time to first reproduction and the intrinsic rate of population increase (R_m) varied significantly between aphid genotypes (pre-reproductive period: $F_{6, 30.808} = 47.635$, P = < 0.001; R_m: $F_{6, 2.188} = 19.017$, P = 0.041; Figure 4.10A and B). Sidak post-hoc tests revealed that genotypes 4, 6 and 7 took longer to begin producing offspring than genotypes 1, 2, 3 and 5. Genotypes 4, 6 and 7 also exhibited smaller mean intrinsic rates of population increase compared to genotypes 1, 2 and 5 due to a combination of low numbers of offspring produced in the time equal to the pre-reproductive period and relatively high mortality rates. Aphids from genotype 3 on average both developed faster and produced offspring earlier than aphids from genotypes 4, 6 and 7, but their poor survival meant that the number of offspring produced and therefore their overall rate of population increase was not significantly different from these three poorly-performing lines.

Neither time to first reproduction nor intrinsic rate of population increase varied significantly with the presence of endosymbiont infection (Time to first reproduction: P = 0.270; R_m: P = 0.654; Figure 4.10C and D), although the low statistical power of these tests reduces the likelihood of detecting a difference when one actually exists. The

differences between aphid lines from the same genotype and with the same endosymbiont infections were also not significant (Time to first reproduction: P = 0.900; R_m: P = 0.396). However, alate aphids exhibited significantly longer time to first reproduction and smaller intrinsic rate of population increase than apterous aphids (Time to first reproduction: $F_{1, 117} = 79.391$, P = <0.001; R_m: $F_{2, 140} = 17.640$, P = <0.001).

Finally, the time to first reproduction did not vary either temporally with trial or spatial position in the experiment (P = 0.076 and 0.168, respectively), whilst the intrinsic rate of population increase was significantly higher in aphids from the first trial compared with the second trial ($F_{1, 140} = 7.357$, P = 0.008) but did not vary significantly with position in the experiment (P = 0.790).

The time to adulthood and time to first reproduction of the 10 *M. euphorbiae* clonal lines were strongly and significantly positively correlated, whilst both time to adulthood and time to first reproduction were strongly and significantly negatively correlated with intrinsic rate of population increase (Table 4.6).

Table 4.6: Pearson's product moment correlation coefficients, R, and statistical significance of the relationships between three fitness parameters measured in the performance assay of 10 *M. euphorbiae* clonal lines.

	Time to adulthood	Time to first reproduction	R_m
Time to adulthood	R = 1	R = 0.856 P = <0.001	R = -0.760 P = <0.001
Time to first reproduction	R = 0.856 P = <0.001	R = 1	R = -0.799 P = <0.001
R_m	R = -0.760 P = <0.001	R = -0.799 P = <0.001	R = 1

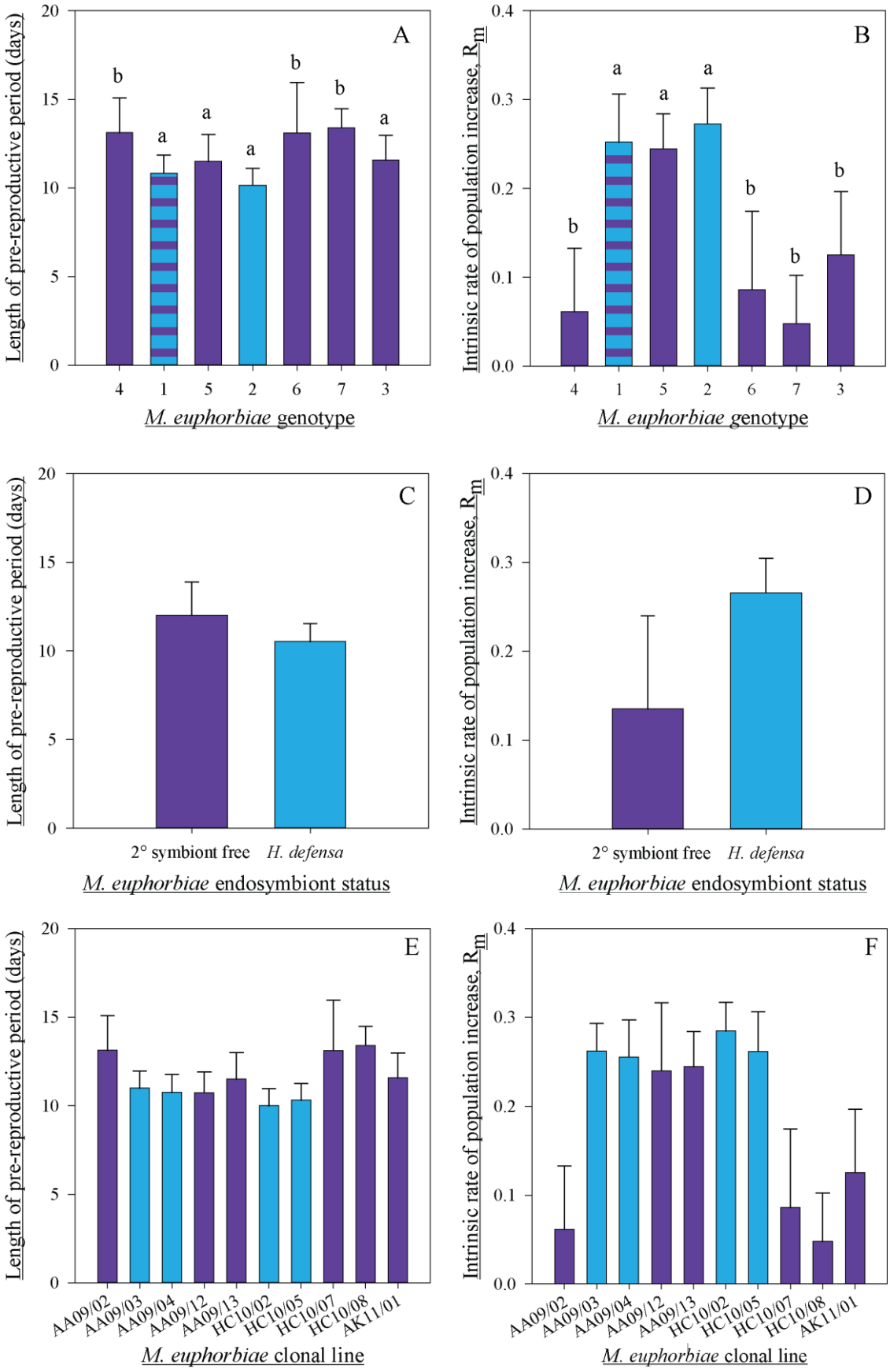

Figure 4.10: Length of pre-reproductive period (left) and intrinsic rate of population increase (right) of the 10 *M. euphorbiae* clonal lines, when grouped by secondary endosymbiont status and when grouped by genotype. Error bars show +1 s. d. Light blue bars represent the presence of *H. defensa*; purple bars represent no secondary endosymbiont infections. Columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

Analysing the time to adulthood, time to first reproduction and intrinsic rate of population increase of the 10 *M. euphorbiae* lines in a multivariate GLM to account for these relationships confirmed that genotype and wing morph significantly affected all three fitness parameters measured (P = <0.001), whilst the presence or absence of *H. defensa* was not a significant factor (P = 0.599).

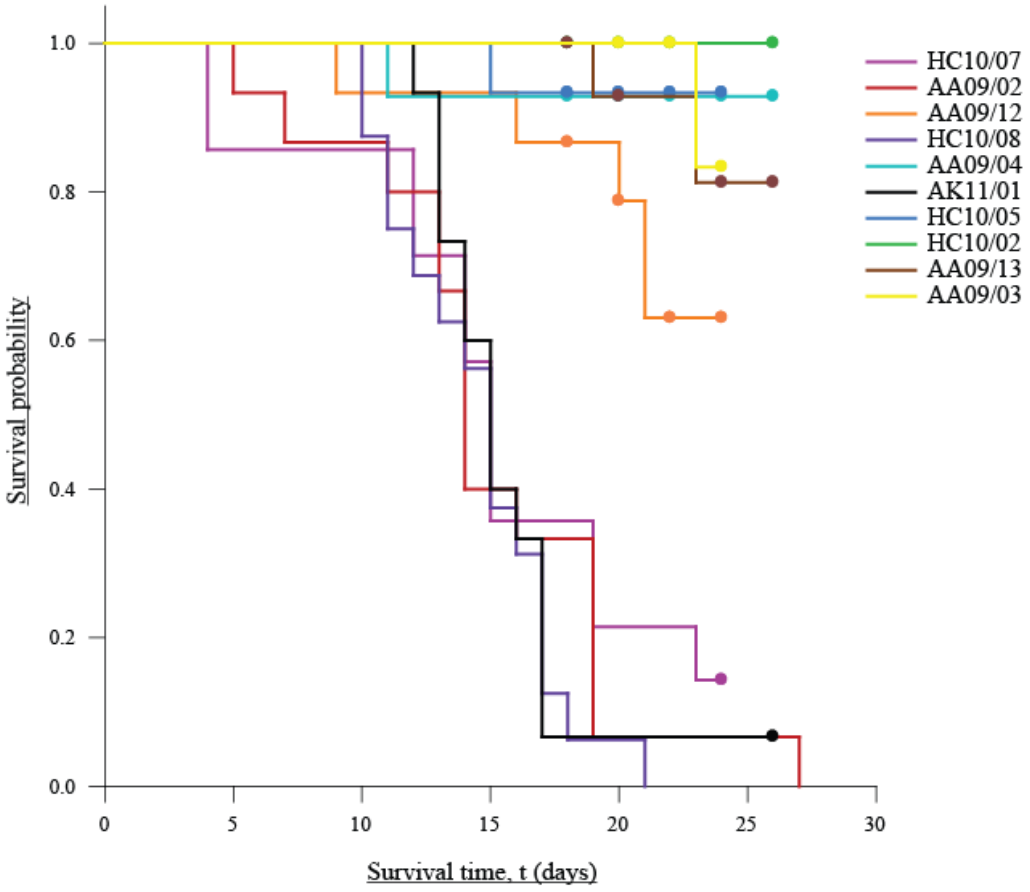

Figure 4.11: Kaplan-Meier survival curves for the ten *M. euphorbiae* clonal lines used in the performance assay. Censored subjects are indicated by the coloured circular symbols.

With all time points weighted equally, comparisons between the survival curves using the log-rank test divided the ten *M. euphorbiae* aphid lines into two distinct groups (Figure 4.11). The survival of the AA09/03, AA09/04, AA09/12, HC10/02 and HC10/05 clonal lines, representing genotypes 1, 2 and 5, did not differ from each other but each differed significantly at the 5% level from those of the AA09/02, AK11/01, HC10/07 and HC10/08 clonal lines from genotypes 3, 4, 6 and 7. These clonal differences in survival reflected differences in the other fitness parameters, with lower survival probabilities associated with longer development times and lower intrinsic rates of population increase. The Breslow and Tarone-Ware tests, both of which place greater emphasis on

the earlier period of the survival curves, also showed that the survival of the aphid lines from genotypes 1, 2 and 5 differed significantly from those of genotypes 3, 4, 6 and 7. However, with this greater weighting of earlier mortalities there was also a significant difference at the 5% level between the clonal lines AA09/03 and AA09/12 (genotype 1) in both tests.

It was not possible to construct models of the survival data that incorporated both aphid genotype and the presence or absence of *H. defensa*, as the natural presence of the secondary endosymbiont in only two of the genotypes led to the two variables confounding. Instead, both a Cox proportional hazards (PH) model and a Weibull distribution model were fitted to the survival data from the aphid lines that harboured only *B. aphidicola* in order to determine the intrinsic genetic variation and the effects of wing morph on survival rates, as well as any differences resulting from the collection of survival data from two sequential trials (Table 4.7). The effects of harbouring *H. defensa* on survival estimates within a given genotype of potato aphid, using five potato aphid lines and sub-lines from genotype 1, were modelled separately (see section *4.3.4.2*).

Table 4.7: Cox proportional hazards model fitted to the survival data from six *M. euphorbiae* aphid lines, excluding individuals not scored for wing morph. Genotype 2 aphids, all of which harboured *H. defensa*, and genotype 1 aphids harbouring the secondary endosymbiont were excluded from the analysis to prevent the two factors (genotype and *H. defensa* presence) confounding.

Factor		**Model co-efficient (β)**	**Hazard ratio (exp(β))**	**P-value**	**Significance (level)**
Genotype	Genotype 1	----------Reference----------			
	Genotype 3	2.697	14.842	8.16×10^{-5}	Significant (0.1%)
	Genotype 4	2.270	9.676	0.000577	Significant (0.1%)
	Genotype 5	-0.686	0.504	0.455026	Not significant
	Genotype 6	1.802	6.060	0.008658	Significant (5%)
	Genotype 7	2.719	15.161	8.49×10^{-5}	Significant (0.1%)
Wing morph	Alate	----------Reference----------			
	Apterous	-0.306	0.993	0.979894	Not significant
Trial	1	----------Reference----------			
	2	-0.007	0.736	0.464139	Not significant

The Cox proportional hazards model is given as:

Equation 5) $h(t) = h_0(t) \exp(\beta_1 x_1 + \beta_2 x_2 + \ldots + B_n x_n)$

where h(t) is the hazard function at time t, $h_0(t)$ is the (unknown) baseline hazard function and β are the predictor coefficients. As the baseline hazard is unspecified, the exponential of each model coefficient β therefore gives the hazard ratio of one covariate relative to the reference covariate. In the model above, for example, the mortality rate of an aphid from genotype 3 is nearly 15 times greater at any instant in time than an aphid from the reference genotype, genotype 1.

As indicated by the Kaplan-Meier curves in Figure 4.11, the instantaneous hazard rate of aphids of genotypes 3, 4, 6 and 7, represented by the aphid lines AK11/01, AA09/02, HC10/07 and HC10/08, were significantly higher than from aphids of genotypes 1 and 5 (aphid lines AA09/12 and AA09/13). Although the model co-efficients indicated that apterous aphids had a slightly lower hazard rate than their winged counterparts (hazard ratio of 0.993, P = 0.9799), and aphids from trial 2 a lower hazard rate than those from trial 1 (hazard ratio = 0.736, P = 0.4641), neither effect was significant at the 5% level.

Analyses of variance determined genotype was the only significant factor affecting survival time (Likelihood ratio (LR) χ^2=47.608, P = <0.001), although when the factors were considered sequentially and the effects of wing morph were considered before the effects of genotype, this too was a significant factor (LR χ^2=6.7604, P = 0.00932). Similarly, the most heavily weighted model ranked by AIC scores incorporated only genotype in addition to the intercept (AICc=353.1, weight = 0.546), although the second best model also incorporated wing morph (AICc =354.9. weight =0.219). Given that the proportion of apterous aphids differed between the aphid genotypes, it is not surprising that the significance of wing morph as an explanatory variable diminishes once the effect of genotype is taken into account.

As the aphids that suffered the earliest mortalities did not reach adulthood, they were not scored for wing morphology. Excluding wing morph from the Cox PH model to allow inclusion of the earliest mortalities produced similar model co-efficients and the same levels of significance for both genotype and trial, with the most heavily weighted model again only incorporating genotype.

The second model to evaluate the effects of genotype, wing morph and trial on the survival of *M. euphorbiae* clonal lines that lacked *H. defensa* was fitted to a Weibull distribution (Table 4.8).

Table 4.8: Weibull distribution model fitted to the survival data from six *M. euphorbiae* aphid lines, excluding individuals not scored for wing morph. Genotype 2 aphids, all of which harboured *H. defensa*, and genotype 1 aphids harbouring the secondary endosymbiont were excluded from the analysis to prevent the two factors (genotype and *H. defensa* presence) confounding.

Factor		**Model co-efficient (β)**	**Survival ratio (exp(β))**	**P-value**	**Significance (level)**
Intercept		3.334	-	3.96×10^{-122}	Significant (0.1%)
Genotype	Genotype 1	----------Reference----------			
	Genotype 3	-0.5745	0.562907	0.000318	Significant (0.1%)
	Genotype 4	-0.488	0.613749	0.00153	Significant (5%)
	Genotype 5	0.238	1.268075	0.260	Not significant
	Genotype 6	-0.377	0.686211	0.0169	Significant (5%)
	Genotype 7	-0.643	0.525671	0.0000555	Significant (0.1%)
Wing morph	Alate	----------Reference----------			
	Apterous	0.088	1.091519	0.360	Not significant
Trial	1	----------Reference----------			
	2	0.009	1.009434	0.885	Not significant
Log(scale)		-1.501	-	5.16×10^{-49}	Significant (0.1%)

A survival model fitted to a Weibull distribution in **R** is parameterised to yield an accelerated failure time model rather than a proportional hazards model. Consequently the model produces estimates of survival times rather than of instantaneous hazard rates, and the signs of the model coefficients are the inverse of those given in the Cox PH model above. The intercept is the log of the scale parameter and hence also the log of the survival rate of the reference group, whilst the log (scale) value is the reciprocal of the shape parameter. As the model is expressed on a log scale, it is the exponent of each model coefficient β that gives the effect on survival time relative to the reference group. For example, in the model above, the survival time of an aphid from genotype 5 is increased by a factor of 1.27 compared to that of an aphid from the reference genotype, genotype 1.

As indicated by the Kaplan-Meier curves in Figure 4.11, the survival times of aphids from genotypes 3, 4, 6 and 7, represented by the aphid lines AK11/01, AA09/02, HC10/07 and HC10/08, were significantly lower than of aphids of genotypes 1 and 5, represented by aphid lines AA09/12 and AA09/13 (differing in survival by a factor of 0.526 – 0.686 of genotype 1). Although the model coefficients indicated that apterous aphids had longer survival times than their winged counterparts (survival ratio = 1.0915,

P = 0.360), and aphids from trial 2 exhibited slightly longer survival times than those from trial 1 (survival ratio = 1.009, P = 0.885), neither effect was significant at the 5% level. Analyses of variance to determine which factors were important to the model again found that genotype significantly affected the aphid survival rates (LR χ^2=48.235, P = <0.001), whilst wing morph was only significant if genotypic differences were not considered.

As with the Cox PH model, excluding wing morph from the Weibull distribution model to allow inclusion of aphids with early mortality produced similar model coefficients and the same levels of significance for the factors of genotype and trial, with only genotype as a significant factor in determining survival times.

4.3.4.2 Fitness comparisons between M. euphorbiae *clonal lines naturally infected and artificially cured of* H. defensa

Within genotype 1, there was no significant effect of either harbouring *H. defensa* or of the elimination of *H. defensa* on time to adulthood, time to first reproduction or R_m (endosymbiont status: P = 0.253, 0.119 and 0.141, respectively, Figure 4.12B, D and F), suggesting that the presence of *H. defensa* did not impact on these aspects of aphid performance. There were also no significant differences in these three fitness parameters between individual aphid lines harbouring *H. defensa* or between aphid lines cured of the endosymbiont infection (P = 0.509, 0.864 and 0.460 for time to adulthood, time to first reproduction and R_m, respectively).

None of these three fitness parameters differed significantly between aphids reared in different positions within the glasshouse (P = 0.195, 0.539 and 0.401, respectively), although the intrinsic rate of population increase was significantly lower in aphids reared in the second trial ($F_{1, 65}$ = 5.591, P = 0.021).

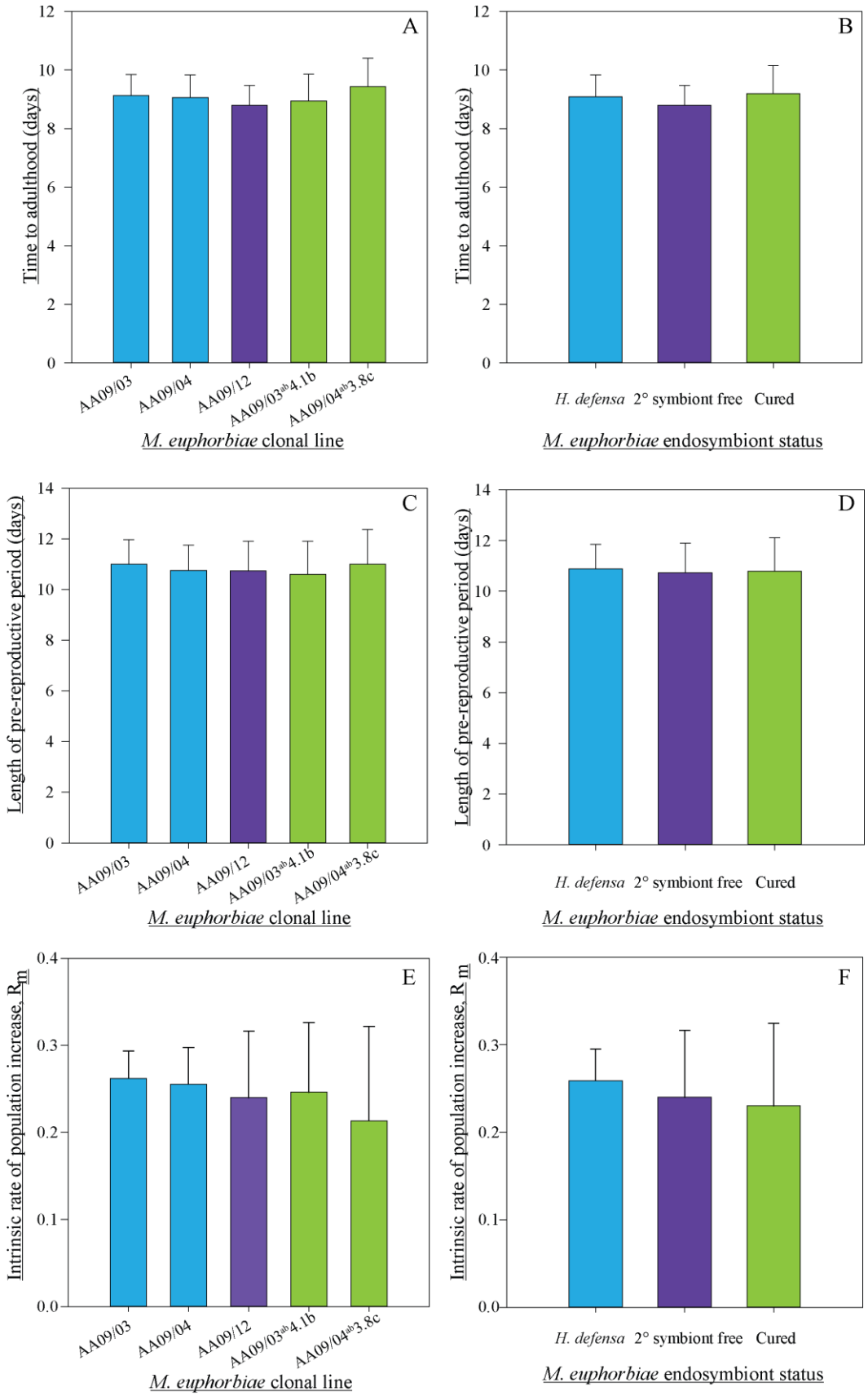

Figure 4.12: Time to adulthood (A and B), length of pre-reproductive period (C and D) and intrinsic rate of population increase (E and F) of the five *M. euphorbiae* clonal lines belonging to genotype 1and when grouped by secondary endosymbiont status. Error bars show +1 s. d. Light blue bars represent the presence of *H. defensa*; purple bars represent a natural absence of secondary endosymbionts and green bars represent artificially eliminated *H. defensa* infection.

As shown above for the full experiment analysis (section *4.3.4.1*), wing morph was the only biological factor that consistently influenced aphid performance. The apterous aphids developed and began producing offspring earlier than the alate morphs and had a higher intrinsic rate of increase, with all differences very highly significant (Time to adulthood: $F_{1, 65} = 37.859$, P = <0.001; Time to first reproduction: $F_{1, 62} = 108.843$, P = <0.001; R_m $F_{2, 65} = 8.675$, P = <0.001).

As with the full performance assay experiment, there was significant positive correlation between time to adulthood and time to first reproduction of the genotype 1 aphids (R = 0.728, P = <0.001), and significant negative correlation between both time to adulthood and time to first reproduction and the intrinsic rate of population increase (R = -0.427 and -0.809, respectively, P = <0.001). A multivariate GLM taking into account these correlations confirmed that the time to adulthood, the length of the pre-reproductive period and the intrinsic rate of increase did not differ significantly between the *M. euphorbiae* aphids grouped by their secondary endosymbiont status and treatment (Pillai's Trace P = 0.169, Wilks' Lambda P = 0.161), whilst the differences in all three fitness parameters between apterous and alate morphs were very highly significant (P = <0.001).

Using the log-rank test, there were no significant differences between the five aphid lines in survival (Figure 4.13), but both the Breslow and Tarone-Ware tests identified marginally significant differences between aphid lines AA09/03 and AA09/12 (χ^2 = 4.126, P = 0.042 and χ^2 = 3.926, P = 0.048, respectively).

The Cox PH model (Table 4.9) showed that the aphids still harbouring *H. defensa* had a lower instantaneous hazard rate than their cured counterparts, although the differences were not significant (hazard ratio = 0.2868, P = 0.141). In contrast, the hazard rate of the potato aphid line naturally free of secondary endosymbionts was slightly higher than the aphids harbouring and cured of *H. defensa*, although again this difference was not statistically significant (hazard ratio = 1.1410, P = 0.858). Thus, in a common genetic background, the presence or absence of the endosymbiont *H. defensa* did not have a statistically significant effect on the instantaneous hazard rate of the aphids.

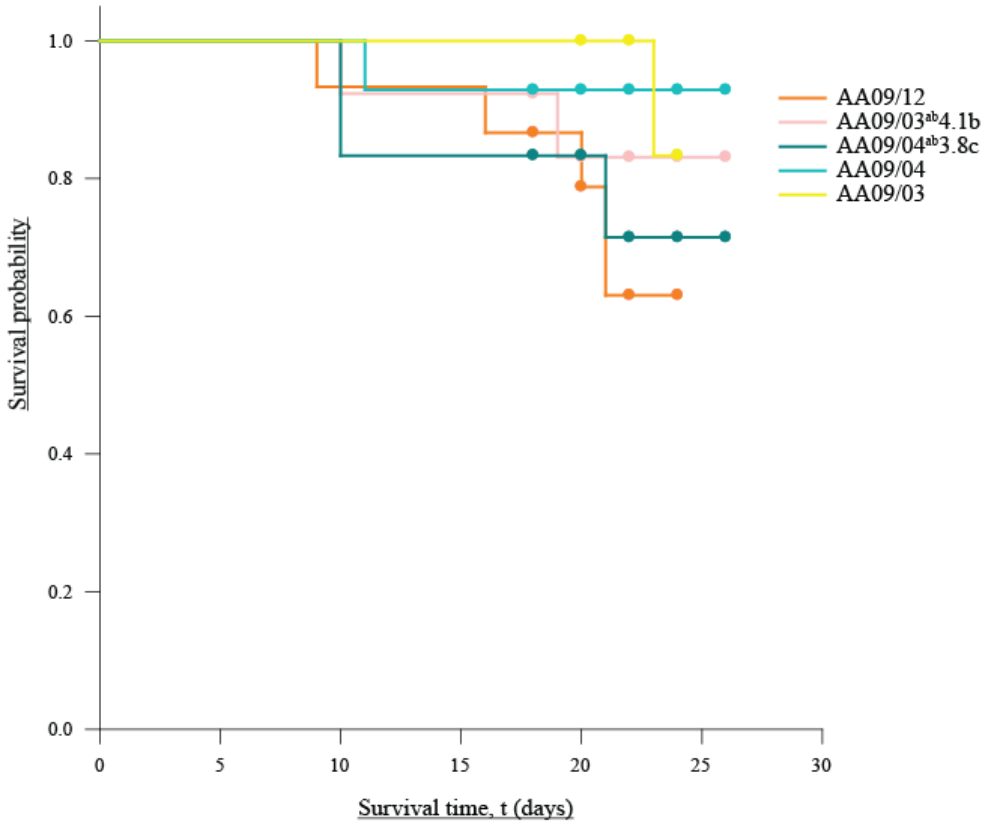

Figure 4.13: Kaplan-Meier survival curves for the five *M. euphorbiae* clonal lines belonging to genotype 1 and differing in their secondary endosymbiont status. Censored subjects are indicated by the coloured circular symbols.

Table 4.9: Cox proportional hazards model fitted to the survival data from *M. euphorbiae* aphids with a common genetic background (genotype 1) but differing in their endosymbiont status.

Factor		**Model co-efficient (β)**	**Hazard ratio (exp(β))**	**P-value**	**Significance (level)**
Treatment	Cured of *H. defensa*	----------Reference----------			
	H. defensa present	-1.249	0.28676	0.141	Not significant
	No 2° endosymbiont	0.132	1.14104	0.858	Not significant
Wing morph	Alate	----------Reference----------			
	Apterous	-1.190	0.30416	0.149	Not significant
Trial	1	----------Reference----------			
	2	-0.079	0.92434	0.906	Not significant

Table 4.10: Weibull distribution model fitted to the survival data from *M. euphorbiae* aphids with a common genetic background (genotype 1) but differing in their endosymbiont status.

Factor		Model co-efficient (β)	Survival ratio (exp(β))	P-value	Significance (level)
Intercept		3.489	-	2.17×10^{-122}	Significant (0.1%)
Treatment	Cured of *H. defensa*	----------Reference----------			
	H. defensa present	0.465	1.592651	0.191	Not significant
	No 2° endosymbiont	-0.692	0.500574	0.806	Not significant
Wing morph	Alate	----------Reference----------			
	Apterous	0.482	1.618968	0.180	Not significant
Trial	1	----------Reference----------			
	2	0.0405	1.041331	0.873	Not significant
Log(scale)		-0.955	-	0.00147	Significant (1%)

These results were reinforced using the Weibull distribution model (Table 4.10), with non-significant trends towards increased survival of aphids harbouring *H. defensa* (survival ratio = 1.5927, P = 0.191) and decreased survival of the aphids naturally free of secondary endosymbionts compared to aphids from which *H. defensa* had been eliminated (survival ratio = 0.5006, P = 0.806). Within this common genetic background, aphid survival was not significantly affected by the presence or absence of the endosymbiont *H. defensa*. Similarly, there was no significant effect of aphid morph on hazard rate and survival time (hazard ratio = 0.3042, P = 0.109; survival ratio = 1.6190, P = 0.180), nor any differences between the two experimental trials (hazard ratio = 0.9243, P = 0.906; survival ratio = 1.0413, P = 0.873).

4.4 Discussion

4.4.1 Elimination of secondary endosymbiont infection, and the effects of oral antibiotic treatment

The successful curing of several sub-lines of *M. euphorbiae* demonstrates that the antibiotics imbibed by the treated aphids effectively targeted the *H. defensa* endosymbiont, as predicted from their known modes of action (Poole, 2005; Koga *et al*.,

2007). Whether the ampicillin, gentamycin and cefotaxime reduced the maternal *H. defensa* titres and thus prevented the transfer of bacteria to the developing embryos, or else eliminated the endosymbiont directly from the developing embryos is unclear.

The transfer of both the obligate endosymbiont *B. aphidicola* and the secondary endosymbiont *S. symbiotica* in asexual *A. pisum* occurs at the blastula stage early in embryonic development (Miura *et al*., 2003; Koga *et al*., 2012). Whilst the latest histological techniques suggest that *B. aphidicola* is selectively transported from a maternal bacteriocyte to the blastulae through a process of exo- and endocytosis, *S. symbiotica* cells appear to be recruited by endocytosis directly from the haemolymph (Koga *et al*., 2012). If the same is true of *H. defensa* infections within *M. euphorbiae*, the successful elimination of the secondary endosymbiont from a clonal line would only require titres within the haemolymph to be reduced, rather than a reduction of bacterial densities within the secondary bacteriocytes. Given that *H. defensa* infections persisted in the treated aphids that produced monosymbiotic offspring, the reduction of *H. defensa* titres predominantly in the haemolymph is a plausible mechanism by which the cured aphids were generated. It is less likely that *H. defensa* was eliminated post-transfer to the embryo due to rapid division of endosymbiont cells immediately following transmission resulting in much larger *H. defensa* populations compared to the founding population inherited from the maternal aphid (Mira & Moran, 2002; Wilkinson *et al*., 2003). Furthermore, the ovariole sheath, a selective barrier between the embryo and the maternal haemolymph, could prevent or reduce the concentration of antibiotics reaching the blastula (Douglas, 1996; Bermingham & Wilkinson, 2009).

The low success rates from the curing trials are likely in part to be the result of different feeding rates between individual aphids, and therefore variation in the amount of antibiotics consumed. Although an undisturbed aphid can feed on phloem from a single sieve element continuously for hours at a time, artificial diets lack the mechanical and olfactory plant cues as well as gustatory cues such as secondary metabolites that are thought to contribute to plant acceptance (Powell *et al*., 2006, and references therein). Furthermore, whilst the components of the diet were kept standardised, some diet sachets were found to oxidise rather faster than others, potentially contributing to depressed feeding rates. Regular observations of *M. euphorbiae* nymphs on the standard diet with or without antibiotics showed that the aphids did not always settle to feed, although this behaviour was not specific to any particular potato aphid line or genotype.

Differences in the success rate of the curing trials may also stem from genotypic variation in the transmission of secondary endosymbionts between potato aphid clones. Both *B. aphidicola* and secondary endosymbionts undergo a severe restriction in their effective population sizes (N_e) as they are horizontally transmitted from one aphid generation to the next. Nevertheless, although the exact molecular and cellular processes involved in inducing transmission are yet to be understood, the primary bacterium is always faithfully transmitted (Miura *et al*., 2003; Wilkinson *et al*., 2003; Koga *et al*., 2012). In contrast, the transfer of secondary endosymbionts such as *S. symbiotica* appears to take advantage of the non-specific nature with which cytoplasmic extensions capture bacterial cells for endocytosis into the developing blastula (Koga *et al*., 2012). Whilst extracellular secondary endosymbionts appear to aggregate around the ovariole tips of the aphid host, this in itself would not ensure transmission to the blastulae and stochastic events could therefore result in the loss of endosymbiont infections occasionally observed in cultured aphids and that are hypothesised to occur in natural aphid populations (Darby & Douglas, 2003; Koga *et al*., 2012).

The two successful curing trials in this study both eliminated the secondary endosymbiont from sub-lines of potato aphids from genotype 1, but not from genotype 2. From the *M. euphorbiae* clonal lines maintained for this study, three of the aphid lines from genotype 1 were naturally free of secondary endosymbionts (AA09/12, AA09/14 and HC11/02), whilst two harboured *H. defensa* with and without the APSE bacteriophage (AA09/04 and AA09/03, respectively). In contrast, all six potato aphid lines from genotype 2 harboured *H. defensa* (AA09/06, HC10/02, HC10/05, HC10/06, HC11/03 and HC11/09). It is therefore possible that transmission of *H. defensa* to the developing embryos of genotype 2 is more robust than in genotype 1 aphids, reducing transmission failures that might otherwise generate monosymbiotic lineages and increasing the difficulty of eliminating the infection artificially. Such a situation could arise as the result of a beneficial interaction between *H. defensa* and genotype 2 potato aphids that has selected for the ensured continuation of the symbiotic association.

Alternatively, the presence of the endosymbiont could be under neutral selection and the apparently ubiquitous presence of *H. defensa* within this aphid genotype purely the result of variation in endocytotic machinery by which *B. aphidicola* and secondary endosymbiont cells alike are taken into the cytoplasm of the blastula. Failure to cure genotype 2 aphids of their secondary endosymbiont infections could also reflect

differences between strains of *H. defensa*, with some variants of the endosymbiont exhibiting higher transmission fidelity to the developing embryos than others. Which, if any, of these scenarios are true would require further investigation.

The normalised *Buchnera* titres of the two cured *M. euphorbiae* sub-lines AA09/03^{ab}4.1b and AA09/04^{ab}3.8 were significantly lower than both those of the parental lines still harbouring *H. defensa*, AA09/03 and AA09/04, and to the *Buchnera* titres of another potato aphid line from the same genotype that naturally lacked *H. defensa*, AA09/12. This suggests that the lower bacterial densities were the result of the curing process, rather than the absence of the *H. defensa* endosymbiont, despite selecting antibiotics with modes of action that should have precluded effects on the *B. aphidicola* bacteria, and allowing ten generations to pass between antibiotic administrations and quantifying endosymbiont titres.

Given that *H. defensa* is a predominantly intracellular endosymbiont, residing in sheath cells and secondary bacteriocytes within the aphid bacteriome, it is possible there is an element of spatial competition between *H. defensa* and the primary endosymbiont, *B. aphidicola*. In addition, the dependency of *H. defensa* on both *B. aphidicola* and the aphid host for certain nutrients could limit the proliferation of the primary endosymbiont (Koga *et al*., 2003). Monosymbiotic aphids would therefore be expected to harbour greater titres of *B. aphidicola* than aphids from the same lineage that also harboured *H. defensa*. Indeed, increased titres of *B. aphidicola* have been observed in *A. pisum* aphids cured of *H. defensa* relative to disymbiotic aphids from the same parental lineage, at least in teneral and early-reproducing aphids (Koga *et al*., 2003). Similar increases in *B. aphidicola* titres have been demonstrated in *A. pisum* following the elimination *of Rickettsia* (Sakurai *et al*., 2005), although limited genotypic analysis of *A. pisum* clones means there is little information on primary endosymbiont densities in naturally monosymbiotic and disymbiotic pea aphids with a common genetic background.

The reduction in *B. aphidicola* titres in *M. euphorbiae* aphids cured of *H. defensa* is in disagreement with these findings; rather than enabling *Buchnera* cells to proliferate or the genome copy number to increase, the elimination of the secondary endosymbiont reduced the genome copy number of *B. aphidicola* several generations after the administration of the antibiotics. Given that *Buchnera* titres do not differ significantly between potato aphids of this genotype with and without natural infections of *H. defensa*,

the presence of the secondary endosymbiont is unlikely to have promoted *Buchnera* genome duplication.

Between 25 and 50 generations post-infection are required for the effects of artificial infection of *A. pisum* clones with *H. defensa* to attenuate and for the localization of the introduced endosymbionts to stabilise (Koga *et al*., 2003). However, studies using *A. pisum*, *A. fabae* and other aphid species experimentally cured of secondary endosymbionts typically allow a minimum of ten generations before commencing experiments (Koga *et al*., 2003, 2007; Sakurai *et al*., 2005; Douglas *et al*., 2006a; Chandler *et al*., 2008). The decreased densities of primary endosymbiont populations approximately 25 generations after antibiotic administration may therefore have implications for the performance of potato aphids from these cured sub-lines, although no negative effects of these reduced *Buchnera* titres on development or reproduction were apparent in the performance assay (see section *4.4.2*).

By contrast, when sub-lines of the naturally monosymbiotic *M. euphorbiae* line AA09/12 were exposed to oral administration of the same antibiotics at the same dosages, there was no reduction in *B. aphidicola* titre. It is possible that the quantity of the diet solution imbibed by AA09/12 aphids was insufficient to expose aphids to an antibiotic dose comparable to that of the cured AA09/03 and AA09/04 aphids. Future curing experiments that quantified the primary bacteria of aphid sub-lines both successfully and unsuccessfully cured following antibiotic administration would confirm whether a decrease in *B. aphidicola* titres is a general phenomenon in potato aphids treated with antibiotics.

These findings highlight the need for caution when techniques such as the administration of antibiotics in artificial diets, established in *A. pisum*, are applied to alternative aphid species, as there may be subtle differences in the outcome. Furthermore, the reduction in titres of the primary endosymbiont in cured potato aphid sub-lines may have unforeseen consequences on fitness aspects beyond those measured here, particularly given the vital role that *Buchnera* plays in nutrient provisioning. Nonetheless, the antibiotic curing of potato aphid lines is a useful approach to enable comparison with potato aphid lines from the same genotype that naturally harbour *H. defensa*, and therefore to allow genotypic variation in fitness to be separated from endosymbiont-mediated heritable traits.

4.4.2 Variation in performance of **M. euphorbiae** *genotypes and of* **M. euphorbiae** *aphids differing in their endosymbiont status*

The range of values produced for the development time, pre-reproductive period, intrinsic rate of population increase, R_m, and survival estimates of the *M. euphorbiae* lines investigated here are comparable to those measured under similar conditions published in other studies (Barlow, 1962a, 1962b; Karley *et al*., 2002; Davis *et al*., 2007; Le Roux *et al*., 2007). The natural occurrence of *H. defensa* in only two of the seven genotypes of *M. euphorbiae* increased the difficulty in separating effects of the endosymbiont infection from those attributable to the genetic background of the aphid host. Nevertheless, within the *M. euphorbiae* lines used for this study, there were no apparent costs or benefits attributable to secondary endosymbionts to the inherent fitness of the aphid clones, but there were significant differences between aphid genotypes.

Trade-offs between survival and reproduction are commonly observed, as the allocation of finite resources to maximise one trait often occurs to the detriment of the other trait (Stearns, 1989). However, there was no evidence for such a trade-off within the potato aphid clones tested here. The two genotypes that exhibited the most rapid development and the highest intrinsic rates of increase (genotypes 1 and 2) also displayed the highest survival probabilities. Aphids from the clonal line AA09/13 (provisionally designated genotype 5) showed similar fitness characteristics, providing support to the molecular evidence that these aphids may be descended from a genotype 1 aphid in which a mutation had altered the size of one allele at a single microsatellite locus (see section *3.3.2.1*). In contrast, the aphids from genotypes 4, 6 and 7 took longer to develop, produced fewer offspring in the time equal to the pre-reproductive period and had the lowest survival probabilities. Only genotype 3 aphids from the clonal line AK11/01 displayed intermediate properties between these two groups, with relatively short development times but low R_m and survival probabilities akin to those from genotypes 4, 6 and 7.

It is possible that the relatively poor intrinsic fitness of some of the *M. euphorbiae* genotypes reflects trade-offs with other physiological traits, as has been observed in other aphid species. For example, in the peach-potato aphid *Myzus persicae*, clones in which insecticide resistance is conferred through modified acetylcholinesterase (MACE) exhibit lower intrinsic rates of increase, whilst a clone homozygous for knock-down resistance

(*kdr*) displays a concomitant reduction in reproductive output (Foster *et al*., 2003; Fenton *et al*., 2010, but see Castañeda *et al*., 2011; Silva *et al*., 2012). Negative correlations between fecundity and two other aspects of defence, parasitoid resistance and off-plant survival time, have been reported for *A. pisum* clones (Gwynn *et al*., 2005), although other studies have found little evidence of inherent fitness costs to parasitoid resistance (Ferrari *et al*., 2001; von Burg *et al*., 2008; Vorburger *et al*., 2009). Alternatively, the poorly performing *M. euphorbiae* lines may represent specialized clones, races in which performance is maximised on one host plant to the detriment of fitness on other hosts. Host-adapted races have been identified in several species of aphid (Sunnucks *et al*., 1997; Vialatte *et al*., 2005; Frantz *et al*., 2006; Simon *et al*., 2003; Ruiz-Montoya *et al*., 2003), and genetic evidence for host-plant utilization trade-offs identified in *A. pisum*, *Aphis gossypii* and *Sitobion avenae* (Mackenzie, 1996; Leonardo & Muiru, 2003; Leonardo, 2004; Carletto *et al*., 2009). Should such trade-offs exist in *M. euphorbiae* populations, the intrinsic fitness of specialised clones reared on potato plants to which they are maladapted would be relatively low.

Resource investment in particular aphid life history traits, be it increased survival through defence against natural enemies or pathogens or through maximising survival and reproductive output on a given host plant, could be mediated by secondary endosymbiont bacteria (Tsuchida *et al*., 2004; Scarborough *et al*., 2005; Oliver *et al*., 2005; Vorburger *et al*., 2010; Łukasik *et al*., 2013a). As these bacteria are not ubiquitous, it is often presumed that there are also costs to the aphid hosts of such bacterial infections (Tsuchida *et al*., 2002; Simon *et al*., 2003; Oliver *et al*., 2006). Within this study, however, the clonal potato aphid lines harbouring *H. defensa* were amongst the fittest, exhibiting fast development times to adulthood and first reproduction, and high intrinsic rates of population increase and survival probabilities.

All of the genotype 2 *M. euphorbiae* aphids successfully reared in culture naturally harboured *H. defensa*, and attempts to cure sub-lines of the secondary endosymbiont infection were unsuccessful. It is therefore not possible to infer from these results whether the presence of *H. defensa* impacted upon the fitness characteristics measured here other than to observe that the overall fitness of genotype 2 aphid lines with their natural *H. defensa* infection was greater than that of the three aphid lines from genotypes 4, 6 and 7, all of which harboured only *B. aphidicola*. In contrast, clonal lines of genotype 1 aphids that harboured *H. defensa*, that were naturally free of *H. defensa* and

that were artificially cured of *H. defensa* were available for comparison. Within this common genetic background, there were no significant differences between time to development, time to first reproduction, rate of population increase and survival probabilities, despite the variation in endosymbiont status, and all had a greater overall fitness than the aphids from genotypes 4, 6 and 7. It therefore appears that, for genotype 1 aphids at least, there were no inherent costs to harbouring the *H. defensa* bacterium, nor were there any fitness costs apparent in clonal line AA09/03 to harbouring *H. defensa* without the APSE phage. In *A. pisum* the deleterious effects of such an association stem from the uncontrolled proliferation of the *H. defensa* in the absence of the lytic phage (Weldon *et al*., 2013), an effect not apparent when the natural secondary endosymbiont complements were quantified (see section *3.3.2.3*). It is possible that detrimental effects of *H. defensa* infection on the fitness of the aphid host may only be apparent when aphids are competing for resources or when exposed to natural enemy pressures, as has been demonstrated in population cage experiments using sub-lines of an *A. pisum* clone with and without *H. defensa* infection (Oliver *et al*., 2008).

Few deleterious effects have been demonstrated clearly to explain the distribution and maintenance of facultative endosymbionts such as *H. defensa*. Both *S. symbiotica* and *H. defensa* have been shown to reduce longevity in clones of *A. pisum* and *A. fabae*, respectively (Koga *et al*., 2007; Vorburger & Gouskov, 2011). However, such effects are not always apparent, with both the genotype of the aphid host and the strain of the endosymbiont affecting the expression of inherent aphid fitness traits (Chen *et al*., 2000; Vorburger & Gouskov, 2011; Łukasik *et al*., 2013b). For example, one recent study on the grain aphid, *Sitobion avenae*, found that lines that naturally harboured *H. defensa* had the greatest fecundity, even after the secondary endosymbiont infection had been eliminated (Łukasik *et al*., 2013b). Consequently, correlative studies such as that by Vorburger *et al*. (2009) and Castañeda *et al*. (2010), in which *A. fabae* clones naturally infected with *H. defensa* displayed greater overall fitness relative to uninfected lines might be more informative, reflecting selection against combinations of endosymbiont strain and aphid genotype that are detrimental to the fitness of the endosymbiont partners. Similarly, the occurrence of *H. defensa* in only two of the six distinct *M. euphorbiae* genotypes identified here might represent favourable combinations of *H. defensa* strain and potato aphid genotype. Empirical evidence for such a hypothesis would require the successful transfection of *H. defensa* strains into a range of *M. euphorbiae* genotypes.

In addition to genotype, the effect of wing morph was very highly significant on development time, pre-reproductive period and intrinsic rate of population increase, reflecting the reduction in reproductive investment concomitant with increased resource allocation to wing muscle production (Zera & Denno, 1997; Ishikawa & Muira, 2009). The R_m also differed significantly between trials, with shortening day lengths and cooler outdoor temperatures potentially reducing the quality of the plants used in the second temporal block, which in turn may have had a negative impact on the fitness of the aphids that were feeding upon them. Similarly, the significant variation in development times between aphids reared in different positions in the performance assay probably reflects environmental variation within the glasshouse.

The differences in life history characteristics observed here may in part explain the predominance of aphid genotypes 1, 2 and 3 in the potato aphid lines collected for this study, although the sampling effort was too restricted to form conclusions about the clonal population structure of potato aphids in eastern Scotland. Despite the clear disparities in inherent fitness parameters between *M. euphorbiae* genotypes under the conditions investigated, these differences alone are unlikely to determine the prevalence of each genotype in the field. Numerous extrinsic ecological factors are known to curb exponential aphid population growth and shape the spatial and temporal structure of aphid populations, including fluctuating pressures from natural enemies, plant distribution and quality, thermal tolerances and, with recent agricultural practices, insecticide resistance (Karley *et al*., 2003; Vorburger, 2004, 2006; Fenton *et al*., 2005; Kasprowicz *et al*., 2008). Even when natural enemies are excluded, field experiments have demonstrated that both biotic and abiotic factors can diminish or obscure differences in aphid fitness observed in more controlled conditions (Stadler, 1998, Darby *et al*., 2003).

Parasitoids have been shown to contribute, by varying degrees, to the dynamics of potato aphid populations on crop plants (Shands *et al*., 1965; Sullivan & van den Bosch, 1971; Walker *et al*., 1984; Nakata, 1995; Karley *et al*., 2003), and the final section of this study therefore investigates the outcome of interactions between the various *M. euphorbiae* genotypes and the parasitoid *Aphidius ervi*. Furthermore, whilst natural infections of the *H. defensa* endosymbiont do not negatively affect the development time, rate of population increase or survival of the *M. euphorbiae* aphid hosts, as yet there is little evidence to explain what might maintain the endosymbiont infection within these potato

aphid genotypes. Given the role that *H. defensa* and particular strains of the associated APSE bacteriophage have been shown to play in parasitoid resistance in certain pea aphid clones, any degree of resistance bestowed by *H. defensa* on the potato aphid host would further our understanding of the selection pressures conserving the endosymbiosis.

4.4.3 Summary and conclusions

The oral administration of a combination of antibiotics, following a method piloted in *A. pisum* and other aphid species, successfully eliminated *H. defensa* from several clonal lines of *M. euphorbiae*. Based on current understanding of primary and secondary endosymbiont transfer within parthenogenetic aphid lineages, endosymbiont titres were most likely decreased within the haemolymph of the treated aphid, restricting the transmission of *H. defensa* to the developing embryo to titres below which bacterial infections could not become established. Although sub-lines free of secondary endosymbionts were generated, the success rate for generating cured *M. euphorbiae* lineages was low; it is unclear whether this was a result of genotypic differences or a consequence of natural variation in feeding rates between individuals leading to different dosages of antibiotics being imbibed. In addition, despite allowing for a minimum of ten generations for the effects of the antibiotics to attenuate, titres of *B. aphidicola* were lower in the cured potato aphid sub-lines compared with the disymbiotic parental lineages and compared with aphids from the same genotype naturally free of secondary endosymbionts. Whilst no fitness costs associated with depressed *B. aphidicola* densities were detected in the performance assays of the cured sub-lines, there may be unanticipated effects on other aspects of aphid fitness, particularly during times of nutrient stress when demands on the primary endosymbiont may be increased. Nevertheless, the ability to generate such cured sub-clonal lineages is a useful tool for disentangling endosymbiont-mediated heritable traits from those attributable to aphid genetic variation.

Within the sub-set of *M. euphorbiae* clonal lines reared in culture for which inherent fitness parameters were measured, traits associated with both survival and reproduction were highest in those from genotypes 1, 2 and, to a lesser extent, genotype 3. Although these three genotypes predominated in the clonal lines collected for this study, any conclusions about the contribution of genotypic differences in fitness to the genetic

structure of *M. euphorbiae* populations remains speculative. The poor overall fitness seen in the potato aphid clones representing genotypes 4, 6 and 7 may reflect costs associated with investment in insect adaptations to specific environmental factors, such as host plant specialization or insecticide resistance.

All of the *M. euphorbiae* clonal lines naturally infected with *H. defensa* were from genotypes 1 and 2 (see section *3.3.2.2*), the two genotypes with the greatest overall fitness in the performance assay. Harbouring the endosymbiont therefore did not reduce aphid clonal fitness below that of aphids from other genotypes, all of which lacked *H. defensa*. Furthermore, comparisons of sub-clonal potato aphid lines differing in their endosymbiont status from genotype 1, including lines both naturally free of secondary endosymbionts and artificially cured of *H. defensa*, showed no significant differences in time to development and reproduction, rate of population increase or survival probabilities, suggesting there were no inherent costs or benefits to these aphids of carrying *H. defensa*. Transfection of the *H. defensa* bacterium into a range of aphid genotypes would be required to establish whether there are costs to the aphid host of harbouring *H. defensa* in other *M. euphorbiae* genotypes that prohibit stable infections.

This study found little evidence that *H. defensa* infected potato aphids are under strong selection pressure, either positive or negative, due to their impact on aphid fitness, and thus the factors underlying the maintenance of *H. defensa* at intermediate frequencies in natural potato aphid populations remain unexplained. However, measuring aphid performance under less benign conditions might reveal costs and/or benefits to harbouring *H. defensa* on which selection is acting, and could elucidate further factors shaping clonal frequencies within *M. euphorbiae* populations. One potential factor is the presence of natural enemies, and the remainder of this study focusses on the effect of endosymbiont complement and aphid genotypic background on the interaction between *M. euphorbiae* aphids and the parasitoid wasp *Aphidius ervi*.

Chapter 5: Interactions between *M. euphorbiae*, *H. defensa* and the parasitoid *Aphidius ervi*

5.1 Introduction

Numerous biotic and abiotic factors alter the dynamics of aphid populations, amongst which pressure from parasitoids can be a major influence (Singh & Sinha, 1983; Snyder & Ives, 2003; Schmidt *et al*., 2003; Liu *et al*., 2004; Miao *et al*., 2007). However, several aspects of aphid ecology, behaviour and physiology affect the reproductive success of parasitoids, which must find patches of acceptable hosts and overcome aphid defensive behaviours in order to oviposit in hosts suitable for the developing parasitoid larvae.

5.1.1 Parasitoid optimal reproductive strategies

5.1.1.1 Locating hosts

A number of stimuli may be utilised by aphidiine parasitoids searching for hosts in a heterogeneous environment, both from the aphids themselves and from the plants on which they feed. To first locate habitats likely to contain suitable aphid hosts, some parasitoids rely initially on constitutive chemical cues from the plants (Lo Pinto *et al*., 2004). Far more informative to searching parasitoids and the predominant method of host location in the Aphidiinae are the volatile semiochemicals produced by plants in response to aphid feeding (Reed *et al*., 1995; Blande *et al*., 2007). The complex olfactory cues produced by plants in response to herbivore damage can allow some parasitoids to distinguish between plants infested with host and non-host aphid species, and between current infestations and past damage (Du *et al*., 1996, 1998; Powell *et al*., 1998).

Parasitoids such as *Aphidius colemani* and *Diaeretiella rapae* initially show preferences in their searching behaviour for the aphid species and/or plant on which they developed, primed by chemical cues on the mummy from which they eclosed (Wickremasinghe & Emden, 1992; Storeck *et al*., 2000; Pope *et al*., 2008). However, for these and other parasitoids, responses to infochemicals emanating from aphid hosts or aphid–plant

complexes can be modified by oviposition experience and can alter host preference (Grasswitz & Paine, 1993; Du *et al*., 1997; Storeck *et al*., 2000; Micha *et al*., 2000).

5.1.2.2 Host acceptability and suitability

Having located a patch of potential hosts, the female parasitoid must decide whether to attack and oviposit one or more eggs. This requires her to assess the quality of the aphids in terms of their relative suitability for a developing larva, alongside the likely availability of alternative hosts in other patches and to take account of any handling risks involved during attack (Godfray, 1994). Haplo-diploidy is the principal mechanism of sex determination in the hymenoptera, whereby the sex of the eggs is under the behavioural control of the female parasitoid; unfertilised haploid eggs develop into males and fertilised diploid eggs into females (Quick, 1997). Parasitoids developing in poorer quality hosts suffer increased mortality, and surviving offspring are smaller. As the fitness costs of developing in poor quality hosts are proportionately greater to female offspring, whose fecundity is directly proportional to their size, it is thought that aphidiine parasitoids are more likely to lay male eggs in poor quality aphids (Cloutier *et al*., 1991; Pandey & Singh, 1999). It cannot be ruled out, however, that some of the observed bias in sex ratios is the result of differential male and female larval survival in aphids of differing quality (Wellings *et al*., 1986; Jarošík *et al*., 2003).

Both external and internal assessments may be used by parasitoids to recognise and assess potential hosts. Visual cues, evaluated before contact is made, can affect host selection, with several parasitoid species demonstrating disparate attack rates on different colour morphs of otherwise suitable aphid species (Ankersmit *et al*., 1986; Michaud & Mackauer, 1994, 1995; Battaglia *et al*., 1995), although again this can be altered by experience (Langley *et al*., 2006). Given that developing larvae are entirely dependent on the resources available from their aphid host, the size and/or age of a potential host also affects host acceptability (Rakhshani *et al*., 2004; Tahriri *et al*., 2007). Although adult aphids contain the largest quantity of resources for developing larvae, development time in adult aphids is often longer and there is a greater risk of aphid behavioural or physiological defences preventing successful parasitism, whilst the incidence of host mortality resulting from parasitoid attack is often greatest in the smallest aphid hosts. For koinobiont parasitoids in which the hosts continue to feed as the larvae grow, the

developmental stage and feeding ecology of the host also greatly affect host quality (Shu-sheng, 1985; Sequeira & Mackauer, 1992, 1994; Stadler & Mackauer, 1996; Colinet *et al*., 2005). It is therefore hosts of intermediate age and/or size that are preferentially attacked by many Aphidiinae parasitoids (Kouamé & Mackauer, 1991; Chau & Mackauer, 2001; Tsai & Wang, 2002; Colinet *et al*., 2005; but see Lin & Ives, 2003).

Cornicle secretions of suitable hosts, honeydew and species-specific cuticle kairomones, perceived on antennal receptors from short range or upon contact, provide another means by which parasitoids can discern information regarding their hosts (Grasswitz & Paine, 1992; Powell *et al*., 1998). Such semiochemicals, as well as visual cues such as host movement and aphid shape and colour, also act singly or in combination as attack stimuli (Michaud & Mackauer, 1994, 1995; Battaglia *et al*., 1993, 1995, 2000; Powell *et al*., 1998).

The internal chemistry of a potential aphid host may also influence oviposition decisions, with various mechano-, proprio- and chemo-sensory sensillae identified on parasitoid ovipositors (Ralec *et al*., 1996). Ovipositor probing can also reveal the presence of secondary endosymbionts within potential aphid hosts, a further factor influencing host acceptance (Oliver *et al*., 2012; Łukasik *et al*., 2013b). For parasitoids such as *A. ervi*, both internal and external cues are required to elicit a strong oviposition response in pea aphid hosts; the presence of cornicle secretions can stimulate an attack, but oviposition requires the detection of suitable host haemolymph (Larocca *et al*., 2007). Other aphid parasitoids appear predominantly reliant on visual and tactile cues to assess their host, or are almost entirely dependent on internal chemosensory information acquired through ovipositor probing (Pennacchio *et al*., 1994; Michaud & Mackauer, 1995; De Farias & Hopper, 1999).

The physiology of the parasitoid and in particular both current egg load and, in synovigenic parasitoids, the rate at which egg reserves can be replenished can also influence oviposition decisions (Mangel, 1989). For example, the rate at which the parasitoid *Monoctonus pseudoplatani* oviposits in its sycamore aphid host decreases and discrimination against poorer-quality hosts increases as egg load diminishes (Collins & Dixon, 1986). Egg maturation may also be responsive to perceived future reproductive opportunities, with the aphid parasitoid *Aphelinus albipodus* maturing greater numbers of eggs in the presence of early instar aphid hosts (Wu *et al*., 2010).

In addition, the probability of a given host being accepted is also likely to be affected by the quality and quantity of alternative hosts available. Both type II and type III functional responses in response to increasing host density are common within the Aphidiinae, with parasitism rates affected by searching efficiency and handling time (Singh & Sinha, 1983; Jones *et al*., 2003; Rakhshani *et al*., 2004; Fathipour *et al*., 2006; Tahriri *et al*., 2007). The presence of other parasitoids, meanwhile, negatively impacts searching efficiency through mutual interference, and can lead to shorter patch time to avoid superparasitism (Fathipour *et al*., 2006; Tahriri *et al*., 2007). Ovipositing in an already parasitized aphid results in larval competition, and usually the younger larva is eliminated through physiological suppression or physical attack (Bai, 1991). Consequently parasitoids will avoid laying an egg in a host already attacked by a conspecific parasitoid (superparasitism) or by another species of parasitoid (multiparasitism) when better quality hosts are available (Bai, 1991; Bai & Mackauer, 1991). Host discrimination may be based on changes to the host physiology following attack, or the result of external or internal host marking by the parasitoid (Hofsvang, 1988; Outreman *et al*., 2001; van Baaren *et al*., 2009). In certain circumstances, however, superparasitism may be an advantageous reproductive strategy for solitary parasitoids (Van Alphen & Visser, 1990; Bai & Mackauer, 1992; Mackauer *et al*., 1992). Self-superparasitism may also help overcome host defences; the added venom and teratocytes resulting from multiple attacks increases the successful parasitism by *A. ervi* of pea aphids harbouring protective *H. defensa* (Oliver *et al*., 2012).

To summarise, the optimal patch use and oviposition strategies used by aphid parasitoids to maximise their offspring production, and hence their fitness, depends on many factors. Furthermore, oviposition strategies are adaptive, with prior experiences of encountering suitable hosts either modulating or enhancing cue responses (Li *et al*., 1997). Also called the preference-performance hypothesis (Jaenike, 1978), the supposition that female insects will evolve to oviposit within hosts on which their offspring most prosper is supported by some empirical evidence (Chau & Mackauer, 2001), and yet is also contradicted by work in which the perceived and realised quality of potential hosts are inconsistent (Henry *et al*., 2005; Desneux *et al*., 2009). The work presented here focuses on two aspects of oviposition; investigating whether the presence of *H. defensa* affects either (i) the acceptability or (ii) suitability of *M. euphorbiae* hosts to the parasitoid *A. ervi*. Genetic variation in both the quality of *M. euphorbiae* hosts to developing

parasitoid larvae and in the decisions shown by *A. ervi* in whether to oviposit in genetically distinct *M. euphorbiae* lines under no-choice situations is also examined.

5.1.2 Parasitoids as potential vectors

The incongruent phylogenies of aphids and their secondary bacteria connotes both the occasional failure in vertical transmission from one aphid generation to the next and the rare horizontal transfer of endosymbionts, both within and between aphid species (Sandström *et al*., 2001; Russell *et al*., 2003; Tsuchida *et al*., 2006). For holocyclic aphid populations, endosymbionts may be transmitted during sexual reproduction. Secondary bacteria are located in the accessory glands of male reproductive organs of pea aphids, and sexual crosses have produced paternally inherited stable infections in their parthenogenetic offspring, accompanying or often replacing maternally inherited endosymbionts (Moran & Dunbar, 2006). Outwith sexual reproduction, the lateral transfer of aphid endosymbionts may be mediated by other organisms with which aphids interact.

Successful transfection experiments using endosymbiont-laden artificial diets illustrate that secondary bacteria such as *H. defensa* are able to cross the aphid gut wall to colonise new hosts (Darby & Douglas, 2003). With *H. defensa* found in both the siphuncular fluid and the honeydew exuded by infected pea aphids, endosymbionts could be acquired orally, taken up either from plant tissues or from the leaf surface as the aphid feeds. Whilst the transmission of a *Rickettsia* species is plant-mediated in white-fly (Caspi-Fluger *et al*., 2011), there is no evidence that the phloem of plants serve as reservoirs for other known aphid endosymbionts. Furthermore, several experiments allowing infected and uninfected aphids to feed either simultaneously or subsequently on plants have yet to show horizontal transmission (Chen & Purcell, 1997; Chen *et al*., 2000; Darby & Douglas, 2003).

Higher trophic levels may too facilitate the transfer of endosymbionts. Ectoparasitic mites feeding on insect haemolymph have been shown to transfer *Spiroplasma* from infected to uninfected *Drosophila* hosts (Jaenike *et al*., 2007), although similar experiments using *A. fabae* infected with *H. defensa* or *R. insecticola* failed to demonstrate lateral transfer (Gehrer & Vorburger, 2012). Parasitoids may also act as

vectors, with endosymbiont bacteria transferred from the haemocoel of one aphid host to another as female aphidiine wasps insert their ovipositor, either to probe for further information regarding the suitability of a host or to oviposit. Stable, inheritable infections of *H. defensa* and *R. insecticola* have been established in *A. fabae* in this way, transmitted by *A. colemani* and *L. fabarum* parasitoids (Gehrer & Vorburger, 2012), although such transfection events are yet to be replicated at a larger scale in caged aphid population experiments (Oliver *et al*., 2008).

The intimate interaction between parasitoids and their hosts can also result in the parasitoids internally harbouring endosymbionts. Adult parasitoids that feed on the honeydew or haemolymph of the whitefly *Bemisia tabaci* can acquire transient infections of *H. defensa* and *Rickettsia*, whilst feeding on the tissues of their host by developing larvae results in detectable endosymbiont levels within the pupae (Chiel *et al*., 2009). However, such endosymbiont acquisition has not been demonstrated in adult parasitoids eclosing from aphids harbouring secondary bacteria (Gehrer & Vorburger, 2012).

Given the protective role against parasitoids that several *H. defensa* strains have been shown to confer to their aphid hosts, by mediating the horizontal transfer of these endosymbionts the parasitoids could be said to facilitate their own destruction. The final experiment of this chapter therefore seeks to investigate the possibility of lateral transfer of the *H. defensa* endosymbiont between *M. euphorbiae* lines carried on the ovipositor of *A. ervi*. In conjunction with the results of the host acceptability and susceptibility experiments, this will indicate not only the capability of the parasitoids to vector the endosymbionts, but also whether by doing so they are acting against their own interests.

5.1.3 Study objectives and hypotheses

The objectives of the study presented in this chapter are:

1. To quantify the susceptibility of potato aphid clones to parasitism by *A. ervi* parasitoids, thus exploring the hypothesis that there would be variation in the degree of resistance exhibited by different aphid holobionts. With parasitoids one of mulitple mortality factors acting upon *M. euphorbiae* populations, aphid resistance to endoparasitoids such as *A. ervi* should be under positive selection yet may be constrained by the costs of such traits, resulting in variation in parasitoid

susceptibility amongst potato aphid clones. A secondary hypothesis was to test whether the endosymbiont *H. defensa* can increase the resistance of *M. euphorbiae* to parasitism by *A. ervi*.

2. To determine whether variations between *M. euphorbiae* lines in mummification originates from differences in parasitoid oviposition rates based on the perceived quality of the aphid host by the parasitoid (host acceptability), or from differences in the realised quality of the aphid hosts for parasitoid development (host suitability). Based on the aphid literature, I hopothesised that reduced susceptibility to parasitism would be due to decreased aphid suitability for supporting wasp larval development. To this end, aphids were dissected at set time points following parasitoid attack to determine the presence and fate of developing parasitoid larvae. The *M. euphorbiae* lines used were selected to represent different genotypes and endosymbiont complements, allowing the contribution of genetic and endosymbiont-mediated variation to parasitoid resistance to be determined.

3. To ascertain whether *H. defensa* could be transmitted to uninfected aphid clones through failed or aborted attacks by *A. ervi* parasitoids. In addition to recognising the ecologically important heritable traits that secondary endosymbiont bacteria confer on their hosts, to fully comprehend their impacts on aphid population dynamics also requires the frequency and manner by which stable endosymbiont infections are lost and gained to be understood. The hypothesis that parasitoids can act as vectors for horizontal transfer of aphid secondary endosymbionts was therefore investigated by allowing *A. ervi* parasitoids to sequentially attack infected and non-infected *M. euphorbiae* lines, the latter of which were then screened for novel and heritable acquisitions of *H. defensa*.

5.2 Materials and Methods

5.2.1 Preliminary assays to assess parasitism rates of M. euphorbiae *aphids*

Five *M. euphorbiae* clonal lines, differing in their endosymbiont complement, were used in preliminary experiments to quantify aphid susceptibility to parasitism (Table 5.1).

M. euphorbiae line	Genotype	Secondary endosymbionts present
AA09/02	4	None found
AA09/03	1	*H. defensa*
HC10/02	2	*H. defensa* (+ APSE)
HC10/05	2	*H. defensa* (+ APSE)
HC10/08	7	None found

Table 5.1: The genotypes and endosymbiont complement of the five *M. euphorbiae* lines initially assessed for parasitism suitability.

To assess parasitism rates on whole potato plants, 50 small *S. tuberosum* (cv. Désirée) plants aged approximately 3 weeks were confined within a mesh cage (see section *2.1.2*). A group of 25 *M. euphorbiae* nymphs aged 2–4 days was transferred to each plant, replicated 10 times for each of the 5 aphid lines (Table 5.1). The plants were transferred to two growth cabinets set to 20°C ± 1°C, 60% humidity and with a light regime of 16 h light: 8 h dark in a randomised block design. The following day, two female and one male *A. ervi* wasp were added to each cage along with a ball of cotton wool soaked in a 50% (v/v) honey solution, and were left for approximately 72 hours before being removed. 14 days after the parasitoids were first introduced, the numbers of live and parasitized aphids present on each plant were counted.

Parasitism rates were also assessed on excised potato leaves in culture cups (section *2.1.1*), to minimise the effects of the plant architectural complexity on insect behaviour. A group of 30 *M. euphorbiae* nymphs aged 2–4 days was transferred to each leaf, replicated 6 times for each of the 5 *M. euphorbiae* lines (Table 5.1), and maintained at 20°C ± 1°C, 60% humidity and with a light regime of 16 h light: 8 h dark. After 24 h, one male and one female *A. ervi* parasitoid aged 3 days were added to each cup for 6 hours and then removed. The numbers of live and parasitized aphids present in each cup were counted after a further 14 days, with the leaf cuttings changed as necessary.

For both assays, parasitism rates were calculated as the proportion of aphids present 14 days after exposure to the parasitoids that had been successfully mummified. Aphids unaccounted for or that had died post-assay were excluded from the analysis. Analyses of variance (ANOVAs) were applied to compare parasitism rates between aphid lines using the software programme **R** (v. 2.14.0, www.R-project.org).

5.2.2 Assessing the acceptability and suitability of *M. euphorbiae* aphids to *A. ervi* parasitoids

5.2.2.1 Generating cohorts of aphids each singly attacked by A. ervi

9 *M. euphorbiae* clonal lines, selected to represent different secondary endosymbiont complements and a range of aphid genotypes, along with two of the sub-lines cured of the *H. defensa* endosymbiont, were used to investigate the acceptability and suitability of *M. euphorbiae* aphids to *A. ervi* parasitoids (Table 5.2).

***M. euphorbiae* line**	**Genotype**	**Secondary endosymbionts present**
AA09/03	1	*H. defensa*
AA09/04	1	*H. defensa* (+ APSE)
AA09/12	1	None found
AA09/03^{ab}4.1b	1	Cured of *H. defensa*
AA09/04^{ab}3.8c	1	Cured of *H. defensa* (+ APSE)
HC10/02	2	*H. defensa* (+ APSE)
HC10/05	2	*H. defensa* (+ APSE)
AK11/01	3	None found
AA09/02	4	None found
HC10/07	6	None found
HC10/08	7	None found

Table 5.2: The genotype and endosymbiont complement of the 11 aphid clonal lines and sub-lines used to investigate the acceptability and suitability of *M. euphorbiae* to parasitoids.

Experimental arenas were prepared by fixing round potato leaves (var. Désirée), into 1% agar gel in Petri dishes (as detailed in section *2.3.2.1*). Thirty nymphs aged 3–4 days from a given *M. euphorbiae* line were transferred to the arena, and left to settle for at least 10 minutes. A single female *A. ervi* parasitoid aged 2–5 days and presumed mated was then introduced. The parasitoid was observed and, every time an attack was made on a nymph, the aphid was removed and transferred to one of two culture cups set up as given in section *2.1.1* until a total of 60 aphids had been attacked. The aphid density was maintained in the arena by replacing attacked aphids with fresh nymphs.

If the parasitoid failed to make any attacks for more than 5 minutes, most commonly as a result of becoming coated with aphid cornicle secretions, she was removed and replaced

with a second female parasitoid. The time taken for the parasitoid(s) to attack 60 aphids and the age, generation and batch of the parasitoids used was recorded.

Five replicate assays were conducted for each of the 9 *M. euphorbiae* lines and 2 cured sub-lines to assess the acceptability of each *M. euphorbiae* clone to *A. ervi* parasitoids, and a further five replicates were conducted to assess the suitability of each *M. euphorbiae* clone to the developing parasitoid larva. Culture cups containing the attacked aphids were kept in a controlled environment room at 20°C ± 2 °C, 60% humidity and with 16 h light: 8 h dark.

5.2.2.2 Quantifying host acceptability and suitability

To determine whether there were differences in the parasitoid's acceptance of the various *M. euphorbiae* clones, for each replicate 30 of the 60 attacked aphids were dissected in PBS solution between six and ten hours following the parasitoid attack (see section *2.3.3.1*). The number of aphids that contained a parasitoid egg was recorded. To provide a search image for the parasitoid egg and to ensure aphid embryos, which at a certain stage of development are of a similar size and shape, were not mistakenly counted (as shown in Figure 5.1), the ovaries from a naïve female *A. ervi* parasitoid were removed and ruptured so the eggs could be observed. Efforts were made to confirm that parasitoid eggs were successfully being distinguished from developing *M. euphorbiae* embryos by PCR amplification of parasitoid and aphid DNA. However, this approach was unsuccessful due to insufficient amounts of DNA being recovered from the parasitoid reproductive organs or aphid embryos.

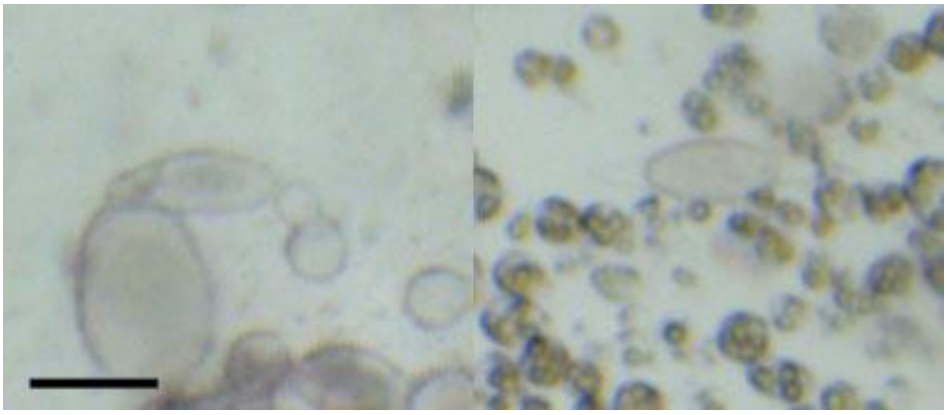

Figure 5.1: Developing embryo from the ovariole of an *M. euphorbiae* aphid aged 3 d (left) and an *A. ervi* egg 8 hours post oviposition (right). The scale bar represents 100 µM.

The 30 attacked aphids in the second culture cup were reared for 12 days, with the leaf material replaced in the cup as necessary. After this time, the number of mummies and the number of live adult or fourth instar aphids were counted. Replicates in which more than five aphids had died or were unaccounted for were disregarded.

The same procedure was followed to determine the suitability of the various *M. euphorbiae* clones for developing *A. ervi* larvae, but for each replicate 30 of the 60 of the aphids were dissected 4 days after parasitoid exposure. Each aphid was dissected as detailed previously (see *2.3.3.1*), and the number containing a parasitoid larva tallied. Live larvae were distinguished from moribund or deceased larvae by the thrashing movement of the abdomen or the apparent peristalsis visible in the developing gut.

The 30 attacked aphids in the second culture cup were reared for 12 days post attack, at which time the number of mummies and the number of live adult or fourth instar aphids were counted. Replicates in which more than five aphids had died or were unaccounted for were discarded.

5.2.2.3 Analysis of host acceptability and suitability data

Analyses of the egg and larval counts and successful mummification rates from the nine *M. euphorbiae* lines harbouring their natural endosymbiont complement were performed using the software programme **R** (v. 2.14.0, www.R-project.org), with graphs plotted in SigmaPlot (v. 12.3, Systat Software).

The results of the host acceptability and host suitability experiments were analysed using two-way analyses of variance (ANOVA), with Tukey's Honest Significant Difference (HSD) tests used to isolate differences between the means. ANOVAs were constructed so as to ascertain any significant differences in the counts between parasitism stages (egg/larval counts against mummy counts), in the total parasitism counts between aphid groups (be it between aphid lines, genotypes or when grouped by endosymbiont status), and in the interactions between aphid group and parasitism stage.

To determine which factors and covariates contributed to any observed differences in either egg, larval or mummy counts, generalised linear models (GLMs) with a Poisson

error distribution (based on dispersion parameter ø ≈ 1), incorporating aphid genotype, endosymbiont status within genotype, the age, generation and batch of the parasitoids, the number of parasitoids used and the time to attack each set of 60 aphids were fitted to the counts from the host acceptability and suitability experiments. Akaike Information Criterion (AIC and AICc) scores and weights were used to assess the relative goodness of fits of various models. The natural occurrence of *H. defensa* in only two of the potato aphid genotypes led to the two variables confounding; endosymbiont status was therefore nested within genotype.

The same analyses were used to compare the egg, larval and mummy counts of the five *M. euphorbiae* lines from genotype 1 that differed in their endosymbiont status: lines AA09/03 and AA09/04 that naturally harboured *H. defensa* with and without APSE, respectively, line AA09/12 that was naturally free of *H. defensa* and lines AA09/03 ^{ab}4.1b and AA09/04^{ab}3.8c, both of which had been cured of their *H. defensa* infection.

5.2.3 Establishment of novel heritable infections through ovipositor attacks

5.2.3.1 Sequential parasitoid attacks on infected and uninfected M. euphorbiae *clones*

Three isofemale *M. euphorbiae* lines were used to investigate the occurrence of horizontal transmission of *H. defensa* from an infected to an uninfected aphid by means of transfer on the ovipositor of the parasitoid *A. ervi* (Table 5.3).

In order to increase the likelihood of detecting novel, heritable infections clone AA09/12 was selected as the potential recipient. As well as being free of known secondary endosymbionts, the parasitoid resistance of this clone was high, thus minimising the proportion of attacked aphids likely to succumb to parasitism and thus curtail the establishment of potential endosymbiont infections. Furthermore, the AA09/12 line belongs to genotype 1, in which stable *H. defensa* infections are found in other clones.

***M. euphorbiae* clonal line**	**Endosymbiont status**	**Role**
HC10/05	*H. defensa* (+ APSE)	Donor line
AA09/12	No secondary endosymbionts	Recipient line
AK11/01	No secondary endosymbionts	Control donor line

Table 5.3: The three clonal lines of *M. euphorbiae* used in the ovipositor transfection experiment, and their endosymbiont complement

Approximately 30 three- and four-day-old *M. euphorbiae* aphids from the donor line HC10/05 were transferred to the underside of a single round potato leaf (var. Désirée), set into 1% agar gel in a Petri dish (as detailed in section *2.3.2.1*). A single *A. ervi* female aged 2–3 days and presumed mated was introduced into the arena, and observed until she had made 5 attacks on the aphid nymphs. The parasitoid was then immediately transferred to another leaf fixed in a second, smaller Petri dish and containing a single three-day-old *M. euphorbiae* nymph from the recipient line AA09/12. After three further attacks (or after a maximum of 10 minutes, if less than three attacks were made) the parasitoid was removed and frozen, whilst the AA09/12 aphid was transferred to an excised leaf in a culture cup and reared in a controlled environment room at 18°C ± 2 °C, 60% humidity and with 16 h light: 8 h dark. (see section *2.1.1*).

A total of 30 replicates in three temporal blocks were conducted in this manner, each using fresh parasitoid arenas, naïve parasitoids and donor and recipient aphids not previously exposed to parasitoid attack.

5.2.3.2 Eliminating other potential sources of novel heritable infections

A series of control replicates were conducted to confirm that any change in endosymbiont status of the attacked AA09/12 aphids could be attributed to the introduction of the *H. defensa* endosymbiont from the ovipositor of the parasitoid.

To ensure the *H. defensa* bacterium had originated from the donor line of aphids rather than from the parasitoid or from the arena in which the aphids were attacked, three replicates were conducted in which a parasitoid first made 5 attacks on aphids from a control donor line, AK11/01, which lacks secondary endosymbiont bacteria. The parasitoid was then transferred to a second arena and allowed to attack a single AA09/12 recipient aphid as described above (section *5.2.3.1*). In addition, five unattacked nymphs aged 3 days from each of the AA09/12, HC10/05 and AK11/01 lines were collected and frozen, enabling the endosymbiont status of the clones at the time of attack to be confirmed. Single nymphs from each of the three lines were also transferred to excised leaves in individual culture cups and reared in the same manner as the attacked AA09/12 aphids to confirm that the rearing process did not affect the endosymbiont status.

5.2.3.3 Determining instances of horizontal transfer and heritable infection

Each attacked AA09/12 aphid was reared under standard conditions (in a controlled environment room at 18°C ± 2 °C, 60% humidity and with 16 h light: 8 h dark) on an excised leaf within a culture cup for 10 days, at which point the aphid was checked for signs of parasitism. Replicates in which the aphid had become mummified were discarded. Each surviving aphid was transferred to a fresh culture cup 10, 13 and 16 days after they were attacked, generating three cohorts of offspring. On day 19, each attacked aphid was collected from the third culture cup and frozen, ready for subsequent molecular analysis of their bacterial endosymbiont complement.

To investigate the possibility of successful transfection events generating heritable infections, the three cohorts of first generation offspring were left to develop under the standard rearing conditions, with the leaf material replaced as necessary. After 14 days, adult aphids were removed from each culture cup and frozen, ready for molecular diagnostic screening.

Genomic DNA was extracted from the attacked aphids that did not succumb to parasitism, and from their three cohorts of offspring (see section *2.2.1*). Diagnostic PCR analysis was then used to determine whether detectable levels of *H. defensa* were present (section *2.2.2*). Any cohorts of offspring from the attacked AA09/12 aphids found to harbour the *H. defensa* endosymbiont were maintained in culture, enabling the stability of any novel heritable infections to be investigated.

Diagnostic PCR was also used to verify the original endosymbiont complement of the three *M. euphorbiae* lines, to determine that the rearing process and collection of cohorts of offspring did not affect these endosymbiont complements, and to confirm that no secondary endosymbiont infections had become established in the aphids attacked by parasitoids previously exposed to a potato aphid line free from *H. defensa*.

5.3 Results

5.3.1 Parasitism rates of M. euphorbiae *aphids on whole plants and in culture cups*

The proportion of mummified aphids differed significantly between five aphids lines (Figure 5.2), both when parasitized on whole plants and when parasitized in culture cups (Whole plants: $F_{4, 45} = 2.84$, P = 0.035; Culture cups $F_{4, 25} = 9.095$, P = >0.001)

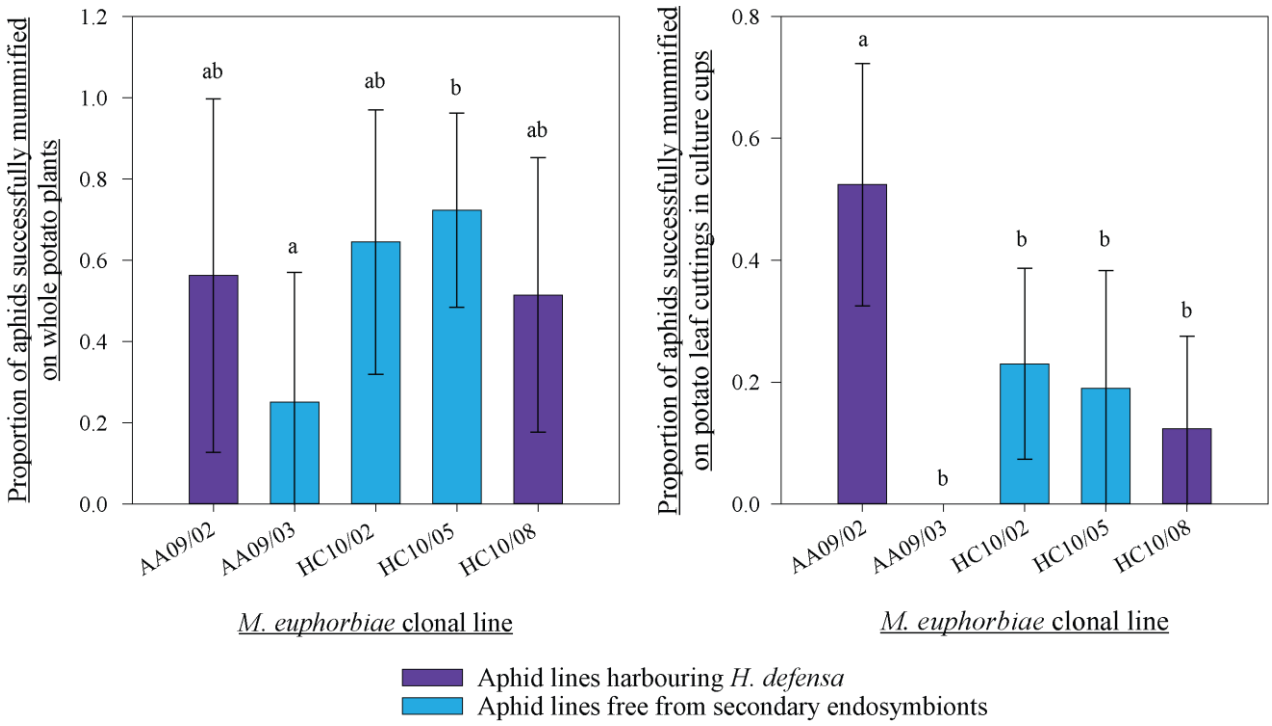

Figure 5.2: Proportion of aphids present 12–14 d following exposure to parasitoids on whole potato plants (left) or on potato leaf cuttings (right) that were successfully mummified. Error bars show ± 1 s. d., whilst columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

The lowest proportion of mummies was found consistently in line AA09/03, although Tukey's post-hoc tests indicated that the only difference in mummy counts significant at the 5% level from the whole plant parasitism assay was between lines AA09/03 and HC10/05, both of which harboured *H. defensa*. In the parasitism assay conducted within culture cups, the mummy counts from line AA09/02 were significantly higher than the remaining four lines.

When grouped by endosymbiont status, the difference in mummy counts between aphids with and without *H. defensa* when parasitized on whole plants was not significant ($F_{1, 48} = 0.00$, P = 0.992). When parasitized in culture cups, the mummy counts from aphids

free from secondary endosymbionts were significantly higher than those infected with *H. defensa* ($F_{1, 28}$ = 5.299, P = 0.029); however, grouping the aphid lines in this way obscures the evident interclonal variations.

5.3.2 The acceptability and suitability of M. euphorbiae *to* A. ervi

5.3.2.1 Host acceptance

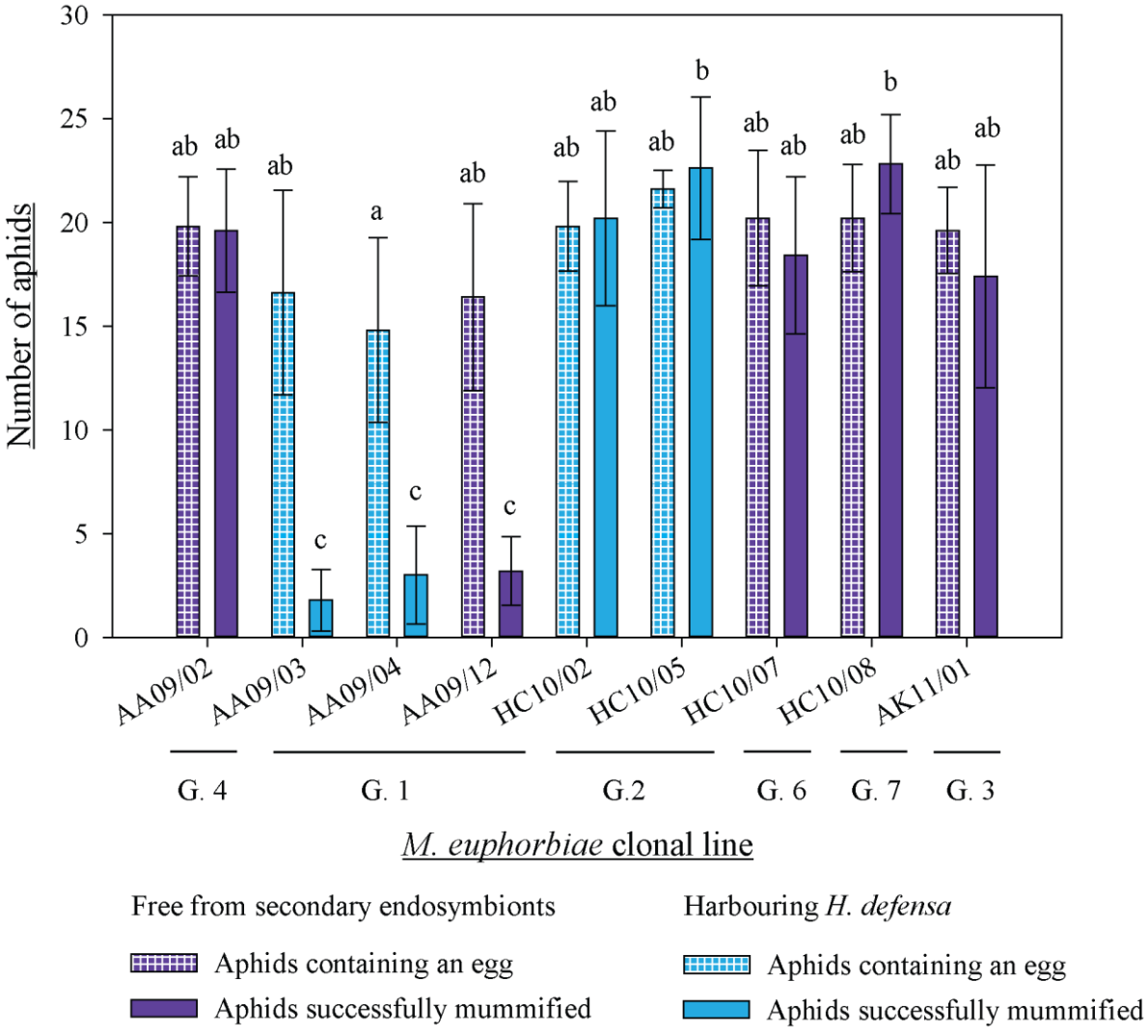

Figure 5.3: Mean egg and mummy counts from singly-parasitized *M. euphorbiae* lines differing in genotype (G. 1, G. 2 etc.) and endosymbiont status. Error bars show ± 1 s. d., whilst columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

There were significant differences in total parasitism rates (egg plus mummy counts) between the 9 *M. euphorbiae* lines ($F_{8, 72}$ = 28.50, P = < 0.001, Figure 5.3), and between egg and mummy counts ($F_{1, 72}$ = 41.09, P = < 0.001); the differences between egg and

mummy counts also varied significantly between aphid lines ($F_{8, 72} = 10.71$, P = < 0.001). The mummy counts from lines AA09/03, AA09/04 and AA09/12 were significantly smaller (at the 0.1% level) than the egg counts of these three lines, and than the egg and mummy counts of the remaining six aphid lines, using Tukey's HSD post-hoc test. In addition, the egg counts from line AA09/04 were significantly smaller than the mummy counts of lines HC10/05 and HC10/08 (P = < 0.05).

These results were reinforced by grouping the aphid lines by genotype. Total counts varied significantly between genotypes ($F_{5, 78} = 46.60$, P = < 0.001), with the number of successfully parasitized genotype 1 aphids, consisting of lines AA09/03, AA09/04 and AA09/12, significantly smaller (at the 0.1% level) than the egg counts from this aphid genotype and the egg and mummy counts of the remaining 5 genotypes. As well as the very highly significant differences between egg and mummy counts ($F_{1, 78} = 42.44$, P = < 0.001), the differences between egg and mummy counts interacted significantly with genotype ($F_{5, 78} = 17.48$, P = < 0.001). Tukey's HSD post-hoc tests identified that the egg counts from the genotype 1 aphids were significantly smaller (at the 1% level) than the egg and mummy counts of the genotype 2 aphids, and the mummy counts of the genotype 7 aphids.

Grouping the aphids by their secondary endosymbiont complement indicated no effect of *H. defensa* on total counts ($F_{1, 86} = 3.556$, P = 0.627). Despite significant differences between the total egg and mummy counts, these differences were similar between aphids with and without *H. defensa* (parasitoid stage: $F_{1, 86} = 9.685$, P = 0.003; endosymbiont group × parasitoid stage: $F_{1, 86} = 1.351$, P = 0.245). Significant differences using Tukey's HSD post-hoc test were found at the 5% level between the mummy counts of aphids harbouring *H. defensa* and the egg counts of aphids either harbouring or lacking the secondary endosymbiont, although this result may have been unduly influenced by the greater proportion of genotype 1 aphids in the *H. defensa*-infected group.

None of the explanatory variables significantly improved the GLM fitted to the egg count data (Appendix 2, section *A2.1.1*), and the most heavily weighted model was described by the intercept only (ln model coefficient for the intercept = 2.933). Therefore, there was no effect of *M. euphorbiae* genotype or endosymbiont complement on the success of parasitoid oviposition, during single attacks.

In contrast, when the contribution of each explanatory variable to the mummification count model were assessed, the effect of genotype was very highly significant (Analysis of deviance genotype: LR χ^2 = 252.165, P = <0.001), whilst the remaining variables were not significant (Appendix 2, section A.2.2). Models including various combinations of these factors and covariates were assessed using AIC and AICc selection criteria. In addition to the intercept, the most heavily weighted model incorporated genotype and parasitoid generation (Table 5.4).

Factor		**ln of model co-efficient**	**Z value**	**P-value**	**Significance (level)**
Intercept		1.3075	5.522	3.35×10^{-8}	Significant (0.1%)
Genotype	Genotype 1	----------Reference----------			
	Genotype 2	2.0611	11.940	2.00×10^{-16}	Significant (0.1%)
	Genotype 3	1.8792	9.836	2.00×10^{-16}	Significant (0.1%)
	Genotype 4	2.0064	10.687	2.00×10^{-16}	Significant (0.1%)
	Genotype 6	1.8757	9.777	2.00×10^{-16}	Significant (0.1%)
	Genotype 7	2.0752	11.061	2.00×10^{-16}	Significant (0.1%)
Parasitoid generation		-0.0416	-1.835	0.0665	Not significant

Table 5.4: Generalised linear model fitted to the mummification count data from the host acceptability experiment. Note that log link function used in the Poisson distribution models returns the natural logarithm of the model co-efficients

The natural log of the co-efficients for genotypes 2, 3, 4, 5 and 7 in the model fitted to the mummification count data were all positive and of similar magnitude, reflecting the low mummification rate of the reference group, genotype 1. Therefore, whilst the acceptability to the *A. ervi* parasitoid of each aphid line was comparable regardless of genotype or secondary endosymbiont infection, the successful development of mummies was strongly affected by aphid genotype.

5.3.2.2 Host suitability

The suitability of *M. euphorbiae* for development of *A. ervi* larvae was assessed for nine *M. euphorbiae* lines, but only 8 of these lines were included in the statistical analyses as the suitability of *M. euphorbiae* aphid line AK11/01 to *A. ervi* was measured at a later date using parasitoids from a genetically distinct parasitoid population.

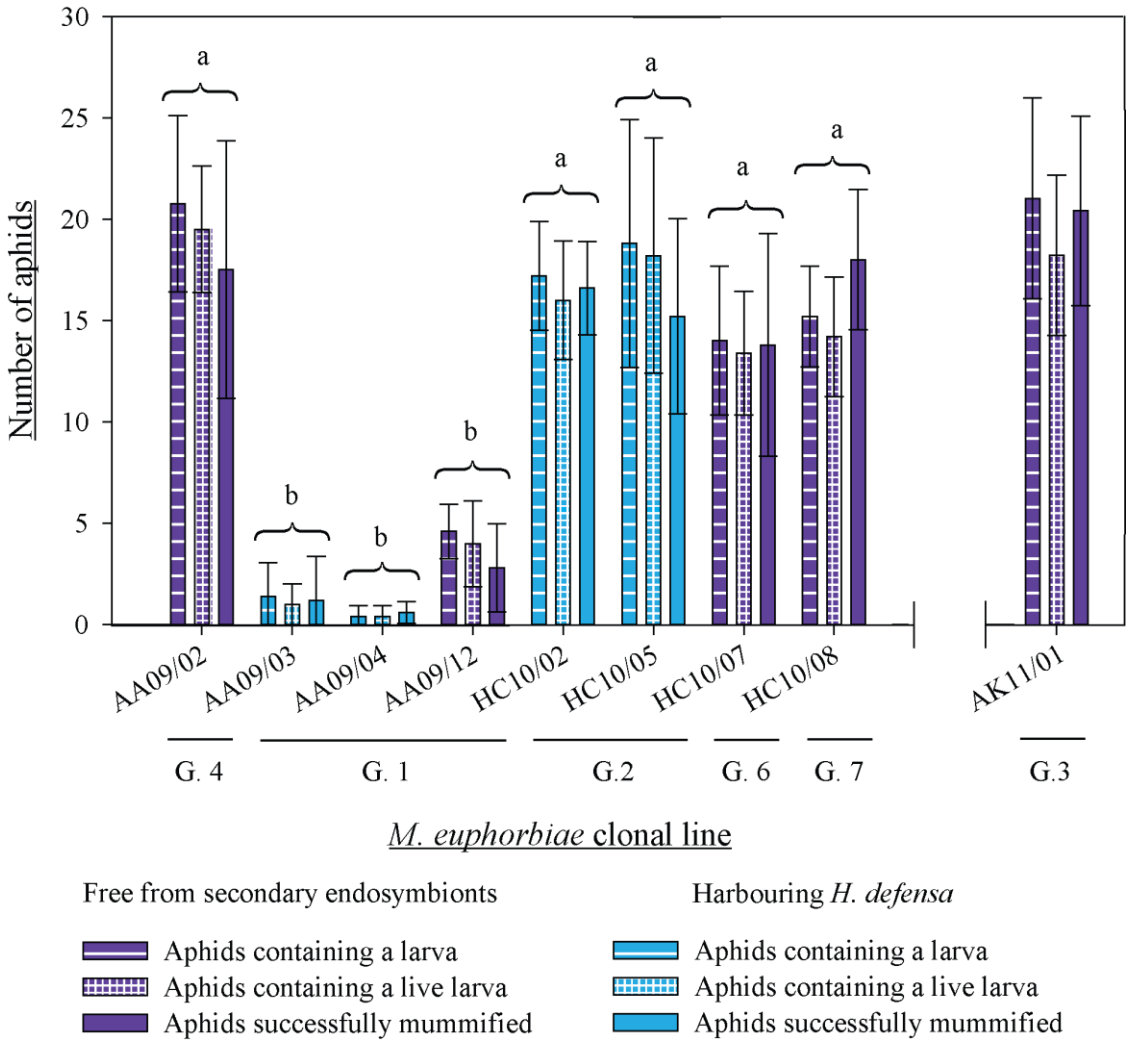

Figure 5.4: Mean larval, live larval and mummy counts from singly-parasitized *M. euphorbiae* lines differing in genotype (G. 1, G. 2 etc.) and endosymbiont status. Note that host suitability for clone AK11/01 was assessed separately. Error bars show ± 1 s. d., whilst sets of columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

The effect of aphid line on total parasitism rates (larval and mummy counts) was very highly significant ($F_{7, 62}$ = 48.090, P = < 0.001); Tukey's HSD post-hoc test identified that the counts from the *M. euphorbiae* lines AA09/03, AA09/04 and AA09/12 were significantly lower (at the 0.1% level) from the remaining five lines. There were no significant differences between larval and mummy counts, either overall or within aphid lines (parasitoid stage: $F_{1, 62}$ = 0.923, P = 0.341; aphid line × parasitoid stage $F_{7, 62}$ = 0.810, P = 00583), and this pattern was maintained when live larval counts were analysed.

Total parasitism rates also differed significantly between the aphid genotypes ($F_{4, 68}$ = 83.309, P = < 0.001), with significantly lower counts (at the 0.1% level) in genotype 1 potato aphids, comprising of lines AA09/03, AA09/04 and AA09/12, compared to

genotypes 2, 4, 6 and 7. As before, there were no significant differences between the larval and mummy counts, either overall or within genotypes (parasitoid stage: $F_{1, 68}$ = 0.926, P = 0.339; genotype × parasitoid stage: $F_{4, 68}$ = 1.084, P = 0.372), indicating that most larval infections resulted in successful mummification.

There were significant differences too in total parasitism counts between the aphids when grouped by the presence or absence of *H. defensa* ($F_{1, 74}$ = 5.132, P = 0.026), although this result was confounded by the uneven representation of different aphid genotypes and the limited distribution of *H. defensa* infection in the *M. euphorbiae* lines studied. No differences were found between the larval and mummy counts, either overall or within endosymbiont groups (parasitoid stage: $F_{2, 74}$ = 0.181, P = 0.672; endosymbiont group × parasitoid stage $F_{1, 74}$ = 0.025, P = 0.874).

Similar results were found when the live larval counts rather than the total larval counts were used. There was a strong and significant relationship both between larval and mummy counts (Linear regression R^2 = 0.851, t = 14.526, P = <0.001, Figure 5.5A), and between live larval and mummy counts (Linear regression R^2 = 0.833, t = 13.583, P = <0.001, Figure 5.5B), with larval counts seemingly a better predictor of the number of aphids likely to succumb to parasitism.

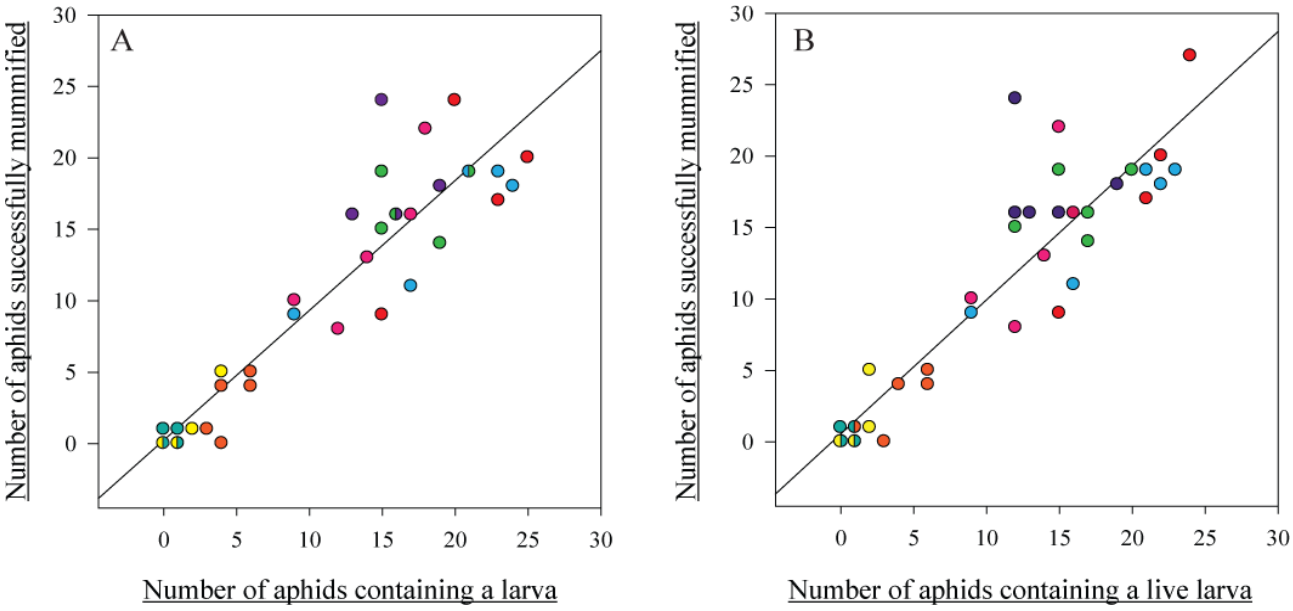

Figure 5.5: The number of aphids containing a larva (left) or a live larva (right) plotted against the number of aphids successfully mummified from each replicate of the host susceptibility experiment. Different colours circles represent different aphid lines.

When a GLM was fitted to the larval counts, both genotype and endosymbiont status within genotype contributed significantly to the model (Analysis of deviance genotype: LR χ^2=212.468, P = <0.001; endosymbiont within genotype LR χ^2=21.689, P = <0.001). The effects of the number, age and generation of the parasitoids used in each replicate were not significant, nor was the time taken for the 60 aphids in each replicate to be attacked (Appendix 2, section *A2.2.1*).

Generalised linear models were fitted to the larval count data with various combinations of the possible explanatory variables, and the goodness of fit assessed. In addition to the intercept, the most heavily weighted model included only genotype, endosymbiont within genotype and time, a summary of which is given in Table 5.5.

Factor		**ln of model co-efficient**	**Z value**	**P-value**	**Significance (level)**
Intercept		-0.0929	-0.237	0.813	Not significant
Genotype	Genotype 1	----------Reference----------			
	Genotype 2	3.1920	8.256	2.00×10^{-16}	Significant (0.1%)
	Genotype 4	3.2852	8.244	2.00×10^{-16}	Significant (0.1%)
	Genotype 6	3.0749	7.476	7.69×10^{-14}	Significant (0.1%)
	Genotype 7	2.9660	7.509	5.98×10^{-14}	Significant (0.1%)
Within genotype	*H. defensa*	----------Reference----------			
	None	1.8934	4.306	1.66×10^{-5}	Significant (0.1%)
Time		-0.0026	-1.492	0.136	Not significant

Table 5.5: Generalised linear model fitted to the larval count data from the host suitability experiment. Note that log link function used in the Poisson distribution models returns the natural logarithm of the model co-efficients

The positive natural logs of the model co-efficients for genotypes 2, 4, 6 and 7 relative to genotype 1 are all fairly similar, reflecting the comparably high larval counts in these susceptible *M. euphorbiae* genotypes. The positive natural log of the model co-efficient for aphid clones free of *H. defensa* relative to lines that harboured *H. defensa* indicates that, within an aphid genotype, the presence of the secondary endosymbiont reduced the number of aphids in which a parasitoid egg successfully developed to the larval stage. Although limited to genotype 1 potato aphids, this effect will be explored further in the following section.

As anticipated from the close correlation between larval counts and mummification rates, genotype and endosymbiont status within genotype were the only significant factors to emerge when the number of aphids successfully parasitized was modelled (see Appendix 2, sections *A2.2.2* and *A2.2.3*). This differed from the model fitted to the mummification data from the host acceptability experiment, in which there was no effect of *H. defensa* within an aphid genotype.

5.3.2.3 Host acceptability and suitability of genotype 1 M. euphorbiae *aphids differing in their endosymbiont complement*

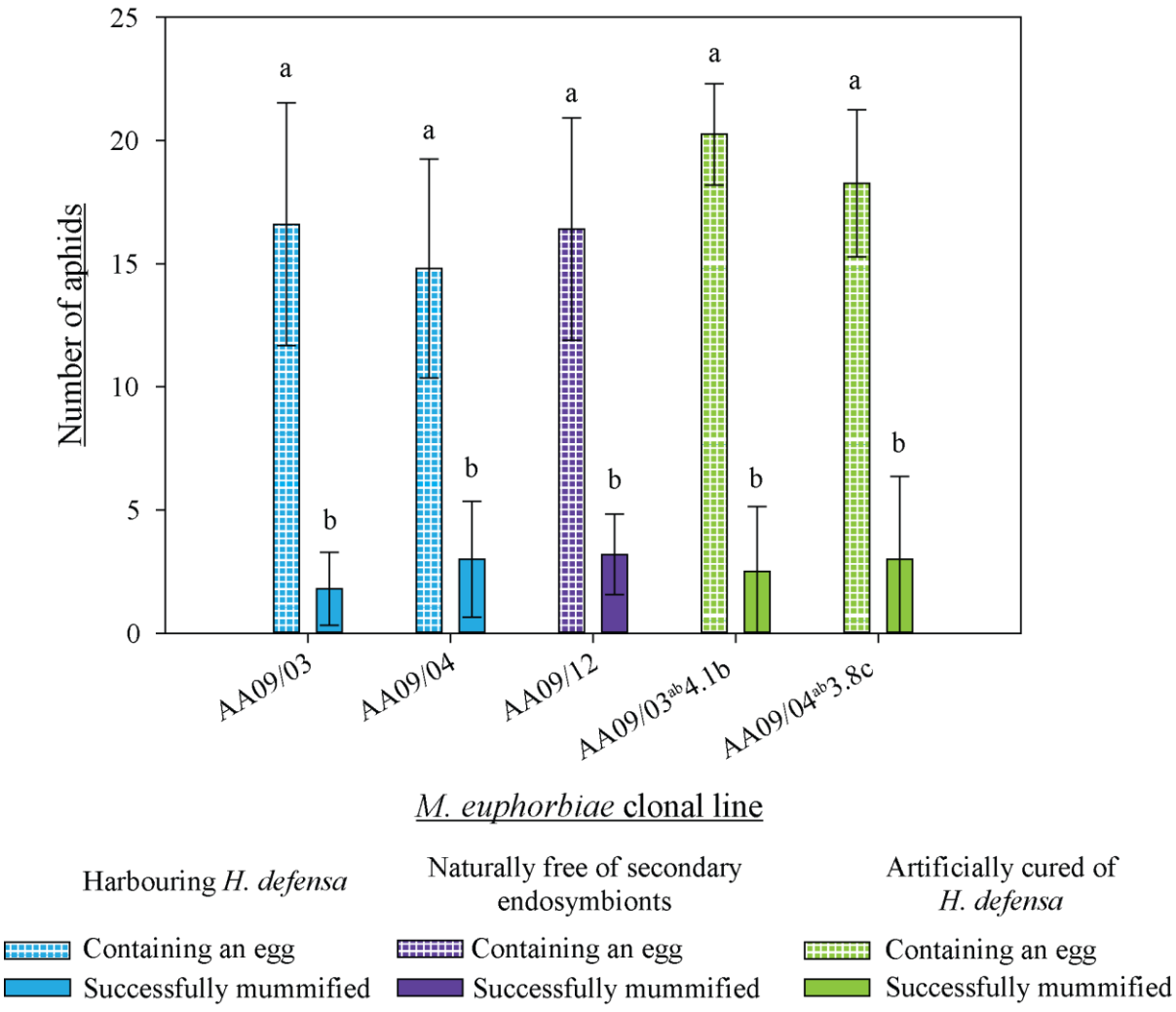

Figure 5.6: Mean egg and mummy counts from singly-parasitized *M. euphorbiae* lines differing in their endosymbiont status. Error bars show ± 1 s. d., whilst columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

Egg counts from the acceptability experiment were significantly higher than mummy counts in genotype 1 aphids ($F_{1, 36}$ = 218.010, P = <0.001, Figure 5.6), but there was no

significant variation between aphid lines in total parasitism rates ($F_{4, 36} = 0.838$, P = 0.510) or in the way egg and mummy counts varied between aphid lines ($F_{4, 36} = 1.025$, P = 0.408). Tukey's HSD post-hoc tests confirmed that the egg counts of each genotype 1 aphid line differed significantly (at the 0.1% level) from the mummy counts of each line.

Endosymbiont status (harbouring *H. defensa*, cured of *H. defensa* or naturally free from secondary endosymbionts) had no significant effect on total parasitism rates ($F_{2, 40} = 1.64$, P = 0.207). Again Tukey's post-hoc tests showed that the egg counts of each endosymbiont group differed significantly (at the 0.1% level) from the mummy counts of each group, but the way in which the egg and mummy counts varied did not differ significantly between the different endosymbiont groups (parasitism stage: $F_{1, 40} = 230.40$, P = <0.001; parasitism stage × endosymbiont group $F_{2, 40} = 1.32$, P = 0.279).

The most heavily weighted GLM fitted to the egg count data included the intercept only (ln model coefficient for the intercept = 2.838), with none of the explanatory variables contributing significantly to the model (Appendix 2, section A2.3.1). Within this experiment the *A. ervi* parasitoids therefore did not discriminate between aphids that differed in their endosymbiont complement within the same genetic background.

When a GLM was fitted to the mummy counts from each genotype 1 aphid line using the same explanatory variables, the age and batch of the parasitoids significantly affected mummy counts (Analysis of deviance parasitoid age: LR $\chi^2 = 4.188$, P = 0.041; parasitoid batch $\chi^2 = 11.437$, P = 0.001). The remaining variables did not significantly contribute to the model (Appendix 2, section *A2.3.2*). When various models containing combinations of these variables were assessed using AIC selection criteria, in addition to the intercept the best fit model contained the time taken for the aphids to be attacked, the number of parasitoids used and the batch from which the parasitoids were bred (Table 5.6).

Factor	ln of model co-efficient	Z value	P-value	Significance (level)
Intercept	4.0955	3.716	0.0002	Significant (0.1%)
Number of parasitoids	-0.6321	-2.312	0.0208	Significant (5%)
Batch of parasitoids	-0.8196	-3.624	0.0003	Significant (0.1%)
Time	0.0413	1.912	0.0559	Not significant

Table 5.6: Generalised linear model fitted to the mummy count data of the genotype 1 aphids from the host acceptability experiment.

The negative natural log of the model co-efficient for parasitoid batch indicates that parasitoids from the latter batch produced fewer mummies. As these parasitoids were only used for 1-2 replicates from each of the AA09/12, AA09/03^{ab}4.1b and AA09/04^{ab}3.8c lines, it is possible that the mummification rates of these lines have been underestimated relative to those of lines AA09/03 and AA09/04. However, when an interaction term between endosymbiont group and parasitoid batch is included in the model, the variable is not significant, and does not increase the predictive power of the model. Therefore, within a given aphid line from the genotype 1 aphids used in the host acceptability experiment, the batch from which the parasitoids used were reared did not affect the number of aphids successfully mummified.

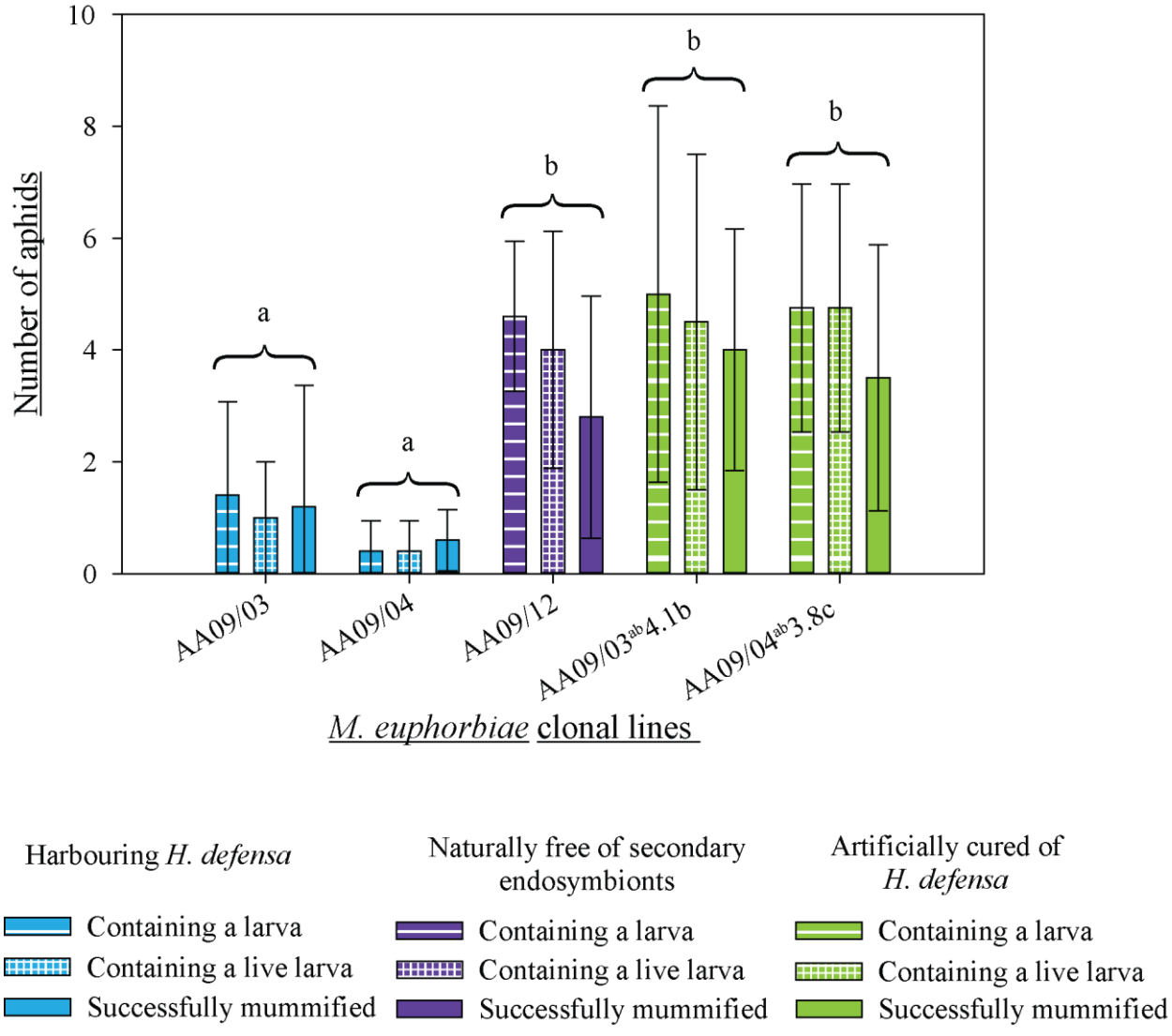

Figure 5.7: Mean larval, live larval and mummy counts from singly-parasitized *M. euphorbiae* lines differing in their endosymbiont status. Error bars show ± 1 s. d., whilst groups of columns labelled with different letters differ significantly at the 5% level (Tukey's HSD post-hoc test).

Contrary to the counts from the genotype 1 aphids in the host acceptability experiment, in the host suitability experiment there were very highly significant differences between the genotype 1aphid lines in total parasitism rates (larval plus mummy counts; $F_{4, 36} = 7.828$, P = <0.001), but not between the larval and mummy counts ($F_{1, 36} = 1.835$, P = 0.184, Figure 5.7). Nor was the interaction between parasitism stage and aphid line significant ($F_{4, 36} = 0.416$, P = 0.766). Tukey's HSD post-hoc tests indicated that the overall parasitism rates of the AA09/03 and AA09/04 aphid lines differed significantly at the 5% level from those of the remaining three lines.

The differences in total parasitism rates were still very highly significant when the aphid lines were grouped by endosymbiont status ($F_{2, 40} = 16.374$, P = <0.001), with Tukey's HSD test indicating that the parasitism rates of aphids infected with *H. defensa* were significantly lower (at the 0.1% level) than for aphids either naturally free or artificially cured of secondary endosymbionts.

When a GLM was fitted to the larval count data, the presence of *H. defensa* was a very highly significant factor in the model (LR $\chi 2 = 31.442$, P =<0.001). The age, number and generation of parasitoids used were also significant, but neither the time taken nor the aphid line contributed significantly (Appendix 2, section *A2.4.1*). When modelling the larval counts using various combinations of these explanatory variables, the only variables included in the most heavily weighted model were endosymbiont group and time, and only the presence of *H. defensa* and the intercept were significant (Table 5.7). Repeating the GLM with the *H. defensa*-infected line as the reference group showed that the presence of *H. defensa* also significantly decreased the number of aphids containing a larva relative to those that naturally lacked the endosymbiont (P = 0.0001). The negative natural log of the model coefficient for harbouring *H. defensa* reflects the lower mean larval counts seen in the infected aphids.

A further GLM was fitted to the mummy counts from each genotype 1 aphid line, using the same factors and covariates. Both *H. defensa* infection and the age of the parasitoid were significant explanatory variables in the model (Analysis of deviance endosymbiont: LR $\chi^2 = 8.894$, P = 0.0117; parasitoid age LR $\chi^2 = 4.983$, P = 0.0256), whilst the remaining variables were not significant (Appendix 2, section *A2.4.2*). However, assessing the goodness of fit of models with various combinations of the possible explanatory variables using AIC and AICc scores produced a best fit model in which the

only significant factor was endosymbiont group (Table 5.8). Two further models nearly as heavily weighted also had the presence *of H. defensa* as the only significant model co-efficient other than the intercept.

Factor		ln of model co-efficient	Z value	P-value	Significance (level)
Intercept		1.2168	2.530	0.011	Significant (5%)
Endosymbiont	Cured of *H. defensa*	----------Reference----------			
	Naturally free of *H. defensa*	-0.1656	-0.455	0.649	Not significant
	Harbouring *H. defensa*	-1.9257	-4.533	5.81×10^{-6}	Significant (0.1%)
Time		0.0075	0.819	0.413	Not significant

Table 5.7: Generalised linear model fitted to the larval count data of the genotype 1 aphids from the host suitability experiment.

Factor		ln of model co-efficient	Z value	P-value	Significance (level)
Intercept		0.1375	0.202	0.840	Not significant
Endosymbiont	Cured of *H. defensa*	----------Reference----------			
	Naturally free of *H. defensa*	-0.0482	-0.098	0.922	Not significant
	Harbouring *H. defensa*	-1.2296	-2.747	0.006	Significant (1%)
Number of parasitoids		0.5508	1.847	0.065	Not significant
Time		-0.0032	-0.226	0.822	Not significant

Table 5.8: Generalised linear model fitted to the mummy count data of the genotype 1 aphids from the host suitability experiment.

As with the larval counts, the model fitted to the mummy counts shows that within the common genetic background of genotype 1 potato aphids, the presence of *H. defensa* significantly decreased the number of aphids successfully mummified relative to those that lacked the endosymbiont through artificial means. Repeating the GLM with the *H. defensa*-infected line as the reference group showed that the presence of *H. defensa* also significantly decreased the number of aphids successfully mummified relative to those that naturally lacked the endosymbiont (P = 0.01478). The same effect was not seen in the host acceptability experiment, in which the number of aphids successfully parasitized did not vary significantly with *H. defensa* presence.

5.3.3 Transfection of secondary endosymbionts by means of parasitoid ovipositor attacks

5.3.3.1 Horizontal transfer of H. defensa *via a parasitoid ovipositor*

Of the thirty AA09/12 nymphs attacked in the experimental replicates, 9 were successfully parasitized by *A. ervi*, two produced offspring before becoming mummified, and two further aphids died. Of the remaining 19 aphids, only two did not develop beyond the fourth instar and so did not produce any offspring.

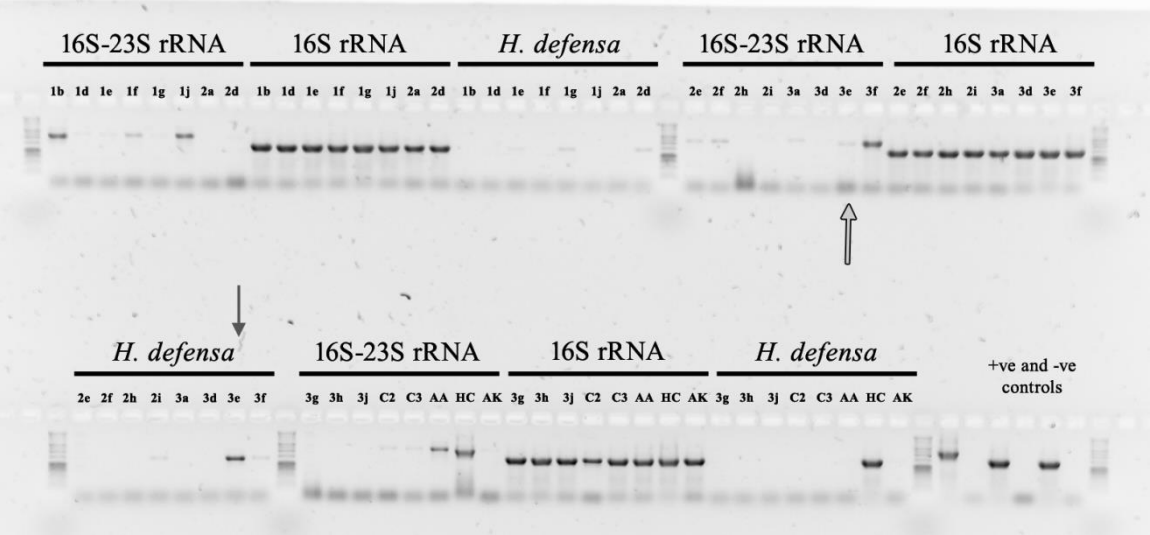

Figure 5.8: Inverted gel electrophoresis image of the diagnostic PCR products generated when the attacked AA09/12 aphids from the ovipositor transfection experiment were screened for *H. defensa* and the presence of other bacteria. The solid arrow indicates the only lane in which a clear product was visible from the aphid designated 3e in the *H. defensa* screen, indicating a single instance of horizontal transmission; the empty arrow indicates the lane in the 16S-23S rRNA screen corresponding to the same aphid template.

All 19 surviving attacked aphids were screened for the presence of bacteria and specifically for *H. defensa* using diagnostic PCR (Figure 5.8). A band of amplified product was visible in the *H. defensa* screen of the attacked aphid designated 3e (the fifth aphid attacked in the third temporal block of the experiment), but with only a faint band in the 16S-23S rRNA screen. This indicated one instance of horizontal transmission from the HC10/05 aphids to the AA09/12 aphid via the ovipositor of the parasitoid, although the weak 16S-23S rRNA result implies that bacterial density was low.

5.3.3.2 Novel infections not sufficiently established for heritable vertical transmission

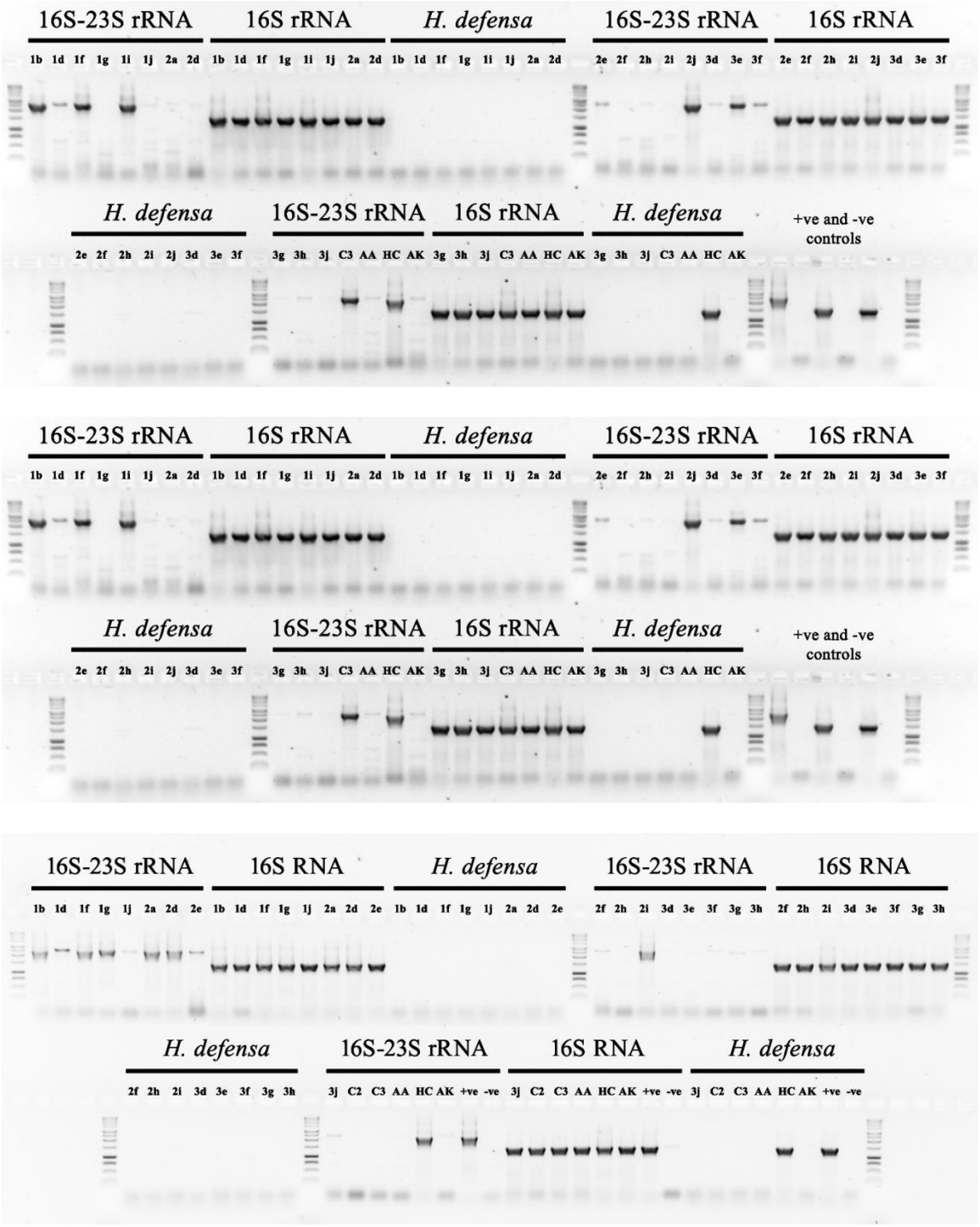

Figure 5.9: Inverted gel electrophoresis images of the diagnostic PCR products generated when the first, second and third cohorts of offspring (upper, middle and lower images, respectively) from the AA09/12 aphids attacked in the ovipositor transfection experiment were screened for *H. defensa* and the presence of other bacteria. Other than the donor line (HC) and the positive control, no products were generated in the *H. defensa* screen, indicating that any horizontally-transmitted *H. defensa* infections were not sufficiently established to be transmitted vertically.

None of the three cohorts of generation 1 offspring produced by the attacked AA09/12 aphids gave positive results when screened for *H. defensa* (Figure 5.9), indicating that the

endosymbiont infection was not sufficiently established for heritable vertical transmission to occur.

5.4 Discussion

5.4.1 Variation in M. euphorbiae *aphids to parasitoid susceptibility*

5.4.1.1. Initial parasitism assays

The initial parasitism assays revealed differences in parasitism rates both between the aphid lines and between the two experimental systems, but no clear effects of endosymbiont infection. Interestingly, the AA09/03 clone harbouring *H. defensa* but without the APSE phage produced the lowest proportion of mummies in both assays, yet in the whole plant assay the greatest proportions of parasitized aphids occurred in the two lines harbouring *H. defensa* and the associated phage.

The parasitism assays conducted on whole plants were complicated by plant architecture, which could have reduced the ability of the parasitoids to locate the aphids. It is therefore not clear whether the number of parasitized aphids reflects those that were found by the parasitoids, or whether more aphids were encountered but either aphid behavioural or physiological defences prevented successful parasitism. There were also high levels of mortality, which may have resulted from the stress of transferring young nymphs from leaf cuttings to whole plants. Alternatively, restricting the parasitoids to a single plant with low aphid densities may have resulted in the young aphid nymphs being attacked multiple times, increasing the rate of mortality.

The parasitism assays conducted within the culture cups used only leaf cuttings, reducing the spatial complexity and increasing the likelihood of parasitoid encounters with aphids. The number of aphids dying in each replicate was also much lower and was comparable across aphid lines (data not shown). Despite this, in only one of the *M. euphorbiae* lines, AA09/02, did the proportion of aphids parasitized exceed 50%. Whilst this aphid line does not harbour any secondary endosymbionts, a second clone also free from secondary

bacteria (HC10/08) exhibited parasitism rates comparable to the three clones infected with *H. defensa*, both with and without the associated APSE phage.

More controlled experiments were therefore conducted to better understand the effects of both the genetic background of the aphid host and the presence of secondary endosymbionts on two aspects of parasitism, oviposition rates and larval development.

5.4.1.2 A. ervi *parasitoids do not clearly discriminate between potato aphid hosts of differing quality*

Unlike the initial parasitism assays, the mummy counts from both the host acceptability and suitability experiments followed the fates of singly-attacked aphids. This excluded the possibility that parasitism susceptibility had been underestimated as a result of aphids avoiding parasitoid encounters in refugia within the plant architecture or through behavioural defences, including any potential behavioural influences of *H. defensa* (Dion *et al*., 2011a). It also removed the possibility of aphid mortality stemming from repeated parasitoid attacks. The parasitism rates observed therefore differed from the initial assays in that the mean numbers of mummies produced were comparable across the aphid lines, with the exception of the three aphid lines from genotype 1 that all displayed significantly lower mummy counts. That the low parasitism rates observed in the lines AA09/03, AA09/04 and AA09/12 were genotypic in origin was reinforced by the equally low mummy counts of the cured aphids derived from the two of these three clones that were infected with *H. defensa*.

The mean egg counts for aphid lines from genotypes 2, 4, 6 and 7 did not differ statistically from the number of aphids successfully mummified, indicating that there was little physiological impediment to prevent an oviposited *A. ervi* egg from hatching and the larva developing. In contrast, the mummy counts from the three aphid lines from genotype 1 were far lower than the egg counts, demonstrating a discordance between the acceptability and suitability of the aphid hosts to *A. ervi* (see section *5.4.1.3*, below).

With neither genotype nor *H. defensa* presence included in the general linear model fitted to the egg count data, the various aphid clones with which the *A. ervi* parasitoids were presented appear to have been equally acceptable. Pairwise comparisons between the egg counts of the different aphid genotypes, however, found the number of genotype 1 aphids accepted by ovipositing *A. ervi* parasitoids was significantly lower than that of

genotype 2 aphids. When the same comparisons were made between aphid clones, fewer aphids from the AA09/04 line (genotype 1) were found to have been parasitized than aphids from the HC10/05 line (genotype 2). Given that the genotype 1 aphids all exhibited significantly lower mummification rates than egg counts, parasitoids could be considered to be wasting reproductive effort by ovipositing within these aphids. That some level of host rejection is apparent is therefore not unexpected, but the lack of previous encounters and the consequent large egg reserves of each parasitoid potentially tempered such selectivity. The oviposition of *A. ervi* eggs within unsuitable hosts does not necessarily refute the preference–performance hypothesis as, in this instance, these unsuitable hosts were all that the parasitoid had encountered, and some viable offspring were still produced (Łukasik *et al*., 2013b). Assessing the oviposition behaviour of *A. ervi* when presented with a choice of hosts of differing quality would illustrate the degree to which assessment of hosts by the female parasitoids is imperfect.

Although two of the three genotype 1 aphids and both of the two genotype 2 aphids used in these experiments harboured *H. defensa*, it is possible that variation in the strains of bacteria or in the associated APSE bacteriophages, or the interactions between the endosymbiont and the aphid genotype, also contributed to the lower egg counts seen in the former group. The capacity of *H. defensa* to affect oviposition behaviour of *A. ervi* parasitoids has been illustrated in other studies, although the responses are not always consistent. Singly attacked pea aphid clones harbouring defensive strains of *H. defensa* are as equally accepted as hosts by naive *A. ervi* parasitoids as those free from secondary endosymbionts when alternative hosts are not presented (Oliver *et al*., 2003; Bensadia *et al*., 2006). However, that the presence of *H. defensa*, or the protection it bestows, is discernible is evident from a later study by Oliver *et al*. (2012), in which *A. ervi* parasitoids were presented with equal numbers of pea aphid clones either harbouring *H. defensa*, or free from secondary endosymbionts. Not only were aphids harbouring a protective strain more likely to be superparasitized than non-infected aphids, but the oviposition of more than one egg increased the successful parasitism rate of the endosymbiont protected aphids. In contrast, *A. ervi* parasitoids have been shown to refrain from attacking and ovipositing within certain clones of *S. avenae* when they harbour *H. defensa*, despite no evidence that the endosymbiont conferred any significant physiological resistance to the developing parasitoid. These parasitoids had prior experience of both infected and uninfected aphids, had consequently partially depleted their egg loads, and were simultaneously offered both infected and uninfected aphids as

hosts. Thus, the English grain aphids harbouring *H. defensa* appear to have been perceived as relatively poorer hosts than uninfected aphids by experienced *A. ervi* wasps (Łukasik *et al*., 2013b).

Whilst the density and age structure of the available aphid hosts are known to influence oviposition decisions in *A. ervi* (He & Wang, 2006; He *et al*., 2006), further work is needed to determine how oviposition rates are affected when the availability of aphids differing in quality (either as perceived by the parasitoid or that actually differ in their physiological responses) are varied. This in turn will allow a better understanding of the influence of such parasitoid behaviour on aphid population dynamics and on the selection pressures that could be maintaining the frequency of *H. defensa* infection. Nevertheless the results presented here show that in the physiological condition in which the *A. ervi* parasitoids were tested, with no alternative hosts available neither the presence of *H. defensa* nor the apparent innate resistance mechanisms rendered the *M. euphorbiae* aphids unacceptable hosts.

5.4.1.3 Genotype as the principal determinant of host suitability

As with the previous experiment, when testing for host suitability the mummy counts from the three genotype 1 aphid clones AA09/03, AA09/04 and AA09/12 were significantly lower than for the remaining aphid lines. A GLM of the mummy counts showed that genotype was an important determinant of successful parasitism, whilst the comparable model coefficients for genotypes 2, 4, 6 and 7 emphasised the susceptibility of these aphids relative to genotype 1. Of the four *M. euphorbiae* lines that naturally harboured *H. defensa*, lines AA09/03 and AA09/04 were relatively resistant to *A. ervi* whilst lines HC10/02 and HC10/05 were more susceptible. Similarly, there were resistant and susceptible lines amongst the clones in which no secondary endosymbionts had been identified. Although there were highly significant differences in total parasitism counts between the aphids when grouped by the presence or absence of *H. defensa,* this is likely to reflect both the limited distribution of *H. defensa* infections in the *M. euphorbiae* lines studied, coupled with an uneven representation of the different aphid genotypes. That the mean mummy counts from the two sub-lines of genotype 1 aphid clones cured of *H. defensa* were still significantly lower than the mummy counts of

the aphid lines representing 5 other genotypes reinforces the likely genotypic rather than endosymbiotic origin of the resistance.

The larval counts or the live larval counts and the mummy counts within each the 9 *M. euphorbiae* lines did not differ significantly, suggesting that parasitoid eggs that have successfully developed into first-instar larvae are likely to succeed in mummifying their host. As the host acceptability experiment demonstrated that an approximately equal number of aphids from each line were likely to have been parasitized, it follows that the physiological suppression of the development of the parasitoid occurred prior to the second hatching of the *A. ervi* embryo. The larval counts were a better predictor of the number of aphids likely to succumb to parasitism than live larval counts, which is initially counter-intuitive. However, it is possible that at least some of the larvae identified as moribund or deceased had died as a result of the dissection process.

The humoral defences of the pea aphid are only now being elucidated from both haematological and genotypic evidence (Schmitz *et al*., 2012, and see section *1.1.2.2*). However, a recent study by Nguyen *et al*. (2008) into the protein regulation of *M. euphorbiae* following parasitism provides one suggestion as to the possible mechanism of resistance exhibited in the genotype 1 potato aphids. The arrested development of the *A. ervi* eggs within the *M. euphorbiae* hosts was accompanied, amongst other changes in the protein profile, by upregulated production of the precursor of phenoloxidase (proPO), which in the activated form is implicated in melanotic encapsulation (Laughton *et al*., 2011; Schmitz *et al*., 2012). The same physiological and metabolic changes were not observed in the potato aphids when attacked by *A. nigripes*, to which they were highly susceptible. Given that the proteomic immune response was only examined for one clonal line, it is not possible to know whether this mechanism of resistance is common across *M. euphorbiae* genotypes and is efficacious against populations of *A. ervi* originating outside of North America, where the study was conducted. Further investigation into the innate immunity of potato aphids will be necessary to understand better the mechanism by which development of the parasitoid egg is halted in genotype 1 aphids, and how the phenotypic differences in resistance are determined within the genomes of the different *M. euphorbiae* clones.

5.4.1.4 Within an aphid genotype, aphid parasitoid resistance may be further improved through harbouring H. defensa

Whilst there was a general consensus between the two experiments as to the relative susceptibility to parasitism of the different *M. euphorbiae* lines harbouring their natural endosymbiont complements, the variation in parasitoid susceptibility between the genotype 1 aphid lines was not consistent. In the host acceptability experiment, each of the five genotype 1 lines and sub-lines was equally acceptable to the *A. ervi* parasitoid (equal numbers of aphids with eggs counted) and equally suitable for parasitoid development (equal numbers of mummies formed), resulting in no significant differences in the parasitism rates across the genotype. In the host suitability experiment, however, the total parasitism rates of the two genotype 1 aphid lines harbouring *H. defensa*, AA09/03 and AA09/04, were significantly lower than the total parasitism rates of both the genotype 1 line that was naturally free of secondary endosymbiont bacteria (AA09/12), and of the two AA09/03 and AA09/04 sub-lines cured of *H. defensa*. Although the differences were not significant when comparing only the mummy counts, the power to detect an effect was limited as a result of the smaller sample sizes.

The use of the same set of clonal lineages in the two experiments should have eliminated genetic differences in either the aphid genome or the endosymbiont strain as a source of the disparate results observed. Given that the two studies were conducted using parasitoids reared from batches purchased almost 10 months apart, however, there may have been genetic differences in the virulence of the parasitoids for overcoming *H. defensa* mediated defences. That the interaction between the strain of endosymbiont present and the genotype of the parasitoid can greatly affect the strength of aphid resistance has been amply demonstrated using the black bean aphid, *A. fabae*, infected with various strains of *H. defensa* and the parasitoid *L. fabarum*, the thelytokous reproduction of which makes it suitable for investigating genotype × genotype interactions (Vorburger *et al*., 2009; Schmid *et al*., 2012; Rouchet & Vorburger, 2012; Cayetano & Vorburger, 2013). Genetic variation has also been implicated in the virulence of *A. ervi* parasitoids to a common clone of pea aphid (Henter, 1995), although none of these assays distinguished between pre-ovipositional defences and the presence of physiological resistance mechanisms. Indeed, although the experimental design used here precluded behavioural defences from preventing an attack, it is not evident whether the lower mummification rates seen in the AA09/03 and AA09/04 aphid lines relative to

other genotype 1 clones in the host suitability experiment resulted from lower acceptance of these aphid hosts by these particular parasitoids, or greater physiological protection.

The mechanistic basis of the parasitoid resistance bestowed by *H. defensa* in *A. pisum* and other aphid species is thought to at least in part result from toxins encoded by the APSE phage integrated within the *H. defensa* genome (Oliver *et al*., 2009). Whilst the *M. euphorbiae* lines AA09/03 and AA09/04 both harbour *H. defensa*, diagnostic PCR screening failed to amplify either of two APSE genes from AA09/03 aphids, leading to the conclusion that the phage was absent (see section *3.3.1.2*). Although one of the two genes used to diagnose the presence of APSE appears highly conserved between phage variants, it is nevertheless possible that the integrative phage may have degraded through mutation and/or recombination such that it is no longer active, yet the *H. defensa* genotype may still contain functioning copies of the toxin-encoding genes, and still endow some degree of protection upon their aphid host (Degnan & Moran, 2008a, 2008b). To better understand the potential contribution of the *H. defensa* bacteria from the AA09/03 and AA09/04 aphid lines to parasitoid resistance would require the establishment of the endosymbiont infections into aphid clones from genotypes that are susceptible to parasitism. Although the effects of any endosymbiont strain × host genotype interaction would be lost, such transfections would allow further investigation into both the contribution of these endosymbiont strains in conferring resistance, and how this is affected by genotypic differences in parasitoid strains.

The two clonal lines of aphid representing genotype 2 also harboured both *H. defensa* and the APSE phage, and attempts to cure these aphids of their secondary bacteria were not successful (section *4.3.1*). There is therefore no direct evidence for a beneficial effect of this endosymbiotic association on parasitism, but the high egg counts and mummification rates observed in lines HC10/02 and HC10/05 suggested that any protective effects, either behavioural or physiological, against *A. ervi* were minimal. It is possible, however, that the presence of *H. defensa* may cause these aphids to be perceived as lower quality hosts when alternative, uninfected clones are available, despite the endosymbiont conferring no obvious physiological protection (Łukasik *et al*., 2013b). The presence of *H. defensa* may also be beneficial in other aspects of parasitoid interactions as yet to be investigated in this aphid species. For example, in interactions between *A. fabae* and *L. fabarum*, the presence of *H. defensa* increased the longevity and reproductive output of singly attacked aphids that did not succumb to parasitism

(Vorburger *et al*., 2013). The presence of *H. defensa* in *A. fabae* has also been correlated with reduced emergence, longer parasitoid development times and smaller parasitoid size, although these effects may have been an indirect consequence of a decrease in host quality resulting from the endosymbiont infection. Nevertheless, these traits may improve the inclusive fitness of the parasitized host by reducing the threat of parasitism to aphid kin in the near vicinity (Schmid *et al*., 2012).

5.4.2 A. ervi *as a vector of* H. defensa

By allowing *A. ervi* parasitoids to attack first aphids from a clonal line harbouring *H. defensa*, then aphids from a second clone naturally free from secondary endosymbionts, a single instance of horizontal transfer of *H. defensa* was observed. Although the extent of the endosymbiont infection in this individual was adequate to be detected using diagnostic PCR screening, it was not sufficiently established to be vertically transmitted to the developing aphid offspring. A horizontal transmission rate as low as 3% has been hypothesised as sufficient to maintain the prevalence of *H. defensa* observed in some pea aphid populations in the absence of strong selection pressures for or against the endosymbiont associations (Darby & Douglas, 2003). Assuming a similar rate of lateral transfer in potato aphids, a study of this scale would not expect to observe the acquisition of *H. defensa* in more than one or two instances, though Gehrer and Vorburger (2012) achieved an 8.6% transmission rate of secondary endosymbionts by *A. colemani* in *A. fabae* hosts. Nevertheless, coupled with the apparent requirement of *A. ervi* to probe potential aphid hosts in order to assess their acceptability, these results suggest that transference on parasitoid ovipositors is a feasible means of horizontal transmission of secondary endosymbionts between *M. euphorbiae* aphids.

If the presence of *H. defensa* within an aphid host is detrimental to parasitoid fitness, selection should act against parasitoids that facilitate their spread (Gehrer & Vorburger, 2012). However, the time lag necessary for a protective *H. defensa* endosymbiont to establish in an aphid clonal lineage would limit the direct fitness costs to the *A. ervi* parasitoid that vectored the bacteria. Given the dispersal capabilities of both aphids and parasitoids, the offspring of the parasitoids acting as vectors are also unlikely to suffer disproportionately from the establishment of new *H. defensa* infections. With no clear defensive role for *H. defensa* evident in the *M. euphorbiae* lines tested, selection against

parasitoids acting as vectors is likely to be very weak indeed. The low incidence of horizontal transmission is more likely to reflect the rarity with which the series of necessary events occur; for the endosymbiont bacteria within the aphid haemolymph to adhere to the parasitoid ovipositor, for the bacteria to remain viable until the next oviposition event into a suitable host, and for the endosymbiont bacteria to proliferate and localise in such a manner as to permit vertical transmission.

The occurrence of *H. defensa* within two of the innately resistant clonal aphid lines did not bestow any clear costs or benefits to the fitness of their aphid hosts, either on innate life history characteristics or resulting from interactions with parasitoids. Instead the infection status of lines AA09/03 and AA09/04 may in part result from the increased likelihood of these aphids surviving an attack from a parasitoid and hence a greater probability of a novel endosymbiont association, introduced on the parasitoid ovipositor, becoming established and persisting.

5.4.3 Summary and Conclusions

Despite stable infections of *H. defensa* in eight of the nineteen *M. euphorbiae* aphids kept in culture for this study, there was little evidence of endosymbiont-mediated protection against parasitoids as denoted by the equal numbers of infected genotype 2 aphids that contained an egg and that formed mummies. There are several possible reasons why the physiological resistance mechanisms seen in *H. defensa* isolates from *A. pisum* may not be manifest in *M. euphorbiae*: the pathogenicity factors produced by the APSE phage may be ineffective against the parasitoids, for example, or the genes that encode them may have been inactivated. Alternatively, genotypic differences in the bacterial or aphid genomes may prevent the APSE-derived toxins from being delivered to the site of the developing parasitoid. Whilst a similar lack of physiological resistance has been documented in populations of *S. avenae* harbouring *H. defensa*, in other aphid species such as *A. pisum* the protective role of *H. defensa* has been hypothesised as a major heritable trait upon which selection may act to maintain the symbiotic association (Łukasik *et al*., 2013b). Whether the *H. defensa* strains in potato aphids confer an indirect benefit against parasitoids that may, by increasing the fitness of their aphid host, further propagate the existence of the bacteria awaits further investigation.

There was some indication that the presence of *H. defensa* could reduce parasitism rates in the genotype 1 aphids in which it was present. However, a far greater indicator of likely parasitoid resistance was the genotype of the aphid, with genotype 1 aphid lines in particular consistently exhibiting significantly lower rates of successful parasitism than those of genotypes 2, 3, 4, 6 and 7. Almost all of this resistance was attributable to physiological suppression of the developing parasitoid, with the majority of single eggs oviposited within genotype 1 aphid hosts failing to become first instar larvae. There may also be an element of reduced host acceptance, although within a no-choice environment genotype 1 aphid clones were only marginally less acceptable hosts compared to clones from genotype 2. Consequently, relative to *M. euphorbiae* aphids from other genotypes, the genotype 1 aphids would be expected to thrive when under strong selection pressure from parasitoids.

The research presented here has not identified parasitoid resistance traits conferred by *H. defensa* that would select for or against the maintenance of the endosymbiotic association in *M. euphorbiae* hosts. Nonetheless, parasitoids may be more directly involved in this association by acting as a means of transferring the bacteria from infected to uninfected hosts. Combined with the information garnered from the previous chapter that harbouring *H. defensa* causes no obvious detriment to the fitness of aphids from the two genotypes in which the infection is found, this may go some way to explain the observed distribution of *H. defensa* within the potato aphid clones collected for this study.

Chapter 6: Summary of findings and future perspectives

6.1 Identifying the determinates of aphid fitness in holobionts of *M. euphorbiae*

Despite being a significant component of aphid populations in agricultural and native vegetation, little research has been undertaken to date to characterise intraspecific variation within natural *M. euphorbiae* populations, or understand the forces and interactions that influence *M. euphorbiae* population processes. The composition of aphid populations will depend on selective forces operating on the heritable variation in fitness of aphid clones, which includes the genetic contribution from the primary and, where present, secondary endosymbionts. Prior to this study, endosymbiotic associations of *M. euphorbiae* were known from the screening of only a few individuals and the degree to which the endosymbionts influenced aphid fitness in general, and parasitism resistance in particular, had not been investigated (Russell *et al*., 2003; Russell & Moran, 2005; Francis *et al*., 2010).

In addition to the obligate endosymbiont *B. aphidicola*, molecular screening revealed over half of the 19 *M. euphorbiae* lines maintained in culture for this study harboured secondary endosymbiont bacteria. Of the several secondary endosymbionts previously characterised from *A. pisum* and other aphid species, however, only two, *H. defensa* and *R. insecticola*, were found within the potato aphid cultures. Furthermore, only the *H. defensa* infections appeared stable; *R. insecticola* was lost from each infected culture within 6 months of clonal lines being established, presumably due to the changes in selective pressures imposed by the culture environment.

Seven clonal genotypes were distinguished within the cultured *M. euphorbiae* aphids, three of which were represented by multiple clonal lines. The *H. defensa* infection was only found within aphid clonal lines from two of the more common genotypes in culture, in which it was found at relatively high frequencies. This biased distribution may have been the result of a small sample size, yet may also be indicative of genomic interactions that have resulted in selection maintaining endosymbiotic associations only in certain aphid clones or genotypes.

Under benign testing conditions, both survival and reproductive output were greatest in *M. euphorbiae* clones from genotypes 1, 2 and, to a lesser extent, genotype 3. The potato

aphid lines naturally infected with *H. defensa* were all from these first two genotypes, although the time to development and first reproduction, rate of population increase and survival probabilities were comparable across genotype 1 aphids differing in their endosymbiont status, including lines both naturally free of secondary bacteria and artificially cured of *H. defensa*. These findings suggest that there were no inherent costs or benefits to carrying *H. defensa* in the aphids in which the infection was found that could select against the association. Indeed, the clonal fitness of the holobionts comprising particular aphid genotypes either with or without *H. defensa* was equal to, and in many instances greater than, those from other genotypes, which all lacked the secondary endosymbiont.

Whether harbouring *H. defensa* or free from secondary endosymbiont bacteria, *M. euphorbiae* lines of genotype 1 were consistently parasitized at a far lower rate than those of the other genotypes identified. With the majority of single eggs oviposited within genotype 1 aphid hosts failing to develop to the first instar larval stage, the observed parasitoid resistance was attributable to physiological suppression, possibly through the upregulation of pro-phenoloxidase and consequent melanotic encapsulation observed by Nguyen *et al*. (2008) in a single parasitoid-resistant *M. euphorbiae* line. Although *A. ervi* and other parasitoids may be able to partially overcome certain resistance mechanisms through behavioural or other adaptations (Dion *et al*., 2011b; Oliver *et al*., 2012), these results suggest that selection pressures from this particular parasitoid have the potential to influence the genotypic composition of *M. euphorbiae* populations.

Innate physiological resistance mechanisms are not the only defence available to aphids, however. The enclosed system in which the aphid susceptibility to parasitism was tested meant that defensive behaviours such as kicking and the production of cornicle secretions exhibited by the *M. euphorbiae* aphids were largely ineffectual, when otherwise the parasitoids may have left in search of new patches of hosts. The two-dimensional nature of the parasitoid arenas also precluded plant morphological structures from providing refuge from attack and prevented the aphids from dropping from the plant away from the parasitoid. The disparate results from the parasitism assays conducted in culture cups and on whole potato plants suggest different *M. euphorbiae* genotypes may differ in their spatial utilisation of host plants or in their behavioural defences. Consequently the host acceptability and suitability of the *M. euphorbiae* aphid genotypes may not fully reflect the realised parasitism rates of the potato aphid lines following encounters with *A. ervi*.

Over the past decade, the role of *H. defensa* in conferring protection against parasitoids such as *A. ervi* has been increasingly better understood. There is both correlative and empirical evidence from *A. pisum*, *A. fabae* and some other aphid species to show that particular strains of *H. defensa*, coupled with the APSE bacteriophage, confer on the aphid host varying levels of physiological resistance against developing *A. ervi* eggs (Ferrari *et al*., 2004; Oliver *et al*., 2003, 2005; Guay *et al*., 2009; Vorburger *et al*., 2009). The presence of *H. defensa* can also influence the behaviour of attacking parasitoids, leading to avoidance or superparasitism of infected aphid hosts (Oliver *et al*., 2012; Łukasik *et al*., 2013b). In contrast, there has been scant evidence for endosymbiont-mediated protection against parasitoids conferred by the strains of *H. defensa* (and the APSE phage) infecting *M. euphorbiae*. In particular, *A. ervi* parasitoids exhibited consistently high rates of both host acceptance and successful parasitism when presented with genotype 2 potato aphid hosts naturally infected with *H. defensa*. Why, unlike in those found in *A. pisum*, the strains of *H. defensa* found in potato aphids do not contribute to the defences of their host is unclear; it is possible that the APSE phage(s) present within these strains of *H. defensa* did not produce toxins effective against developing parasitoid larvae, or transporters in the bacterial or aphid cell membranes required to deliver the APSE-derived toxins may have been defunct or absent. Whilst the protective role of *H. defensa* has been hypothesised as a major heritable trait upon which selection could act to maintain the symbiotic association in *A. pisum* (Oliver *et al*., 2008), potential selection pressures acting to maintain *H. defensa* infections in hosts to which they confer no physiological resistance, such as in populations of *S. avenae* and now in *M. euphorbiae*, are as yet unknown (Łukasik *et al*., 2013b).

6.2 Future perspectives: aphid resistance to parasitism and effects on population dynamics

The results presented here represent an initial study into some of the genotypic and endosymbiont–mediated variation in fitness traits exhibited by clonal lines of *M. euphorbiae*. There is still far more to be learned regarding the range of factors that maintain adaptive genetic diversity in natural aphid populations, even with respect to interactions with a single group of natural enemies, parasitoid wasps.

To further elucidate the role that secondary endosymbionts play in the population dynamics of *M. euphorbiae* would first require a greater comprehension of their potential and realised distribution, principally in terms of the genotypic backgrounds of their hosts. Whilst correlative evidence would enable greater statistical inference of an association between *H. defensa* and genotype 1 and 2 potato aphids, transfection experiments of the *H. defensa* bacterium into a range of *M. euphorbiae* genotypes would also be desirable. This would establish whether there are energetic demands to the aphid hosts of harbouring *H. defensa* that prohibit stable infections in *M. euphorbiae* genotypes other than the two identified in this research, therefore limiting the capacity with which *H. defensa* can be a source of heritable beneficial traits.

The founding aphids for this study were collected from a single plant species, and so might have biased the findings towards particular endosymbiont associations and omitted to detect clones of *M. euphorbiae* adapted to host plants other than *Solanum tuberosum*. Larger scale, unbiased screening would not only determine the physical distribution of *M. euphorbiae* clones across heterogenic landscapes rather than monocultures of potato crops, but could also potentially identify less common endosymbiont infections. Such screening would also provide initial information from which to investigate host-plant associations, and whether they are mediated by facultative endosymbionts (Simon *et al*., 2003; Tsuchida *et al*., 2004, 2011; Ferrari *et al*., 2012).

Throughout this investigation the focus in terms of endosymbionts has been on *H. defensa*, although *R. insecticola* was also detected in some of the potato aphid lines when first maintained in culture. The regular loss of this endosymbiotic association in culture suggests either a severe fitness costs to the aphid host of harbouring *R. insecticola* and/or poor vertical transmission. How the establishment of *R. insecticola* may negatively impact potato aphid fitness and how this varies between aphid genotypes merits further investigation, as do any benefits to the infected aphid harbouring the bacteria that, in the right environmental context, could result in selection for the association. As an increasing number of aphid species have been studied for their bacterial endosymbiont associations, a range of symbionts have been detected such as *Arsenophonus*, *Wolbachia* and *Spiroplasma*, strains of which grant defensive or otherwise beneficial traits that appear to have arisen independently (Augustinos *et al*., 2011; Łukasik *et al*., 2013a; Jousselin *et al*., 2013; Russell *et al*., 2013). With wider screening, the population genetic structure of *M. euphorbiae* may yet be revealed to include additional or novel

endosymbiotic associations that also contribute to the adaptive genetic diversity of the potato aphid holobionts.

One of the most intriguing outcomes of this study is the revelation that innate immune mechanisms, rather than defences conferred by *H. defensa*, are responsible for the high rates of parasitoid resistance observed in a single potato aphid genotype. The same species of parasitoid, *A. ervi*, is able to overcome much of the endosymbiont-mediated protection bestowed on *A. pisum* after just a few generations (Dion *et al*., 2011b). An experiment initiated in the present study (but not reported) into the evolutionary effects on parasitoid fitness of being reared on different *M. euphorbiae* genotypes failed to produce enough data to draw robust conclusions. Nevertheless, should *A. ervi* increase in virulence with exposure to resistant, genotype 1 potato aphids, such resistance mechanisms may cease to be beneficial when these clones dominate. More work is needed to understand the range of physiological and environmental conditions under which *A. ervi* parasitoids are likely to oviposit in the various *M. euphorbiae* clones available, including the effects of aphid genotype on aphid defensive behaviour and wasp attack behaviour, in order for the circumstances in which parasitism will be successful to be understood.

A minor role for *H. defensa* in reducing parasitism rates in *M. euphorbiae* aphids from genotype 1 was implicated from the results of the host suitability assay presented here, though not from the host acceptability assay. Whilst the methodologies were similar, the two experiments used parasitoids that were probably sourced from different genetic pools. The reduction in parasitism rates in the *H. defensa*-infected clones may have been the result of selective behavioural avoidance by the parasitoid, or of enhanced physiological resistance mechanisms conferred by the endosymbiont. Regardless of the mechanism, these results suggest a possible genetic specificity to *A. ervi* parasitoid resistance mediated by *H. defensa*. This in turn has the capacity to help maintain genetic variation in natural populations of both infected aphids and attacking parasitoids through negative frequency dependence (Vorburger *et al*., 2009).

Whilst *A. ervi* is thought to be the principal parasitoid of *M. euphorbiae* in Britain and is the parasitoid sold commercially as a biological control agent for potato aphid infestations, natural *M. euphorbiae* populations are part of a complex insect community that include other parasitoids capable of utilising them as hosts (e.g. see Starý *et al*.,

2007). It is not yet understood, however, whether the resistance exhibited by the genotype 1 *M. euphorbiae* aphids in this study is the result of a generalist defence mechanism that would extend to parasitization by other species. The almost total resistance to parasitism by *A. ervi* of one North American clone of *M. euphorbiae*, for example, that is susceptible to the closely related *Aphidius nigripes* implies that resistance mechanisms specific to certain parasitoid species have evolved in at least one instance (Nguyen *et al*., 2008). Also, the *M. euphorbiae* clones for this research were collected largely from arable fields, where the composition of aphid populations may additionally be influenced by the use of insecticides. With trade-offs between insecticide resistance and parasitoid resistance known in clones of the peach-potato aphid *M. persicae* (Foster *et al*., 2011), further potato aphid genotypes with a degree of resistance against parasitism may be found from *M. euphorbiae* assemblages on non-cultivated host plants.

It should also be noted that genotypic variation in both aphid resistance and parasitoid virulence has been reported in several aphid-parasitoid systems, including systems involving *A. ervi* (Henter, 1995; Henter & Via, 1995; Ferrari *et al*., 2001; Vorburger *et al*., 2009; Sandrock *et al*., 2010). Although genomic variation is more difficult to control experimentally in parasitoids that do not reproduce by parthenogenesis, half-sib breeding designs could test for genetic variation in successful parasitism rates of *M. euphorbiae* within these parasitoid species, and also search for genotype-by-genotype interactions between aphids and parasitoids (Henter, 1995; Khudr *et al*., 2013). Just as selection pressures from *A. ervi* are thought to influence populations of *M. euphorbiae*, so the relative frequencies of different *M. euphorbiae* genotypes have the potential to affect the composition and dynamics of *A. ervi* populations.

In order to understand the impact of variation in *M. euphorbiae* holobionts, including variation in parasitoid resistance, on both aphid and parasitoid populations it would be beneficial to combine the results gained from experimental studies with theoretical approaches. Many early ecological models incorporating aphid interactions with other trophic levels focussed on the population as a whole and ignored the behaviour of the individual; more recent models often take an individual-based approach (e.g. see Lombaert *et al*., 2006; Parry *et al*., 2006; Kindlmann & Dixon, 2010). As the responses of various holobionts to parasitism differ, it is now clear that the compositions of aphid populations, both in terms of aphid genotype and of their endosymbiotic complement,

and of their natural enemies are integral to population processes (Kwiatowski & Vorburger, 2012). As knowledge of fine-scale genetic variation in interactions between *M. euphorbiae* and its parasitoids improves, so too will the ability to accurately incorporate the effect of parasitoids into wider models for predicting aphid population dynamics.

6.3 General conclusions

With the aphid life cycle at most exhibiting a single generation of sexual reproduction each year, there is negligible scope for heritable symbionts to persist and spread in host populations through reproductive manipulation (though see Leonardo & Mondor, 2006; Simon *et al*., 2011). Instead, maternally-transmitted microorganisms can facilitate their own propagation by increasing the fitness, and therefore the reproductive success, of their host (Moran *et al*., 2008). Assuming the short-term costs of harbouring novel endosymbionts are not too severe, facultative symbionts that confer a beneficial role will be selected for, as too will adaptations by the aphid host to reduce the energetic cost of maintaining the symbiosis (Ferrari & Vavre, 2011). A continuum in the degree of association between aphids and microbes results; from pathogenic or opportunistic microbes that are detrimental or commensal to the aphid host, through the numerous facultative endosymbiotic associations currently being identified that benefit their host in a context-dependent manner, and culminating in the obligate, mutualistic partnerships established between aphids and the primary endosymbiont *B. aphidicola*.

The opportunity for bacteria to infect aphids may be facilitated by the adaptations evolved by the host to harbour the obligate primary endosymbiont, such as a reduction in immunity and the development of specialist bacteriocyte cells (Braendle *et al*., 2003, Gerado *et al*., 2010). Nevertheless, many of the endosymbionts found in aphids have independently evolved to confer a degree of protection or other fitness benefit to their host, and in doing so presumably further their own propagation. It is possible that the lack of a strong protective function reflects a less advanced association between *M. euphorbiae* and *H. defensa*, with the effect of harbouring the endosymbiont not being sufficiently detrimental to the fittest host genotypes to select against the establishment of the symbiosis.

The genomes of aphid facultative endosymbionts studied to date are far more dynamic than that of the obligate symbiont *B. aphidicola*, exhibiting extensive genome rearrangements, mobile genetic elements and evidence of recombination events (Moran *et al*., 2005a; Degnan & Moran, 2008a, 2008b; Degnan *et al*., 2009a). With at least one secondary endosymbiont bacterium able to establish a stable infection within certain *M. euphorbiae* genotypes, the stage is set for adaptations beneficial to both endosymbiont and aphid host to arise that, given the right environmental context, may result in net positive selection for the association. Given the huge number of extraneous variables that can impact on the fitness of an aphid holobionts, such context-dependent benefits to *M. euphorbiae* of harbouring *H. defensa* may already have emerged and await detection.

It is well understood that the realised fitness of aphids depends on complex interactions between both biotic and abiotic factors, which in turn affects the dynamics of aphid populations in the field. More recently, the contribution of vertically transmitted facultative endosymbiont bacteria as a source of heritable traits has been recognised. For aphids such as *M. euphorbiae*, only by comprehending the degree to which different selection pressures act on both the aphid hosts and the endosymbionts they carry will it be possible to understand the dynamic distribution of various holobionts within heterogeneous environments, and manage infestations on crops accordingly. The work presented in this study has begun this process by identifying *M. euphorbiae* clonal genotypes and their associated facultative endosymbionts, and by quantifying both differences in intrinsic fitness characteristics between the genotypes and differences in susceptibility to a common parasitoid wasp. The disparities in the susceptibilities of American and British *M. euphorbiae* clones to various *Aphidius* parasitoids warn against making generalisations regarding the outcomes of aphid responses across geographic regions. In addition, this research has highlighted the need for caution when applying our current understanding of the factors shaping the fitness of *A. pisum*, a model organism for study, to even closely related aphid species such as *M. euphorbiae*.

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Appendix 1

A1.1 Diagnostic PCR conditions

A1.1.1 Diagnostic PCR primer sequences

Gene	Primer name	Direction	5'-3' primer sequence	Source
16S rRNA	16F27	Forward	AGAGTTTGATCMTGGCTCAG	Lane, 1991
	1494R	Reverse	GCTCTAGAGCGGYTACCTTGTTACGACTT	Lane, 1991
16S-23S rRNA	10F	Forward	AGTTTGATCATGGCTCAGATTG	As given in Sandström *et al*., 2001
	480R	Reverse	CACGGTACTGGTTCACTATCGGTC	As given in Sandström *et al*., 2001
S. symbiotica 16S rRNA	16SA1	Forward	AGAGGTTGATCMTGGCTCAG	As given in Fukatsu & Nikoh, 2000
	PASScmp	Reverse	GCAATGTCTTATTAACACAT	Fukatsu *et al*., 2000
H. defensa 16S rRNA	PABSF	Forward	AGCGCAGTTTACTGAGTTCA	Darby & Douglas, 2003
	16SB1	Reverse	TACGGYTACCTTGTTACGACTT	As given in Fukatsu & Nikoh, 2000
R. insecticola 16S rRNA	U99F	Forward	ATCGGGGAGTAGCTTGCTAC	Sandström *et al*., 2001
	16SB1	Reverse	TACGGYTACCTTGTTACGACTT	As given in Fukatsu & Nikoh, 2000
PAXS 16S rRNA	PAXSF	Forward	GAAGCAATGCAAAGAGTGTTGC	Guay *et al*., 2009
	1507R	Reverse	TACCTTGTTACGACTTCACCCCAG	As given in Sandström *et al*., 2001
Rickettsia 16S rRNA	16SA1	Forward	AGAGGTTGATCMTGGCTCAG	As given in Fukatsu & Nikoh, 2000
	Rick16SR	Reverse	CATCCATCAGCGATAAATCTTTC	Fukatsu *et al*., 2000
Spiroplasma 16S rRNA	16SA1	Forward	AGAGGTTGATCMTGGCTCAG	As given in Fukatsu & Nikoh, 2000
	TKSSsp	Reverse	TAGCCGTGGCTTTCTGGTAA	Fukatsu & Nikoh, 2000
Rickettsiella 16S rRNA	RCL16S-211F	Forward	GGGCCTTGCGCTCTAGGT	Tsuchida *et al*., 2010
	RCL16S-470R	Reverse	TGGGTACCGTCACAGTAATCGA	Tsuchida *et al*., 2010
APSE P35	APSE20.8F	Forward	GCCGCGGGGCGTGTTATTGACG	Degnan & Moran, 2008
	APSE21.7R	Reverse	TTAAGGCCCGCTCATAAGCTG	Degnan & Moran, 2008
APSE P51	APSE34.0F	Forward	AGGTGCGATTACCCTGTTTG	Degnan & Moran, 2008
	APSE34.9R	Reverse	GATAAAACATCGCCGTTTGC	Degnan & Moran, 2008

A1.1.2 Diagnostic PCR reaction mix contents

Reagent	Amount per reaction (µL)
5× Green GoTaq® reaction buffer	5.0
dNTPs (12.5mM total)	0.5
Milli-Q ultrapure water	16.2
GoTaq® DNA polymerase	0.2
Hha 1	0.1
Forward primer (10µM)	1.0
Reverse primer (10µM)	1.0
DNA template	1.0
Total	**25.0**

A1.1.3 Diagnostic PCR thermocycling conditions

GoTaq diagnostic PCR (for amplifying 16S rRNA, 16S-23S rRNA, *S. symbiotica* 16S rRNA, *H. defensa* 16S rRNA and *R. insecticola* 16S rRNA)

Time	Temperature	Repeat	Action
2 minutes	95°C	-	Initial denaturation
30 seconds	95°C	35 cycles	Denaturation
30 seconds	55°C		Annealing
3 minutes	72°C		Extension
7 minutes	72°C	-	Final extension

GoTaq diagnostic PCR (for amplifying *PAXS* 16S rRNA and *Rickettsiella* 16S rRNA; also used for amplification of short gene sequences for purification, direct sequencing and cloning)

Time	Temperature	Repeat	Action
2 minutes	95°C	-	Initial denaturation
30 seconds	95°C	35 cycles	Denaturation
30 seconds	55°C		Annealing
1.5 minutes	72°C		Extension
5 minutes	72°C	-	Final extension

GoTaq diagnostic PCR (for amplifying *Rickettsia* 16S rRNA and *Spiroplasma* 16S rRNA)

Time	Temperature	Repeat	Action
2 minutes	95°C	-	Initial denaturation
1 minute	95°C	30 cycles	Denaturation
1 minute	55°C		Annealing
2 minutes	72°C		Extension
5 minutes	72°C	-	Final extension

GoTaq APSE screen (for amplifying APSE P35 and P51)

Time	Temperature	Repeat	Action
2 minutes	95°C	-	Initial denaturation
30 seconds	94°C	11 cycles	Denaturation
50 seconds	56°C - 46°C		Annealing (touchdown)
1.5 minutes	72°C		Extension
30 seconds	94°C	25 cycles	Denaturation
50 seconds	46°C		Annealing
1.5 minutes	72°C		Extension
5 minutes	72°C	-	Final extension

A1.2 Quantitative PCR conditions

A1.2.1 Primer sequences used to amplify genes for sequencing and quantitative PCR standards

Gene	Primer name	Direction	5'-3' primer sequence	Source
M. euphorbiae EF-1α	Aphidef1aF	Forward	GGCTGATTGTGCTGTGCTTA	Designed for this study
	Aphidef1aR	Reverse	GCGAAAACCACAACCATACC	
M. euphorbiae RpL7	AphidRpL7F1	Forward	GCGCGTGAAGAAAACTAAGAA	Designed for this study
	AphidRpL7R1	Reverse	CCCCAAGTCACATATGGTTCA	
B. aphidicola groEL	BuchGroELF1	Forward	GTATCCGTAGCCCGTGAAAT	Designed for this study
	BuchGroELR1	Reverse	TACGACGATCACCAAATCCA	
H. defensa gyrB	gyrB87F	Forward	ATTTCATACTCATCCCCAGGC	Degnan & Moran, 2008
	gyrB729R	Reverse	TCTACCGCATCTCCCATCAAC	

A1.2.2 Quantitative PCR reaction mix contents

Reagent	Amount per reaction (µl)
2× MESA Blue reaction buffer	12.5
Forward primer (3uM)	2.5
Reverse primer (3µM)	2.5
Milli-Q ultrapure water	6.5
DNA template	1.0
Total	**25.0**

A1.2.3 Quantitative PCR primer sequences

Gene	Primer name	Direction	5'-3' primer sequence	Source
M. euphorbiae EF1-α	ApisEF1-422F	Forward	CTCTGGATGGAATGG AGACAACA	Sakurai *et al*., 2005 (modified)
	ApisEF1-522Rm	Reverse	ATTTACCGTCAGCCTT TCCT	
M. euphorbiae RpL7	RpL7qF1m	Forward	ACGCGCCGAGGCTTAT	Nakabachi *et al*., 2005 (modified)
	RpL7qR1	Reverse	CCGGATTTCTTTGCAT TTCTTG	
B. aphidicola groEL	BuchGroEL-AF1m	Forward	CAGCGACATTATTAGC CCAATCTATAGTAAAT	Wilkinson *et al*., 2007 (modified)
	BuchGroEL-AR1m	Reverse	TAATAACAGCTTTATC AATTCCACGT	
H. defensa gyrB	HdefGyrBqF2	Forward	CGCAAGGCAGTCATT ATATTTTTG	Designed for this study
	HdefGyrBqR2	Reverse	GACAGATTTTTTGATA TTCGCTACTTTG	

A1.2.4 Quantitative PCR thermocycling conditions

Time	Temperature	Repeat	Action
5 minutes	95°C	-	Meteor*Taq* activation
3 seconds	95°C	40 cycles	Denaturation
45 seconds	60°C		Annealing and extension*
15 seconds 1 minute 15 seconds	95°C 60°C 95°C	-	Melt curve analysis

* denotes data collection step

A1.2.5 Media used for the propagation of transformed bacterial cells

<u>SOC</u>

Select peptone	20g l^{-1}	Adjust to pH 7.0 with 1M NaOH.
Select yeast	5g l^{-1}	
NaCl	0.5g l^{-1}	
250mM KCL	10ml l^{-1}	

Autoclave, and then add the following:

2M $MgCl_2$	5ml l^{-1}
1M filter-sterilised glucose solution	20 ml l^{-1}

<u>LB broth</u>

Select peptone	10g l^{-1}	Adjust to pH 7.5 with 5M NaOH
Select yeast	5g l^{-1}	
NaCl	10g l^{-1}	
Select agar	15g l^{-1}	

A1.3 Microsatellite PCR conditions

A1.3.1 Microsatellite primer sequences

Locus	Primer name	Direction	5'- 3' primer sequence	Source
Me1	Me1F	Forward	([6-FAM])-TTCGCGAAAAACTTTATGACC	Raboudi *et al.*, 2005
	Me1R	Reverse	TCGCTGCGTTCCTATACTACC	
Me5	Me5F	Forward	([6-FAM])-GCAAATATTAAGGGTACAG	
	Me5R	Reverse	CCAATTAAAACAACTTCGTGG	
Me7	Me7F	Forward	([6-FAM])-TTAAGTCACTGCCGGTTCG	
	Me7R	Reverse	ATTAGCTCGAGCTCGTAC	
Me9	Me9F	Forward	([6-FAM])-AGCGAAACCTCCCCTAATAG	
	Me9R	Reverse	GCACAAATAAGCTCGAGTGC	
Me10	Me10F	Forward	([6-FAM])-TCGCTGCGAGACTCGTATTG	
	Me10R	Reverse	GACGACGACGTGTACAATG	
Me11	Me11F	Forward	([6-FAM])-CGTTTTCTACCCAAAGGAGG	
	Me11R	Reverse	ATTGTCCGTATACCACGACG	
Me13	Me13F	Forward	([6-FAM])-GAACTCACTCAGACTCGTGTGG	
	Me13R	Reverse	CAGCCGGAATACCAAGAGC	

A1.3.2 Microsatellite reaction mix contents

Reagent	Amount per reaction (µl)
5× Green GoTaq® reaction buffer	3.0
dNTPs (12.5mM total)	0.48
Milli-Q ultrapure water	9.47
GoTaq® DNA polymerase	0.05
Forward primer ± 5'[6-FAM] labelling (10µM)	0.75
Reverse primer (10µM)	0.75
DNA template	0.5
Total	**15.0**

A1.3.3 Microsatellite thermocycling conditions

Time	Temperature	Repeat	Action
2 minutes	95°C	-	Initial denaturation
1 minute	94°C	40 cycles	Denaturation
1 minute	54°C/62°C		Annealing
1 minute	72°C		Extension
5 minutes	72°C	-	Final extension

Appendix 2

A2.1 Host acceptability of 9 *M. euphorbiae* aphid lines harbouring their original endosymbiont complement

A2.1.1 Assessment of factors contributing to the egg count GLM in the host acceptability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Genotype	8.6129	5	0.1255
Endosymbiont within genotype	0.1689	1	0.6811
Age of parasitoids	0.0248	1	0.8749
Number of parasitoids	0.0341	1	0.8536
Parasitoid generation	0.2266	1	0.6340
Parasitoid batch	0.2958	1	0.5865
Time taken	0.2685	1	0.6043

A.2.1.2 Assessment of factors contributing to the mummy count GLM in the host acceptability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Genotype	252.165	5	2.0×10^{-16}
Endosymbiont within genotype	0.884	1	0.3471
Age of parasitoids	0.196	1	0.6578
Number of parasitoids	0.151	1	0.6977
Parasitoid generation	0.581	1	0.4460
Parasitoid batch	0.180	1	0.6717
Time taken	0.044	1	0.8336

A2.2 Host suitability of 9 *M. euphorbiae* aphid lines harbouring their original endosymbiont complement

A2.2.1 Assessment of factors contributing to the larval count GLM in the host suitability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Genotype	212.468	4	2.2×10^{-16}
Endosymbiont within genotype	21.689	1	3.206×10^{-6}
Age of parasitoids	1.014	1	0.3140
Number of parasitoids	1.070	1	0.3009
Parasitoid generation	0.124	1	0.7249
Time taken	1.641	1	0.2002

A2.2.2 Assessment of factors contributing to the mummy count GLM in the host suitability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Genotype	204.750	4	2.0×10^{-16}
Endosymbiont within genotype	9.055	1	0.0026
Age of parasitoids	0.037	1	0.8485
Number of parasitoids	0.344	1	0.5576
Parasitoid generation	0.633	1	0.4261
Time taken	1.073	1	0.3002

A2.2.3 Generalised linear model fitted to the mummy count data from the host suitability experiment

Factor		**ln of model co-efficient**	**Z value**	**P-value**	**Significance (level)**
Intercept		-0.0125	-0.034	0.973	Not significant
Genotype	Genotype 1	----------Reference----------			
	Genotype 2	2.9178	8.016	1.09×10^{-15}	Significant (0.1%)
	Genotype 4	2.8415	7.416	2.00×10^{-16}	Significant (0.1%)
	Genotype 6	2.9432	7.553	7.69×10^{-14}	Significant (0.1%)
	Genotype 7	3.0040	8.142	5.98×10^{-14}	Significant (0.1%)
Within genotype	*H. defensa*	----------Reference----------			
	None	1.3183	2.930	0.0034	Significant (1%)
Time		-0.0017	-0.956	0.3391	Not significant

A2.3 Host acceptability of 5 genotype 1 *M. euphorbiae* aphid lines differing in their endosymbiont complement

A2.3.1 Assessment of factors contributing to the egg count GLM of genotype 1 aphids in the host acceptability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Endosymbiont group	1.29167	2	0.5242
Aphid line within endosymbiont group	1.0225	2	0.5997
Age of parasitoids	0.0607	1	0.8054
Number of parasitoids	0.6291	1	0.4277
Parasitoid generation	0.0058	1	0.9391
Parasitoid batch	0.1418	1	0.7065
Time taken	0.9429	1	0.3315

A2.3.2 Assessment of factors contributing to the mummy count GLM of genotype 1 aphids in the host acceptability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Endosymbiont group	0.7435	2	0.6895
Aphid line within endosymbiont group	0.7709	2	0.6801
Age of parasitoids	4.1881	1	0.0407
Number of parasitoids	2.7992	1	0.0943
Parasitoid generation	0.0001	1	0.9905
Parasitoid batch	11.4369	1	0.0007
Time taken	3.4407	1	0.0636

A2.4 Host suitability of 5 genotype 1 *M. euphorbiae* aphid lines differing in their endosymbiont complement

A.2.4.1 Assessment of factors contributing to the larval count GLM of genotype 1 aphids in the host suitability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Endosymbiont group	31.4422	2	1.49×10^{-7}
Aphid line within endosymbiont group	0.0751	2	0.9631
Age of parasitoids	5.1645	1	0.0231
Number of parasitoids	5.2694	1	0.0217
Parasitoid generation	3.8700	1	0.0492
Time taken	1.8868	1	0.1696

A2.4.2 Assessment of factors contributing to the mummy count GLM of genotype 1 aphids in the host suitability experiment using analysis of deviance

	LR χ^2	**d. f.**	**P**
Endosymbiont group	8.8935	2	0.0117
Aphid line within endosymbiont group	2.6490	2	0.2660
Age of parasitoids	4.9828	1	0.0256
Number of parasitoids	0.2430	1	0.6221
Parasitoid generation	0.1690	1	0.6810
Time taken	0.3506	1	0.5538