PEA APHIDS, PARASITOIDS, AND PROTECTIVE SYMBIONTS: EXAMINING SOURCES OF VARIATION IN A HOST-PARASITOID-MICROBE INTERACTION

by

ADAM JAVIER MARTINEZ

(Under the Direction of Kerry M. Oliver)

ABSTRACT

Insects, including important pest species, are often attacked and eventually killed by internally-developing parasitoid wasps; a process that leads to strong selection for host resistance. The pea aphid, *Acyrthosiphon pisum*, is a model for studying variation in resistance to its most important parasitoid, *Aphidius ervi*, its variation in survival ranging from nearly 0 – 100% following parasitism. 1) Originally, it was assumed that this variation was due to host genotype, but the discovery of the defensive bacterial symbiont, *Hamiltonella defensa* and its bacteriophage APSE, which show tremendous strain variation in levels of protection conferred, led to the view that this aphid relies primarily on *H. defensa* for protection. By considering a large number of aphid genotypes uninfected with *H. defensa* or other symbionts, however, I discovered that aphids also encode strong intrinsic protection against this wasp, with no apparent trade-offs between intrinsic protection and aphid fitness. 2) I also found that some aphids harbor both symbiont-based *and* intrinsic resistance. In most cases tested, *H. defensa* is at least somewhat beneficial to aphid survival after parasitism but, depending on aphid genotype, can be costly on fecundity and longevity compared to uninfected aphids and is ultimately

not beneficial to carry the symbiont in some cases. 3) I then examined how parasitism by *A. ervi* influences aphid nutritional and defensive symbioses. While effects were highly variable, parasitism generally led to increase in abundance of APSE, decrease of *H. defensa*, and resistance-dependent changes in the nutritional symbiont, *Buchnera*. These are consistent with wasp manipulation of the nutritional symbiont and may signify changes in the defensive symbiosis related to resistance. 4) Finally, *Aphidius ervi* has largely displaced competing parasitoids in North America, but the related aphidiine braconid *Praon pequodorum* persists on this host. Surprisingly, I found that both symbiont-based and intrinsic aphid defenses against *A. ervi* are completely ineffective against *P. pequodorum*, which does not suffer sub-lethal fitness costs from developing in even the most resistant hosts. Moreover I found differences in embryonic development between both wasp species that may influence their parasitism success. In summary, my work shows that pea-aphid parasitoid interactions are much more complex than previously thought, a finding that likely generalizes to other systems as defensive symbiosis is increasingly recognized as a common phenomenon.

INDEX WORDS: Symbiosis, natural selection, trade-off, host, natural enemy, resistance, competition

PEA APHIDS, PARASITOIDS, AND PROTECTIVE SYMBIONTS: EXAMINING SOURCES OF VARIATION IN A HOST-PARASITOID-MICROBE INTERACTION

by

ADAM JAVIER MARTINEZ

B.Sc., Oregon State University, 2009

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2015

© 2015

Adam Javier Martinez

All Rights Reserved

PEA APHIDS, PARASITOIDS, AND PROTECTIVE SYMBIONTS: EXAMINING SOURCES OF VARIATION IN A HOST-PARASITOID-MICROBE INTERACTION

by

ADAM JAVIER MARTINEZ

Major Professor:	Kerry M. Oliver
Committee:	Michael R. Strand
	Kenneth G. Ross

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2015

DEDICATION

I dedicate this dissertation to my wife, Katrina Mounlavongsy, and to my parents, Robin and Javier Martinez. Katrina is fun, loving, and most importantly, supportive. She is a main driving force behind my success. My mother taught me to value my education and was the first to teach me the word ‘entomologist,’ recognizing my delight as I caught grasshoppers in our flower garden. My father’s appreciation of nature taught me how to be patient, observant, and persistent on our many outings in search of insects, snakes, and lizards.

ACKNOWLEDGEMENTS

The research presented here was made possible through my numerous interactions with fellow students, lab researchers, and professors. Without their guidance and/or support, I could not have developed the skills and mentality necessary to undertake and complete my various projects.

First and foremost, I acknowledge my major professor and primary mentor, Dr. Kerry Oliver, for providing guidance when needed, but also for encouraging my intellectual independence which will be necessary for my future success and confidence as a research scientist. Kerry also taught me how to recognize my most interesting results and to follow them up with relevant new questions that would help to distinguish my research from others in our field. I am also thankful for my committee members, Dr. Michael Strand and Dr. Ken Ross, who are both highly regarded scientists in their fields. Their guidance helped me to recognize my limitations and allowed me to broaden my knowledge and the scope of my research.

I have also benefitted greatly from the other members of the Oliver lab, especially our lab technician, Kyungsun Kim, who not only provided lab infrastructural support, but also shared her delicious food and recipes with me. Other important lab members include: Stephanie Weldon, Hannah Dykstra, Jayce Brandt, Matthew Doremus, Clesson Higashi, Vilas Patel, Laura Kraft, Nicole Lynn-Bell, Amiri Banks, Kira Pollack, and our undergraduate researchers. I look forward to keeping up with them and following each of their careers for the years to come.

Finally, I thank Dr. Jake Russell and Dr. Jason Harmon for editing early versions of my manuscripts; our graduate coordinator, Dr. Mark Brown, and the Entomology Department office staff, Sherry Wrona, Sam Waychoff, Nancy Jordan, and Melanie Wood; and the following funding sources: NSF, UGA Graduate School, and The Department of Entomology.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES x

LIST OF FIGURES xii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

1.1 How resident microbes modulate ecologically-important traits of insects 1

1.2 Microbe-modulated resource acquisition 2

1.3 Microbe-modulated insect defense 6

1.4 Literature review conclusions 11

1.5 Introduction to study system and specific dissertation goals 12

1.6 Dissertation format 16

1.7 References 17

2 APHID-ENCODED VARIABILITY IN SUSCEPTIBILITY TO A PARASITOID 26

2.1 Introduction 28

2.2 Methods 31

2.3 Results 36

2.4 Discussion 39

2.5 Conclusions....46
2.6 References....47
3 CONDITIONAL BENEFITS OF INFECTION WITH A PROTECTIVE BACTERIAL SYMBIONT....56
3.1 Introduction....58
3.2 Methods....61
3.3 Results....65
3.4 Discussion....71
3.5 Conclusions....74
3.6 References....74
4 EFFECTS OF PARASITISM ON APHID NUTRITIONAL AND PROTECTIVE SYMBIOSES....80
4.1 Introduction....82
4.2 Methods....86
4.3 Results....92
4.4 Discussion....100
4.5 Conclusions....106
4.6 References....107
5 SPECIALIZATION OF MULTI-MODAL APHID DEFENSES AGAINST TWO PARASITIC WASPS....117
5.1 Introduction....119
5.2 Methods....122
5.3 Results....128

5.4 Discussion 136

5.5 Conclusions 141

5.6 References 142

6 CONCLUSIONS 149

LIST OF TABLES

Page

Table 1.1: Major types of insect-microbe interactions 2

Table 2.1: Genetically distinct aphid clonal lines used in this study 32

Table 2.2: Effect of aphid genotype and resistance phenotype on fecundity and longevity 39

Supplemental Table 2.1: Allele sizes for four aphid microsatellite loci 55

Table 3.1: Experimental aphid lines used in this study 63

Table 3.2: GzLM showing effects of aphid genotype and *H. defensa* infection on aphid survival, mummification, and mortality after parasitism 65

Table 3.3: Factors influencing aphid fecundity (GLM) 67

Table 3.4: Factors influencing aphid longevity (GzLM) 69

Supplemental Table 3.1: GLM examining overall effects of parasitism, aphid genotype, and *H. defensa* infection on aphid fecundity 79

Table 4.1: Experimental aphid lines including symbiont infection status and levels of resistance to parasitism by *A. ervi* 87

Table 4.2: General linear model (GLM) analyzing variables affecting *Buchnera* abundance 95

Table 4.3: GLM analyzing variables influencing defensive symbiont abundance 97

Supplemental Table 4.1: Target genes and primer sequences for qPCR assays 115

Supplemental Table 4.2: GLMs analyzing variables affecting *Buchnera* symbiont densities.....115

Supplemental Table 4.3: GLMs analyzing variables influencing defensive symbiont abundances.....116

Table 5.1: Aphid clonal lines used in this study.....123

Table 5.2: Generalized linear model (GzLM) with factorial design, showing effects of wasp species and aphid line on aphid susceptibility to parasitism 129

Table 5.3: GzLM showing effects of 'protective' symbiont infection and infecting bacteriophage APSE strain on aphid susceptibility to parasitism.....133

Table 5.4: Fitness measures of *P. pequodorum* wasps emerging from aphid lines AS3ø, CJ113ø, CJ113+APSE2, and CJ113+APSE3.....135

LIST OF FIGURES

Page

Figure 1.1: Mechanisms associated with microbe-mediated resource acquisition and insect defense 4

Figure 1.2: The functions provided by aphid protective symbioses may be conditioned by biotic and abiotic interactions, transfer to novel hosts, and have effects that extend to trophic levels above and below 7

Figure 2.1: Survival, mummification, and mortality rates of *A. pisum* counted nine days after parasitism by *A. ervi* 36

Figure 2.2: Aphid fecundity and longevity among lines showing low and high resistance to parasitism 38

Supplemental Figure 2.1: Mortality rates (excluding mummification) between parasitized and control (unparasitized) aphid lines, nine days after parasitism 54

Supplemental Figure 2.2: Average fecundity per aphid per day, among lines showing high and low resistance to parasitism 55

Figure 3.1: Survival, mummification, and mortality of pea aphids counted ten days after parasitism by *A. ervi* 66

Figure 3.2: Average total fecundity of unparasitized and parasitized pea aphids after twenty-four days 69

Figure 3.3: Average longevity of unparasitized and parasitized aphids after 18 days 70

Figure 4.1: Wasp (*A. ervi*) development and timing of mortality inside pea aphids 85

Figure 4.2: Percent resistance to parasitism by *A. ervi* for each aphid line 93

Figure 4.3: Effects of parasitism on *Buchnera* 96

Figure 4.4: *H. defensa* and APSE copy numbers between parasitized and unparasitized aphids over time 98

Figure 4.5: APSE toxin expression relative to *H. defensa gyrB* in parasitized versus unparasitized aphids at several time-points after parasitism.....100

Supplemental Figure 4.1: Parasitized aphid dissections, showing percent aphids found to contain live-developing wasps 113

Supplemental Figure 4.2: Linear regression of *H. defensa* and APSE copy numbers.....113

Supplemental Figure 4.3: Alignment of 82B's *cdtB1* and ZA17, WA4's *cdtB2* allele ...114

Supplemental Figure 4.4: Expression of APSE putative toxins (YDp and cdtB) relative to four reference genes 114

Figure 5.1: Adult female *Aphidius ervi, Praon pequodorum,* and their mummies 121

Figure 5.2: Pea aphid susceptibility to *A. ervi* and *P. pequodorum* 130

Figure 5.3: Effect of *H. defensa* and APSE strain on aphid susceptibility to both parasitoids 132

Figure 5.4: Serial dissections of parasitized pea aphids, revealing differences in early parasitoid wasp development 134

Supplemental Figure 5.1: Comparison of mortality (not resulting in mummification) among aphid lines 148

Supplemental Figure 5.2: Fitness measures of adult *P. pequodorum* emerging from susceptible (AS3) and resistant (CJ113) aphid lines.....148

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 How resident microbes modulate ecologically-important traits of insects

As the most diverse and abundant animals on Earth, insects perform important roles in terrestrial ecosystems as decomposers, recyclers, pollinators, herbivores, and natural enemies (Price *et al.* 2011; Waldbauer 1968). Insects have also evolved for more than 400 myr (Grimaldi *et al.* 2005), and persist today, in a microbial world resulting in an astonishing array of insect-microbe interactions (Engel & Moran 2013; Feldhaar & Gross 2009; Oliver *et al.* 2008; Wernegreen 2002; White *et al.* 2013). Many interactions, including those involving uncultivable microbes and diverse microbial communities, were difficult to study prior to the availability of modern molecular techniques (e.g. (Buchner 1965)), resulting in a worldview that largely emphasized interactions among multicellular eukaryotes. Today, our perspective is undergoing a sea change, and there is a rapidly growing appreciation that microorganisms are key players influencing insect function, ecology, and evolution.

Insects form persistent associations with many microbial groups (Douglas 1989a; Oliver *et al.* 2008), and the major types of interactions are found in **Table 1.1**. In general, surfaces exposed to the environment, including the gut, are colonized by extracellular microbes, but the particular areas infected, the duration and specificity of the association, and effects on the insect are highly variable (Engel & Moran 2013). Many microbial associates are acquired from the environment each generation, but others are maternally

transmitted with high fidelity and persist over long periods of time (Oliver *et al.* 2008). In the latter, insect and symbiont fitness are tightly linked, and many microbes invade and persist in insect populations by conferring net benefits, such as improved host nutrition or defense against natural enemies, although some spread by manipulating insect reproduction in ways that favor infected matrilines, and a few employ both strategies (Himler *et al.* 2011; Xie *et al.* 2014). In this portion of the introductory review, I will focus on the microbial residents of insects that influence resource acquisition and insect defense (Figure 1.1), emphasizing the diversity of conferred phenotypes and recent progress in understanding functional mechanisms.

Table 1.1: Major types of insect-microbe interactions

Symbiont Types	Transmission	Insect Host Range	Major Roles
Obligate intracellular symbionts: Ancient, highly-specialized associations, restricted tissue tropism. Usually bacteria, some fungi.	Strictly vertical	Present in many hemipteran groups feeding exclusively on phloem, xylem, and vertebrate blood. Also found in carpenter ants, cockroaches, tsetse flies, some Psocodea, and Coleoptera.	Nutrient provisioning, possibly defense
Heritable Facultative symbionts: Mostly intracellular, some extracellular, and some both. Usually bacteria.	Primarily vertical, occasionally horizontal	Widespread across insects, although infections are often sporadic within and among host lineages.	Host protection, reproductive manipulation, and some likely mediate herbivore-plant interactions
Gut Microbiota: Extracellular. Mostly bacteria, some fungi and other eukaryotic microbes	Usually environmentally acquired with varying specificity, but also social transmission	Widespread across insects, but highly variable in terms of microbial diversity. Associations are often more specific and specialized in some groups including xylophages and social insects.	Breakdown cellulose and other plant polymers, nutrient provisioning, nitrogen recycling and fixation, detoxify plant defenses and pesticides, protect against ingested pathogens
Environmental associates: Mutualistic associations between insects and environmental microbes, including plant pathogens that confer benefits to insect vectors. Often fungi, bacteria, and viruses.	Environmental and social	Leaf-cutter ants, sap-feeding Hemiptera, xylophagous insects	Food source, mediate resource acquisition, counter plant defenses

1.2 Microbe-modulated resource acquisition

Many of the most successful insect groups are herbivorous or xylophagous, which present major challenges to resource acquisition, including low nitrogen content, indigestible components, and plant chemical defenses (Douglas 2013; Hansen & Moran 2014). Plant tissues vary greatly in nitrogen content and the availability of essential amino acids, and it has long been known that insect groups specializing on nitrogen poor

substrates, such as plant sap, require infection with nutrient-provisioning microbial partners (Douglas 2013; Hansen & Moran 2014). These obligate intracellular symbionts (Table 1.1) have highly reduced genomes, yet retain the specific metabolic machinery, lost ancestrally in animals, allowing for subsistence on restricted diets (Douglas 2013; Hansen & Moran 2014). In some groups multiple obligate symbionts occur, sometimes to an extreme extent (Campbell *et al.* 2015; Van Leuven *et al.* 2014), each with reduced genomes encoding nutritional pathways that complement the capabilities of the other (McCutcheon & Moran 2010). Nutrient provisioning was the sole role assigned to obligate intracellular symbionts until the recent discovery that the Asian citrus psyllid, *Diaphorina citri*, has one obligate symbiont, *Carsonella*, dedicated to nutrient provisioning, and a second, *Profftella armatura,* likely functioning as a protective symbiont, with a large fraction of its small genome dedicated to polyketide toxin biosynthesis (Nakabachi *et al.* 2013).

The gut microbiota (Table 1.1) of insects are associated with more diverse roles in resource acquisition (Engel & Moran 2013). Many groups, but especially xylophages, carry gut associates that, often in conjunction with insect-produced enzymes, break down lignocellulose making insect-microbe collaborations major players in terrestrial carbon cycling (Engel & Moran 2013). In a few cases, gut symbionts are known to recycle nitrogenous waste or fix atmospheric nitrogen for insect nutrition, and some likely provision nutrients directly, including essential amino acids and vitamins (Engel & Moran 2013). For instance, the extracellular gut symbiont *Ishikawaella,* found in the herbivorous plataspid *Megacopta punctatissima,* rivals intracellular nutritional symbionts in degree of specialization, including a sharply reduced genome that retains pathways for

the biosynthesis of essential nutrients (Nikoh *et al.* 2011). Other insects may selectively acquire bacteria, e.g. (Kaltenpoth *et al.* 2009; Kikuchi *et al.* 2007), or form more generalized associations, e.g. (Coon *et al.* 2014), with environmental bacteria that are important for growth, development, and nutrition.

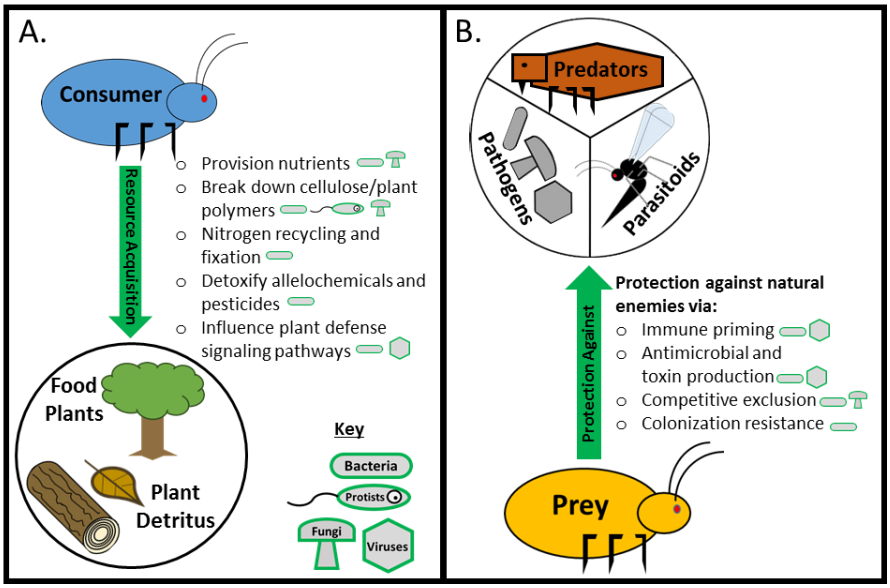


Figure 1.1 Mechanisms associated with microbe-mediated (A) resource acquisition and (B) insect defense.

Plants employ a range of tactics to defend against insect herbivory, including the production of repellant and toxic secondary metabolites. In turn, insects have countered with an array of behavioral and intrinsic physiological responses, including the detoxification of or incorporation of defensive allelochemicals (Dowd *et al.* 1983; Opitz & Muller 2009). The interplay between plant chemical defenses and insect counter-responses impacts herbivore diet breadth and has played a major role in the coevolutionary diversification of plants and phytophagous insects (Ehrlich & Raven 1964; Price *et al.* 2011; Rausher 2001). In addition to intrinsic mechanisms, recent studies hint that microbes may frequently assist insects in the detoxification of plant allelochemicals. For example, several bacterial species found in the gut of the mountain

pine beetle, *Dendroctonus ponderosae*, a major pest of lodgepole pine, encode pathways involved in the detoxification of terpenes, a widespread chemical defense in conifers (Adams *et al.* 2013). Another example occurs in the leaf-cutter ants, which cultivate a symbiotic fungus for consumption of plant material in their nests. In at least one case, laccase enzymes produced by the fungus *Leucocoprinus* are consumed by the ant, passed through gut, and deposited on top of fungus garden where they break down plant phenolics (Licht *et al.* 2013).

Resident microbes have also been shown to modulate inducible plant signaling pathways involved in herbivore defense. The Colorado potato beetle, *Leptinotarsa lycopersicum*, for example, uses orally secreted bacteria to elicit the plant's antibacterial salicylic acid pathway, which interferes with the induction of the jasmonic acid pathway, resulting in the suppression of plant defenses against herbivory (Chung *et al.* 2013). There is also growing evidence that many plant pathogens are simultaneously beneficial symbionts of the insects that vector them. A viral pathogen of tobacco, for example, vectored by the whitefly, *Bemisia tabaci*, suppresses defenses aimed at herbivores, including terpene synthesis (Luan *et al.* 2013). And in tomatoes, herbivore-related plant defensive pathways are suppressed when the plant pathogen *Liberibacter psyllarous* and its vector, the tomato psyllid *Bactericera cockerelli* co-occur, relative to the insect feeding alone (Casteel *et al.* 2012).

Economically important plants are often chemically defended against insect herbivory by human applied insecticides, and again, insects have a very successful history of evolving resistance using intrinsic mechanisms (Despres *et al.* 2007). A role for gut symbionts in insecticide resistance, however, has now been reported in the bean

bug *Riptortus pedestris* (Kikuchi *et al.* 2012). The application of fenitrothion, a widely used organophosphate neurotoxin, results in a dramatic increase in the abundance of fenitrothion-degrading *Burkholderia* strains found in the soil, which are acquired and stored in the midgut crypts of *R. pedestris*, where resistance is conferred.

Microbes also modulate resource acquisition in non-herbivores, but these interactions have generally received less attention. One well-studied exception occurs in some hymenopteran parasitoids carrying ancient, highly-specialized associations with symbiotic polydnaviruses, which aid wasp larvae by compromising their host's immunity and creating an environment more suitable for development (Strand & Burke 2012).

1.3 Microbe-modulated insect defense

Another large component of insect survival and fitness that may benefit from microbial intervention is defense against natural enemies. When microbe-infected insects are attacked, the interests of both host and symbiont become aligned for survival and while insects may use physical defenses or immunity, acquired microbes can present additional hurdles for attacking natural enemies. Indeed, recent findings indicate that symbiont-based defense is a common phenomenon in animals, including insects (Flórez *et al.* 2015; Oliver *et al.* 2014).

The gut microbiota may serve as a primary barrier against ingested pathogens by routinely protecting insects via colonization resistance, although examples to date are limited (Engel & Moran 2013). Some examples though, include worker bumblebees, which are important pollinators, that are protected by an unknown mechanism against pathogenic trypanosomatids by their socially-transmitted gut microbiota (Koch & Schmid-Hempel 2011), also specific components of the gut community of *Anopheles*

gambiae, an important human disease vector, provide refractoriness to the malaria parasite *Plasmodium*, possibly via microbial production of reactive oxygen species (Cirimotich *et al.* 2011).

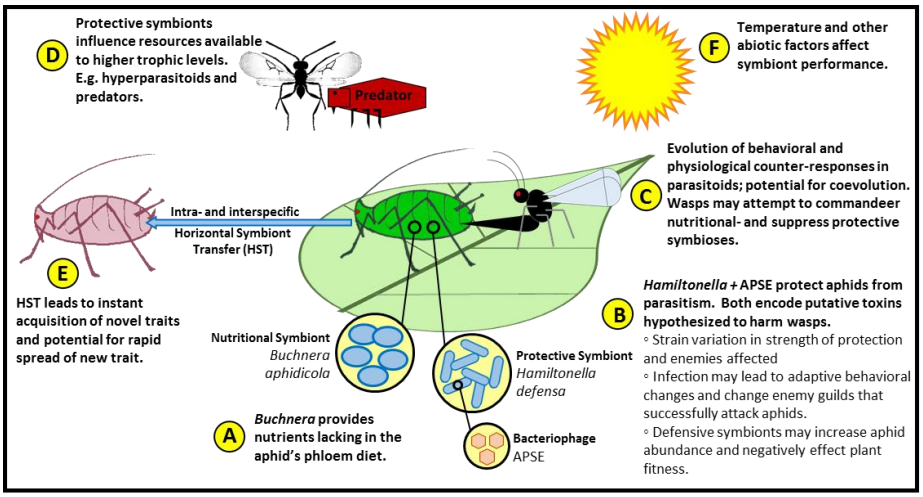


Figure 1.2 The functions provided by aphid protective symbioses may be conditioned by biotic and abiotic interactions, transfer to novel hosts, and have effects that extend to trophic levels above and below.

A wider range of protective roles has been attributed to infection with heritable facultative symbionts (Table 1.1) (Oliver *et al.* 2014). Striking examples of the diverse ecological roles mediated by symbionts occur in the pea aphid, *Acyrthosiphon pisum.* In addition to the obligate, nutrient-provisioning *Buchnera aphidicola* (Figure 1.2A), pea aphids are frequently associated with one or more of seven facultative symbionts (Russell *et al.* 2013). Amazingly, all seven are known or suspected to influence interactions with natural enemies, including parasitoids, predators, and fungal pathogens, while others confer thermal tolerance and one potentially expands diet breadth (Figure 1.2B) (Costopoulos *et al.* 2014; Lukasik *et al.* 2013; Oliver *et al.* 2010). In another example, *Drosophila* are protected against parasitoids and parasitic nematodes by native *Spiroplasma* symbionts, and *Wolbachia* strains infecting *Drosophila* confer protection

against pathogenic bacteria, nematodes, protozoa and viruses, in native or novel insect hosts (Hamilton & Perlman 2013; Xie *et al.* 2014). Additional experimental studies identifying protective effects of infection are needed to better appreciate the scope of defensive symbiosis in insects. However, the fact that so many protective roles have been identified in the first two systems to receive substantial study (*Drosophila* and *Acyrthosiphon*) strongly suggests they are widespread.

Many heritable symbionts are not easily cultivable, hindering elucidation of the functional mechanisms underlying protective services (Oliver *et al.* 2014). For example, mechanisms involved in symbiont-mediated defense in the diverse aphid microbial defenders, and by *Spiroplasma* in *Drosophila,* are largely unknown, although genomic and transcriptomic studies point to putative microbe-encoded toxins (Figure 1.2B) (Hamilton *et al.* 2014; Hansen *et al.* 2012; Moran *et al.* 2005; Oliver *et al.* 2014). Several mechanisms have been proposed for *Wolbachia*-mediated pathogen protection including immune priming, resource competition between symbiont and pathogen, and symbiont effects on an insect methyltransferase-regulating miRNA that correlate with viral abundance (Caragata *et al.* 2013; Hamilton & Perlman 2013; Ye *et al.* 2013; Zhang *et al.* 2013). Mechanisms are *best* understood in interactions where defensive products are released from the insect body, facilitating chemical identification. Examples include the anti-predator polyketide toxins produced by *Pseudomonas*-like symbionts in the rove beetle *Paederus*, and the antimicrobial cocktail produced by specialized *Streptomyces* symbionts in beewolves to protect brood cells from pathogens (Kroiss *et al.* 2010; Piel 2002).

Unlike obligate intracellular symbionts, facultative symbionts are sometimes infected with bacteriophages capable of influencing the biology of both bacterial symbiont and insect host (Moran *et al.* 2005). In the pea aphid, *Hamiltonella defensa* confers protection against parasitic wasps (Oliver *et al.* 2005; Oliver *et al.* 2003), but only if the bacterium is also infected with temperate bacteriophages called *Acyrthosiphon pisum* secondary endosymbionts (APSEs) (Figure 1.2B) (Oliver *et al.* 2009). In addition to their contribution to the protective phenotype, APSEs have also been found to influence symbiont abundance and maintenance. Phage loss, which results in loss of the protective phenotype (Oliver *et al.* 2009), also results in elevated titers of *H. defensa* which, in turn, correlates with poor aphid performance; this likely leads to the rapid removal of phage-free *H. defensa* from natural populations (Weldon *et al.* 2013). Overall, the prevalence and roles of phages in insect symbioses are poorly understood, but initial studies indicate their potential in carrying ecologically important traits, moving traits within and among symbiont lineages, and regulating within-insect bacterial abundance (Kent & Bordenstein 2010; Moran *et al.* 2005; Weldon *et al.* 2013).

Protective symbioses are also proving to be dynamic, with strain variation in the strength of protective phenotypes, in symbiont or strain specificity to particular enemy genotypes or species, and with enemies that can evolve counter-responses to symbiont-based defenses (Asplen *et al.* 2014; Dion *et al.* 2011a; Dion *et al.* 2011b; Oliver *et al.* 2012; Parker *et al.* 2013; Rouchet & Vorburger 2014). Strain variation in enemy specificity, for example, has been reported in *Wolbachia*-mediated pathogen protection (e.g. (Ye *et al.* 2013)) and in several aphid defensive symbionts (Hansen *et al.* 2012; McLean & Godfray 2015; Oliver *et al.* 2010). In the cowpea aphid, *Aphis craccivora*, a

single strain of *H. defensa* confers complete protection against two species of *Binodoxys* wasps, but no protection from two additional aphidiine braconid wasps (Asplen *et al.* 2014), and *Regiella insecticola* infections in pea aphids confer protection against aphid-specific, but not generalist fungal pathogens (Parker *et al.* 2013). Intriguingly, but perhaps not surprisingly, parasitoids have been found to rapidly evolve counter-resistance to symbiont-based defense in aphids and change behavioral tactics associated with attack that increase successful parasitism rates (Figure 1.2C) (Dion *et al.* 2011b; Oliver *et al.* 2012; Rouchet & Vorburger 2014). Aphids infected with protective *H. defensa* also behave differently, reducing defensive and inherently dangerous escape maneuvers, such as dropping from the plant (Figure 1.2B) (Dion *et al.* 2011a). However, symbiont-protected aphids exhibiting fewer defensive behaviors were also more likely to be consumed by generalist predators than their uninfected counterparts (Polin *et al.* 2014). Protective symbionts may also modify the relative costs and benefits of competing mutualisms. Many aphids, for example, engage in defensive mutualisms with ants and protective symbionts may provide redundant services (Erickson *et al.* 2012). More generally, protective symbionts may influence herbivore abundance with effects that extend below to plants, laterally to competitors, and above to higher order enemies including parasites, predators, and hyperparasitoids (Figure 1.2D), likely expanding to community-level processes and beyond.

1.4 Literature review conclusions

Insects maintain a wide range of symbiont associations that aid in their interactions with food plants and natural enemies, including the direct production of nutrients or bioactive compounds and indirect effects stemming from the modulation of

insect and plant defensive responses. While obligate nutritional symbionts clearly played important roles in the evolution of insects allowing novel niche occupation with subsequent diversification (Hansen & Moran 2014), other interaction types that extend insect capabilities or enhance performance are likely to impact diet breadth and species diversity. Facultative symbionts can also move horizontally among insect species, transferring ecologically important traits that facilitate the colonization of novel niches (Figure 1.2E) (Henry *et al.* 2013; Oliver *et al.* 2010). Mechanisms of lateral transfer include sexual reproduction, shared resources, and the contaminated ovipositors of parasitoids (Caspi-Fluger *et al.* 2012; Gehrer & Vorburger 2012). Following acquisition, stable infection with heritable symbionts can rapidly spread beneficial traits through insect populations (Cockburn *et al.* 2013; Himler *et al.* 2011).

Despite the deserved change in perspective emphasizing microbial contributions, it is also important that symbiont roles not be overstated. Recognized attributes of symbiotic microbes, such as nutrition and defense, do not exist in a vacuum and may vary from one infected individual to the next dependent on host genotype, diet, stress, and changing abiotic factors. Symbionts that are not typically considered pathogenic may even *decrease* insect performance in particular interactions (Graham *et al.* 2012). More generally, insects display an impressive reservoir of intrinsic mechanisms as well, which may function alone or in concert with resident microbes, and the importance of their interaction should not be unduly diminished. Another important caveat is that most studies identifying beneficial effects of infection have been conducted under laboratory conditions and outcomes may differ dramatically under natural conditions (Figure 1.2F) (Oliver *et al.* 2014). Further complicating matters, in many plant-insect and insect-enemy

interactions, both macroscopic participants may employ nutritional and defensive symbionts, and these may interact in unexpected ways (Hackett *et al.* 2013). Given the diversity of roles mediated by symbionts, and the potential for extended effects on interacting players and communities, we suggest that ecological studies would generally benefit by incorporating the microbiology of interacting players.

1.5 Introduction to study system and specific dissertation goals

Insects are attacked by a wide range of natural enemies which place strong selective pressures on the development, acquisition, and maintenance of resistance (Boulinier *et al.* 1997; Brinkhof *et al.* 1999; Ebert *et al.* 1998; Fellowes *et al.* 1999; Grosholz 1994; Jaenike *et al.* 2010; Moller 1990; Oliver *et al.* 2008; Smith *et al.* 1999; Spitze 1992). Variation in resistance to natural enemies has been documented in many organisms and can stem from factors encoded in the host's genome or acquired from microbial associates (Carius *et al.* 2001; Evison *et al.* 2013; Martinez *et al.* 2013; Parker *et al.* 2011; Sadd & Barribeau 2013; Sadd & Schmid-Hempel 2009). This variation is important for adaptation via natural selection, can promote the evolution of virulence in natural enemies, and drive host-enemy coevolutionary dynamics (Rolff & Siva-Jothy 2003; Sadd & Schmid-Hempel 2009).

Many herbivorous insects, including most sap-feeding hemipterans, feed exclusively on plant phloem or xylem and require an obligate microbial symbiont to supplement their nitrogen or vitamin poor diets (Baumann 2005; Douglas 1989b; Zientz *et al.* 2004). The pea aphid (*Acyrthosiphon pisum*), which is an important model for symbiosis research, is infected with an obligate nutritional symbiont called *Buchnera aphidicola* which helps them to supplement their nutrient-poor plant sap diet (Douglas

1998; Moran & Degnan 2006). Aphids and *Buchnera,* are co-dependent for survival, however, aphids can also carry one or more facultative symbionts which are not strictly required for aphid survival, but the bacteria require a host for their own survival. These facultative symbionts have been shown to mediate a range of ecological interactions including host plant specialization, increased heat tolerance, and defense against natural enemies (rev. (Oliver *et al.* 2010)). My dissertation work focuses on pea aphid variation in resistance to it most common parasitoid enemy, *Aphidius ervi,* including variation arising from infection with the facultative bacterial symbiont *Hamiltonella defensa* and its associated toxin-encoding bacteriophage APSE (Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2003).

Significant variation in pea aphid resistance to parasitism was first shown by (Henter & Via 1995), and assumed to be due to factors encoded in the aphid genome. It was later shown that infection with the protective symbiont *H. defensa* contributed to much of the observed variability in resistance to parasitism (Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2003). Moreover, variation in protection correlated with the type of APSE found infecting *H. defensa*; those with APSE2 received moderate protection while APSE3 conferred high levels of protection (Degnan & Moran 2008; Oliver *et al.* 2009; Oliver *et al.* 2005). Relative to those infected with *H. defensa*, numerous uninfected aphid genotypes examined to date show high susceptibility and little variation in resistance (Oliver *et al.* 2009; Oliver *et al.* 2005). However, an in-depth analysis of pea aphid intrinsic genetic variation in resistance without facultative symbionts has yet to be performed, so more work is needed to partition the variability arising from aphid, bacterial, and phage genomes.

A proportion of parasitoid-attacked aphids survive parasitism, but the trauma of parasitism likely results in sub-lethal fitness effects relative to unparasitized aphids and may vary depending on the source and amount of resistance. In the absence of facultative symbionts, susceptible pea aphids suffer partial to complete reduction of offspring produced following parasitism (He *et al.* 2005), depending on the nymphal stage attacked, which stems from the castrating effects of wasp venom (Digilio *et al.* 2000; Falabella *et al.* 2007). Parasitized pea aphids protected by *H. defensa* are able to survive and reproduce (Oliver *et al.* 2003) to a greater extent than those without the symbiont meaning that the castrating effects of parasitism are at least partially ameliorated in symbiont protected aphids. However, little is known about how different strains of *H. defensa* or aphid genotypes are impacted by parasitism. Similarly, wasps attacking partially resistant aphids may also suffer sublethal fitness costs, including longer developmental time and lowered weight at emergence (Schmid *et al.* 2012).

Endoparasitic wasps are known to employ a variety of strategies to create a host environment suitable for larval development (Godfray 1994; Vinson & Iwantsch 1980), including avoiding or suppressing host encapsulation responses, limiting damage to critical host tissues, and redirecting host resources for use in wasp development (Beckage & Gelman 2004; Pennacchio & Strand 2006). Wasps developing in insects, including aphids, with obligate nutritional symbionts may also need to protect or commandeer the nutritional symbiosis (Pennacchio *et al.* 1999) and/or suppress any protective symbionts in order to successfully complete development. Most research so far in this symbiont-aphid-parasitoid system has focused on how the aphid symbiosis can influence parasitism, but I am also interested in how parasitism influences both the protective

symbiont, *H. defensa*, and the nutritional symbiont, *Buchnera.* When *A. ervi* parasitizes an aphid, it too must rely on the aphid's nutritional symbiosis with *Buchnera* which needs provide enough nutrition to sustain both aphid survival and wasp development. A few studies have shown that wasps appear to protect or enhance the aphid nutritional symbiosis and show that wasp tissues closely associate with the nutrient-provisioning aphid bacteriocyte cells (Cloutier & Douglas 2003; Falabella *et al.* 2000), avoiding damaging them until later stages in development. A potential mechanism by which *H. defensa* could protect the aphid would be to disrupt the putative wasp takeover of aphid bacteriocytes containing *Buchnera*. Alternatively, parasitism may suppress *H. defensa* and APSE abundance or the expression of symbiont products, including the phage-associated toxins hypothesized to function in defense (Moran *et al.* 2005; Oliver *et al.* 2009). Answering these questions would help further our understanding the mechanisms of symbiont protection and host manipulation of parasitoid wasps.

Finally, protective symbionts and other types of host resistance may vary in the amount of protection conferred against specific natural enemies. For example, *H. defensa* in other aphid species such as the cowpea aphid, *Aphis craccivora*, and the black bean aphid, *Aphis fabae*, have shown some degree of specialization in protection against specific parasitoid wasp species (Asplen *et al.* 2014; Cayetano & Vorburger 2015). Pea aphids in North America, too, are attacked by multiple parasitoids, most commonly *A. ervi* and *P. pequodorum*. Several studies have compared the competitive dynamics of these two parasitoids, of which *A. ervi* is more abundant in natural populations (Danyk 1992; Meisner *et al.* 2007; Schellhorn *et al.* 2002), however pea aphid resistance to *P. pequodorum* has never been examined as a potential variable that could affect the

interaction of these two parasitoids. Host resistance to specific natural enemies may not only affect their abundance, but also potentially affect the abundance and composition of their natural enemies.

1.6 Dissertation format

The focus of my dissertation is on the interactions among pea aphids (*Acyrthosiphon pisum*), their protective bacterial symbiont (*Hamiltonella defensa*/APSE), and two aphid parasitoid wasps (*Aphidius ervi* and *Praon pequodorum*), with the goal of examining several sources of variation in aphid resistance to these common natural enemies and effects that parasitism itself has on aphid symbioses. As such, the format of this dissertation will proceed as follows: 1) I will examine the genotypic variation in pea aphid susceptibility to *A. ervi* by confirming that they are completely devoid of facultative symbionts before subjecting fifteen genetically distinct aphid lines to parasitism; I will also look for costs of intrinsic genotypic resistance to parasitism on aphid fitness. 2) I will determine whether there are additive benefits of maintaining an *H. defensa* infection in an already-resistant aphid genotype; also, how resistance to parasitism affects fitness of aphids parasitized by *A. ervi*. 3) Using quantitative PCR, I will evaluate differences in concentrations of the nutritional symbiont (*Buchnera aphidicola*) and protective symbionts (*H. defensa* and associated bacteriophage APSE) in parasitized aphids; I will also study the effects of *H. defensa* infection on *A. ervi'*s development. 4) I will determine whether pea aphid resistance to *A. ervi* correlates with resistance to another related parasitoid, *P. pequodorum*, and will also examine differences in each wasp's development that may account for differences in ability to overcome aphid defenses.

The collective results of these projects will contribute to the methods we use to understand how protective symbionts, which occur commonly in insects, work with host-encoded factors to overcome natural enemies. They will also help us to better understand the dynamics and distributions of protective symbionts, like *H. defensa* in aphids, which despite conferring important benefits are not fixed in host populations. Finally, this information can be used to understand how symbiont infection and host resistance might directly and indirectly influence populations of interacting insects with effects extending to: host-natural enemy fitness, competition, development, and natural selection and evolution. Note: the general introduction above (sections 1.1 through 1.4) entitled, *How resident microbes modulate ecologically-important traits of insects,* is modified from an existing publication (Martinez & Oliver 2014).

1.7 References

Adams AS, Aylward FO, Adams SM, *et al.* (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and environmental microbiology* **79**, 3468-3475.

Asplen MK, Bano N, Brady CM, *et al.* (2014) Specialization of bacterial endosymbionts that protect aphids from parasitoids. *Ecological Entomology* **Accepted**.

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. In: *Annual Review of Microbiology*, pp. 155-189.

Beckage NE, Gelman DB (2004) Wasp parasitoid disruption of host development: Implications for new biologically based strategies for insect control. *Annual Review of Entomology* **49**, 299-330.

Boulinier T, Sorci G, Monnat JY, Danchin E (1997) Parent-offspring regression suggests heritable susceptibility to ectoparasites in a natural population of kittiwake *Rissa tridactyla*. *Journal of Evolutionary Biology* **10**, 77-85.

Brinkhof MW, Heeb P, Kölliker M, Richner H (1999) Immunocompetence of nestling great tits in relation to rearing environment and parentage. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 2315-2322.

Buchner P (1965) *Endosymbiosis of animals with plant microorganisms* Interscience Publishers, John Wiley and Sons, Inc., New York, London, Sydney.

Campbell MA, Van Leuven JT, Meister RC, *et al.* (2015) Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont Hodgkinia. *Proceedings of the National Academy of Sciences*, 201421386.

Caragata EP, Rances E, Hedges LM, *et al.* (2013) Dietary cholesterol modulates pathogen blocking by *Wolbachia*. *PLoS Pathogens* **9**, e1003459.

Carius HJ, Little TJ, Ebert D (2001) Genetic variation in a host-parasite association: potential for coevolution and frequency-dependent selection. *Evolution* **55**, 1136-1145.

Caspi-Fluger A, Inbar M, Mozes-Daube N, *et al.* (2012) Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proceedings of the Royal Society B-Biological Sciences* **279**, 1791-1796.

Casteel CL, Hansen AK, Walling LL, Paine TD (2012) Manipulation of plant defense responses by the tomato psyllid (*Bactericerca cockerelli*) and its associated endosymbiont *Candidatus* Liberibacter psyllaurous. *PLoS One* **7**, e35191.

Cayetano L, Vorburger C (2015) Symbiont-conferred protection against Hymenopteran parasitoids in aphids: how general is it? *Ecological Entomology* **40**, 85-93.

Chung SH, Rosa C, Scully ED, *et al.* (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 15728-15733.

Cirimotich CM, Dong YM, Clayton AM, *et al.* (2011) Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* **332**, 855-858.

Cloutier C, Douglas AE (2003) Impact of a parasitoid on the bacterial symbiosis of its aphid host. *Entomologia Experimentalis et Applicata* **109**, 13-19.

Cockburn SN, Haselkorn TS, Hamilton PT, *et al.* (2013) Dynamics of the continent-wide spread of a *Drosophila* defensive symbiont. *Ecology Letters* **16**, 609-616.

Coon KL, Vogel KJ, Brown MR, Strand MR (2014) Mosquitoes rely on their gut microbiota for development. *Molecular Ecology* **23**, 2727-2739.

Costopoulos K, Kovacs JL, Kamins A, Gerardo NM (2014) Aphid facultative symbionts reduce survival of the predatory lady beetle *Hippodamia convergens*. *BMC Ecology* **14**, 5.

Danyk TP (1992) *Competitive Interactions Between the Pea Aphid Parasitoids, Aphidius ervi and Praon pequodorum (Hymenoptera: Aphidiidae): Influence on Guild Composition in Southern British Columbia*, Simon Fraser University.

Degnan PH, Moran NA (2008) Diverse phage-encoded toxins in a protective insect endosymbiont. *Applied and environmental microbiology* **74**, 6782-6791.

Despres L, David JP, Gallet C (2007) The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology & Evolution* **22**, 298-307.

Digilio MC, Isidoro N, Tremblay E, Pennacchio F (2000) Host castration by *Aphidius ervi* venom proteins. *Journal of insect Physiology* **46**, 1041-1050.

Dion E, Polin SE, Simon JC, Outreman Y (2011a) Symbiont infection affects aphid defensive behaviours. *Biology Letters* **7**, 743-746.

Dion E, Zele F, Simon JC, Outreman Y (2011b) Rapid evolution of parasitoids when faced with the symbiont-mediated resistance of their hosts. *Journal of Evolutionary Biology* **24**, 741-750.

Douglas A (1989a) Mycetocyte symbiosis in insects. *Biological Reviews* **64**, 409-434.

Douglas A (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annual Review of Entomology* **43**, 17-37.

Douglas AE (1989b) Mycetocyte Symbiosis in Insects. *Biological Reviews of the Cambridge Philosophical Society* **64**, 409-434.

Douglas AE (2013) Microbial brokers of insect-plant interactions revisited. *Journal of Chemical Ecology* **39**, 952-961.

Dowd PF, Smith CM, Sparks TC (1983) DETOXIFICATION OF PLANT TOXINS BY INSECTS. *Insect Biochemistry* **13**, 453-468.

Ebert D, Zschokke-Rohringer CD, Carius HJ (1998) Within–and between–population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265**, 2127-2134.

Ehrlich PR, Raven PH (1964) Butterflies and plants: a study in coevolution. *Evolution*, 586-608.

Engel P, Moran NA (2013) The gut microbiota of insects–diversity in structure and function. *FEMS Microbiology Reviews* **37**, 699-735.

Erickson DM, Wood EA, Oliver KM, Billick I, Abbot P (2012) The effect of ants on the population dynamics of a protective symbiont of aphids, *Hamiltonella defensa*. *Annals of the Entomological Society of America* **105**, 447-453.

Evison SEF, Fazio G, Chappell P, *et al.* (2013) Host-parasite genotypic interactions in the honey bee: the dynamics of diversity. *Ecology and Evolution* **3**, 2214-2222.

Falabella P, Riviello L, Caccialupi P, *et al.* (2007) A γ-glutamyl transpeptidase of *Aphidius ervi* venom induces apoptosis in the ovaries of host aphids. *Insect biochemistry and molecular biology* **37**, 453-465.

Falabella P, Tremblay E, Pennacchio F (2000) Host regulation by the aphid parasitoid *Aphidius ervi*: the role of teratocytes. *Entomologia Experimentalis et Applicata* **97**, 1-9.

Feldhaar H, Gross R (2009) Insects as hosts for mutualistic bacteria. *International Journal of Medical Microbiology* **299**, 1-8.

Fellowes MDE, Kraaijeveld AR, Godfray HCJ (1999) Association between Feeding Rate and Parasitoid Resistance in *Drosophila melanogaster*. *Evolution* **53**, 1302-1305.

Flórez LV, Biedermann PH, Engl T, Kaltenpoth M (2015) Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural product reports*.

Gehrer L, Vorburger C (2012) Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biology Letters* **8**, 613-615.

Godfray HCJ (1994) *Parasitoids: Behavioral and Evolutionary Ecology* Princeton University Press, Princeton.

Graham RI, Grzywacz D, Mushobozi WL, Wilson K (2012) *Wolbachia* in a major African crop pest increases susceptibility to viral disease rather than protects. *Ecology Letters* **15**, 993-1000.

Grimaldi D, Engel MS, Grimaldi D, Engel MS (2005) *Evolution of the insects* Cambridge University Press.

Grosholz ED (1994) The effects of host genotype and spatial distribution on trematode parasitism in a bivalve population. *Evolution*, 1514-1524.

Hackett SC, Karley AJ, Bennett AE (2013) Unpredicted impacts of insect endosymbionts on interactions between soil organisms, plants and aphids. *Proceedings of the Royal Society B-Biological Sciences* **280**, 20131275.

Hamilton PT, Leong JS, Koop BF, Perlman SJ (2014) Transcriptional responses in a *Drosophila* defensive symbiosis. *Molecular Ecology* **23**, 1558-1570.

Hamilton PT, Perlman SJ (2013) Host defense via symbiosis in *Drosophila*. *PLoS Pathogens* **9**, e1003808.

Hansen AK, Moran NA (2014) The impact of microbial symbionts on host plant utilization by herbivorous insects. *Molecular Ecology* **23**, 1473-1496.

Hansen AK, Vorburger C, Moran NA (2012) Genomic basis of endosymbiont-conferred protection against an insect parasitoid. *Genome Research* **22**, 106-114.

He X, Wang Q, Teulon D (2005) The effect of parasitism by *Aphidius ervi* on development and reproduction of the pea aphid, *Acyrthosiphon pisum*. *New Zealand Plant Protection* **58**, 202.

Henry LM, Peccoud J, Simon JC, *et al.* (2013) Horizontally transmitted symbionts and host colonization of ecological niches. *Current Biology* **23**, 1713-1717.

Henter HJ, Via S (1995) The potential for coevolution in a host-parasitoid system. I. Genetic variation within an aphid population in susceptibility to a parasitic wasp. *Evolution*, 427-438.

Himler AG, Adachi-Hagimori T, Bergen JE, *et al.* (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* **332**, 254-256.

Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ (2010) Adaptation via Symbiosis: Recent Spread of a *Drosophila* Defensive Symbiont. *Science* **329**, 212-215.

Kaltenpoth M, Winter SA, Kleinhammer A (2009) Localization and transmission route of Coriobacterium glomerans, the endosymbiont of pyrrhocorid bugs. *Fems Microbiology Ecology* **69**, 373-383.

Kent BN, Bordenstein SR (2010) Phage WO of *Wolbachia*: lambda of the endosymbiont world. *Trends in Microbiology* **18**, 173-181.

Kikuchi Y, Hayatsu M, Hosokawa T, *et al.* (2012) Symbiont-mediated insecticide resistance. *Proceedings of the National Academy of Sciences* **109**, 8618-8622.

Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Applied and environmental microbiology* **73**, 4308-4316.

Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences* **108**, 19288-19292.

Kroiss J, Kaltenpoth M, Schneider B, *et al.* (2010) Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology* **6**, 261-263.

Licht H, Schiott M, Rogowska-Wrzesinska A, *et al.* (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 583-587.

Luan J-B, Yao D-M, Zhang T, *et al.* (2013) Suppression of terpenoid synthesis in plants by a virus promotes its mutualism with vectors. *Ecology Letters* **16**, 390-398.

Lukasik P, van Asch M, Guo HF, Ferrari J, Godfray HCJ (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters* **16**, 214-218.

Martinez AJ, Weldon SR, Oliver KM (2013) Effects of parasitism on aphid nutritional and protective symbioses. *Molecular Ecology*, n/a-n/a.

McCutcheon JP, Moran NA (2010) Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. *Genome Biology and Evolution* **2**, 708-718.

McLean AHC, Godfray HCJ (2015) *Evidence for specificity in symbiont-conferred protection against parasitoids*.

Meisner M, Harmon JP, Ives AR (2007) Presence of an unsuitable host diminishes the competitive superiority of an insect parasitoid: a distraction effect. *Population ecology* **49**, 347-355.

Moller AP (1990) Effects of a haematophagous mite on the barn swallow (*Hirundo rustica*): a test of the Hamilton and Zuk hypothesis. *Evolution*, 771-784.

Moran NA, Degnan PH (2006) Functional genomics of *Buchnera* and the ecology of aphid hosts. *Molecular Ecology* **15**, 1251-1261.

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16919-16926.

Nakabachi A, Ueoka R, Oshima K, *et al.* (2013) Defensive bacteriome symbiont with a drastically reduced genome. *Current Biology* **23**, 1478-1484.

Nikoh N, Hosokawa T, Oshima K, Hattori M, Fukatsu T (2011) Reductive evolution of bacterial genome in insect gut environment. *Genome Biology and Evolution* **3**, 702-714.

Oliver KM, Campos J, Moran NA, Hunter MS (2008) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B-Biological Sciences* **275**, 293-299.

Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology* **55**, 247-266.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325**, 992-994.

Martinez AJ, Oliver KM (2014) How resident microbes modulate ecologically-important traits of insects. *Current Opinion in Insect Science* **4**, 1-7.

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12795-12800.

Oliver KM, Noge K, Huang EM, *et al.* (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC Biology* **10**, 11.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences* **100**, 1803-1807.

Oliver KM, Smith AH, Russell JA (2014) Defensive symbiosis in the real world - advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Functional Ecology* **28**, 341-355.

Opitz SEW, Muller C (2009) Plant chemistry and insect sequestration. *Chemoecology* **19**, 117-154.

Parker BJ, Barribeau SM, Laughton AM, de Roode JC, Gerardo NM (2011) Non-immunological defense in an evolutionary framework. *Trends in Ecology & Evolution* **26**, 242-248.

Parker BJ, Spragg CJ, Altincicek B, Gerardo NM (2013) Symbiont-mediated protection against fungal pathogens in pea aphids: a role for pathogen specificity? *Applied and environmental microbiology* **79**, 2455-2458.

Pennacchio F, Fanti P, Falabella P, *et al.* (1999) Development and nutrition of the braconid wasp, *Aphidius ervi* in aposymbiotic host aphids. *Archives of Insect Biochemistry and Physiology* **40**, 53-63.

Pennacchio F, Strand MR (2006) Evolution of developmental strategies in parasitic Hymenoptera. In: *Annual Review of Entomology*, pp. 233-258.

Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14002-14007.

Polin S, Simon JC, Outreman Y (2014) An ecological cost associated with protective symbionts of aphids. *Ecology and Evolution* **4**, 826-830.

Price PW, Denno RF, Eubanks MD, Finke DL, Kaplan I (2011) *Insect ecology: behavior, populations and communities* Cambridge University Press, New York.

Rausher MD (2001) Co-evolution and plant resistance to natural enemies. *Nature* **411**, 857-864.

Rolff J, Siva-Jothy MT (2003) Invertebrate ecological immunology. *Science* **301**, 472-475.

Rouchet R, Vorburger C (2014) Experimental evolution of parasitoid infectivity on symbiont-protected hosts leads to the emergence of genotype specificity. *Evolution* **68**, 1607-1616.

Russell JA, Weldon S, Smith AH, *et al.* (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. *Molecular ecology* **22**, 2045-2059.

Sadd BM, Barribeau SM (2013) Heterogeneity in infection outcome: lessons from a bumblebee-trypanosome system. *Parasite Immunology* **35**, 339-349.

Sadd BM, Schmid-Hempel P (2009) Principles of ecological immunology. *Evolutionary Applications* **2**, 113-121.

Schellhorn NA, Kuhman TR, Olson AC, Ives AR (2002) Competition between native and introduced parasitoids of aphids: nontarget effects and biological control. *Ecology* **83**, 2745-2757.

Schmid M, Sieber R, Zimmermann YS, Vorburger C (2012) Development, specificity and sublethal effects of symbiont-conferred resistance to parasitoids in aphids. *Functional Ecology* **26**, 207-215.

Smith JA, Wilson K, Pilkington JG, Pemberton JM (1999) Heritable variation in resistance to gastro-intestinal nematodes in an unmanaged mammal population. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 1283-1290.

Spitze K (1992) Predator-mediated plasticity of prey life history and morphology: *Chaoborus americanus* predation on *Daphnia pulex*. *American Naturalist*, 229-247.

Strand MR, Burke GR (2012) Polydnaviruses as symbionts and gene delivery systems. *PLoS Pathogens* **8**, e1002757.

Van Leuven JT, Meister RC, Simon C, McCutcheon JP (2014) Sympatric speciation in a bacterial endosymbiont results in two genomes with the functionality of one. *Cell* **158**, 1270-1280.

Vinson SB, Iwantsch G (1980) Host regulation by insect parasitoids. *Quarterly Review of Biology*, 143-165.

Waldbauer GP (1968) *The consumption and utilization of food by insects* Beament, J. W. L., Treherne, J. E. & Wigglesworth, V. B. Advances in insect physiology. Volume 5. Academic Press, London & New York.

Weldon SR, Strand MR, Oliver KM (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. *Proceedings of the Royal Society B-Biological Sciences* **280**.

Wernegreen JJ (2002) Genome evolution in bacterial endosymbionts of insects. *Nature Reviews Genetics* **3**, 850-861.

White JA, Giorgini M, Strand MR, Pennacchio F (2013) Arthropod endosymbiosis and evolution. In: *Arthropod Biology and Evolution*, pp. 441-477. Springer.

Xie J, Butler S, Sanchez G, Mateos M (2014) Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. *Heredity* **112**, 399-408.

Ye YXH, Woolfit M, Rances E, O'Neill SL, McGraw EA (2013) *Wolbachia*-associated bacterial protection in the mosquito *Aedes aegypti*. *PLoS Neglected Tropical Diseases* **7**, e2362.

Zhang GM, Hussain M, O'Neill SL, Asgari S (2013) Wolbachia uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 10276-10281.

Zientz E, Dandekar T, Gross R (2004) Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiology and Molecular Biology Reviews* **68**, 745-+.

CHAPTER 2

APHID-ENCODED VARIABILITY IN SUSCEPTIBILITY TO A PARASITOID [1]

[1] Martinez AJ, Ritter SG, Doremus MR, Russell JA, Oliver KM. 2014. *BMC Evolutionary Biology*. 14(1):127. Reprinted here with permission of the publisher.

Abstract

Many animals exhibit variation in resistance to specific natural enemies. Such variation may be encoded in their genomes or derived from infection with protective symbionts. The pea aphid, *Acyrthosiphon pisum,* for example, exhibits tremendous variation in susceptibility to a common natural enemy, the parasitic wasp *Aphidius ervi*. Pea aphids are often infected with the heritable bacterial symbiont, *Hamiltonella defensa,* which confers partial to complete resistance against this parasitoid depending on bacterial strain and associated bacteriophages. That previous studies found that pea aphids without *H. defensa* (or other symbionts) were generally susceptible to parasitism, together with observations of a limited encapsulation response, suggested that pea aphids largely rely on infection with *H. defensa* for protection against parasitoids. However, the limited number of uninfected clones previously examined, and our recent report of two symbiont-free resistant clones, led us to explicitly examine aphid-encoded variability in resistance to parasitoid. After rigorous screening for known and unknown symbionts, and microsatellite genotyping to confirm clonal identity, we conducted parasitism assays using fifteen clonal pea aphid lines. We recovered significant variability in aphid-encoded resistance, with variation levels comparable to that contributed by *H. defensa.* Because resistance can be costly, we also measured aphid longevity and cumulative fecundity of the most and least resistant aphid lines under permissive conditions, but found no trade-offs between higher resistance and these fitness parameters. These results indicate that pea aphid resistance to *A. ervi* is more complex than previously appreciated, and that aphids employ multiple tactics to aid in their defense. While we did not detect a tradeoff, these may become apparent under stressful conditions or when resistant and

susceptible aphids are in direct competition. Understanding sources and amounts of variation in resistance to natural enemies is necessary to understand the ecological and evolutionary dynamics of antagonistic interactions, such as the potential for coevolution, but also for the successful management of pest populations through biological control.

2.1 Introduction

Insects and other animals face attack from a wide range of natural enemies which place strong selective pressures on the development, acquisition, and maintenance of resistance (Boulinier *et al.* 1997; Brinkhof *et al.* 1999; Ebert *et al.* 1998; Fellowes *et al.* 1999; Grosholz 1994; Jaenike *et al.* 2010; Moller 1990; Oliver *et al.* 2008; Smith *et al.* 1999; Spitze 1992). Intraspecific variation in resistance to natural enemies has been documented in many organisms and can stem from factors encoded in the host's genome or those acquired from microbial associates (Carius *et al.* 2001; Evison *et al.* 2013; Martinez *et al.* 2013; Parker *et al.* 2011; Sadd & Barribeau 2013; Sadd & Schmid-Hempel 2009). Such variation is important for adaptation via natural selection, can promote the evolution of virulence in natural enemies, and drive host-enemy coevolutionary dynamics (Rolff & Siva-Jothy 2003; Sadd & Schmid-Hempel 2009). Resistance, however, may also be expected to carry costs as resources allocated to the defense are unavailable for other functions (Schmid-Hempel 2005), such that resistance may result in decreased fitness under enemy-free conditions (Armitage *et al.* 2003; Boots & Begon 1993; Kraaijeveld & Godfray 1997; Sutter *et al.* 1968; Webster & Woolhouse 1999; Yan *et al.* 1997). Furthermore, selection for host resistance can reduce enemy prevalence or encourage increased virulence, which in turn, can result in negative frequency-dependent selection against the now potentially costly, resistant phenotypes

(Carius *et al.* 2001; Chaboudez & Burdon 1995; Koskella & Lively 2009). Variability in resistance may also be maintained by fluctuations in enemy pressure, variation in enemy virulence, host-enemy specificity, and mediating environmental factors (Bryan-Walker *et al.* 2007; Foster *et al.* 2007; Luong & Polak 2007; Martinez *et al.* 2013; Sanders *et al.* 2005; Triggs & Knell 2012). Thus, quantifying variation and costs, and properly attributing the sources of variation in resistance to natural enemies, are often required to understand the ecological and evolutionary dynamics associated with antagonistic interactions, including those of economic concern, such as the successful deployment of biological control programs targeting pest organisms.

Insect-parasitoid interactions are among the most common antagonistic interactions in nature, where the survival of one player ultimately leads to the death of the other, resulting in strong selection for host resistance and parasite counter-resistance (Godfray 1994; Kraaijeveld & Godfray 2009). Interactions between the phloem-feeding aphids (Hemiptera) and their hymenopteran parasitoids have received considerable attention (rev. (Le Ralec *et al.* 2010)). Many aphids, including the pea aphid, *Acyrthosiphon pisum*, reproduce parthenogenetically for most of the year, such that variation in resistance among clonal lines to particular natural enemies can be examined. An important study by Henter and Via (Henter & Via 1995) found that some North American pea aphid clonal lines were totally resistant to attack by the prevalent parasitoid *Aphidius ervi*, while other clones were highly susceptible. Subsequent work found that European pea aphid clones also varied in resistance to the wasp *A. ervi*, as well as the more host-specific congener *A. eadyi* (Ferrari *et al.* 2001; Gwynn *et al.* 2005; Stacey & Fellowes 2002). It was first assumed that variation in resistance resulted from

immunological pathways encoded in the aphid genome, but later studies found that infection with heritable bacterial symbionts, was responsible for a substantial portion of the observed variation (Oliver *et al.* 2003). A number of studies identified correlations between infection with *Hamiltonella defensa* and increased clonal resistance to parasitism in the laboratory, but did not explicitly disentangle host- and symbiont-based effects (e.g. (Bensadia *et al.* 2006; Ferrari *et al.* 2004; Guay *et al.* 2009)). Simultaneous experimental studies, comparing aphid clones with and without symbionts, found that most *H. defensa* strains, and a single *Serratia symbiotica* strain, provided defense against the wasp *A. ervi* (Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2006; Oliver *et al.* 2003). Further investigation of this interaction found that bacteriophages called APSEs were required for *H. defensa* to confer protection to pea aphids (Degnan & Moran 2008; Moran *et al.* 2005; Oliver *et al.* 2009) and that levels of resistance to the wasp varied greatly and correlated with symbiont strain and associated virus type; uninfected aphid clones (i.e. no facultative symbionts), on the other hand, exhibited limited variation in resistance and were highly susceptible to attack (Oliver *et al.* 2009; Oliver *et al.* 2005). Together, this work suggested that pea aphids primarily rely on infection with *H. defensa* and APSE to thwart attack from this common natural enemy. This hypothesis was bolstered by the observation that pea aphids have a weak encapsulation response to parasitism (Carver & Sullivan 1988; Laughton *et al.* 2011). A recent study, however, found two pea aphid clones exhibited substantial resistance to *A. ervi* in the absence of *H. defensa* or other facultative symbionts (Martinez *et al.* 2013) indicating that aphid-based resistance persists in North American *A. pisum* populations and may contribute more to the observed variation in susceptibility than is currently appreciated. Aphid-encoded

resistance to parasitism has also been reported in the peach-potato aphid, *Myzus persicae*, and the black bean aphid, *Aphis fabae* (Sandrock *et al.* 2010; von Burg *et al.* 2008; Vorburger *et al.* 2010).

To examine the extent of pea aphid encoded variability in resistance to parasitism by the wasp *A. ervi*, we conducted parasitism assays across a range of aphid clones that were devoid of facultative symbionts. We also estimated the fecundity and longevity of several clonal lines of varying resistance to determine whether increases in resistance correlated with reductions in fitness, which would be expected if resistance is energetically costly.

2.2 Materials and Methods

Study organisms

The pea aphid, *Acyrthosiphon pisum*, has diversified into numerous genetically distinct host races that specialize (i.e. have increased preference for, and performance on) on a variety of cultivated herbaceous legumes, including economically important crops such as alfalfa and clover (Caillaud & Via 2000; Ferrari *et al.* 2006; Ferrari *et al.* 2007; Ferrari *et al.* 2008; Frantz *et al.* 2006; Hufbauer & Via 1999; Peccoud *et al.* 2009; Via *et al.* 2000). This aphid was introduced to North America from Europe in the late 1800s (Eastop 1966), but native and introduced populations exhibit similar patterns of linkage disequilibrium, nucleotide diversity and symbiont diversity; together suggesting bottleneck effects have not limited diversity relative to source populations (Brisson *et al.* 2009; Ferrari *et al.* 2012; Russell *et al.* 2013). At most N. American latitudes this aphid is cyclically parthenogenetic and reproduction is asexual and viviparous for the majority of the year, with sexual morphs arising in the fall in response to shorter day lengths (Lamb

& Pointing 1972). Clonal lines were maintained in the laboratory by rearing them under long day conditions. Each clonal aphid line used in this study (Table 2.1) was initiated from a single parthenogenetic female placed onto a caged broad bean plant, *Vicia faba,* and reared at 20±1˚C with a 16L: 8D photoperiod. We verified that all experimental aphid lines used in this study were free of facultative symbionts by using 1) diagnostic PCR to screen for all known pea aphid facultative symbionts, 2) 'mostly universal' PCR primers that amplify most bacteria, but not the obligate symbiont *Buchnera,* and 3) Denaturing Gradient Gel Electrophoresis (DGGE) with universal 16S rRNA bacterial primers. Primers, PCR cocktails and reaction conditions, and detailed DGGE protocols can be found in Russell et al. (Russell *et al.* 2013); all PCR reactions contained positive and negative controls.

The solitary endoparasitoid, *Aphidius ervi* (Hymenoptera: Braconidae), also introduced from Europe, is the most prevalent parasitic wasp attacking *A. pisum* populations in North America (Angalet & Fuester 1977). The wasps used in this study were obtained from a single, large, laboratory colony containing a mixture of *A. ervi* collected from Wisconsin and North Dakota, as well as commercially produced mummies (Arbico Organics). Wasps were reared continuously on a susceptible aphid line, AS3-AB; adults were provided with constant access to honey and water**.**

Table 2.1 Genetically distinct aphid clonal lines used in this study.

Aphid Clone	Collection locale	Host Plant	Reference
5A	Wisconsin, USA 1999	Alfalfa	(Sandstrom et al. 2001)
AS3-AB	Utah, USA 2007	Alfalfa	(Martinez et al. 2013)
CJ1-13	Utah, USA 2012	Alfalfa	This paper
CJ1-15	Utah, USA 2012	Alfalfa	This paper
CJ2-6	Utah, USA 2012	Alfalfa	This paper
CJ4-2	Utah, USA 2012	Alfalfa	This paper
LSR01	New York, USA 1998	Alfalfa	(Richards et al. 2010)
PB17	Pennsylvania, USA 2011	Alfalfa	This paper
WA4-AB	Pennsylvania, USA 2010	Alfalfa	(Martinez et al. 2013)
WI27	Wisconsin, USA 2011	Alfalfa	This paper
WI48	Wisconson, USA 2011	Alfalfa	This paper
ZA17-AB	Pennsylvania, USA 2010	Alfalfa	(Martinez et al. 2013)
BP14	Georgia, USA 2010	Crimson Clover	(Parker et al. 2014)
G15	Georgia, USA 2008	Mixed Weeds	(Parker et al. 2014)
G6	Georgia, USA 2008	Mixed Weeds	(Barribeau et al. 2010)

Microsatellite analyses to distinguish clonal lines

Microsatellite genotyping was used to confirm the identity and genetic variability between clonal aphid lines used in this study. DNA extractions of each aphid line were performed using an Omega EZNA® Tissue DNA Kit and were stored at -20° C until use. Four microsatellite loci— Ap-02, Ap-03, Ap-05 (Kurokawa *et al.* 2004), and Aph10M (Caillaud *et al.* 2004)—were PCR amplified with Dye Set-30 (DS-30) fluorescent primers using a touchdown reaction as follows: 94° C for 3 min; 45 cycles of 95° C for 30 s, 68–56° C touchdown for 13 cycles, then 55° C for 32 cycles, each cycle for 30 s, 72° C for 30 s; 72° C for final elongation, then held at 4° C. Fluorescent genotyping was then conducted by The Georgia Genomics Facility on an Applied Biosystems 3730xl DNA Analyzer, using the ROX500 size standard. Genotypic data were then analyzed using Geneious® version 6.1 (Biomatters).

Analysis of aphids typed at the four microsatellite loci revealed that all fifteen pea aphid lines used in this study represented distinct genotypes (See Supplemental Table 2.1 for details on loci used and allele sizes).

Aphid parasitism resistance assays to determine protective phenotype

Parasitism assays to determine the resistance phenotype were carried out on all aphid lines used in this study (Table 2.1) as in (Oliver *et al.* 2009). Twenty 2nd to 3rd instar aphids were singly parasitized (each aphid is removed as it is parasitized) for each replicate (at least eight replicates) and placed on a fresh *V. faba* plant in a cup cage and held at 20±1˚C and 50% relative humidity with a 16L: 8D photoperiod. Prior studies have shown that isofemale lines of *A. ervi* wasps can vary in their counter-resistance (i.e. virulence), defined as their ability to successfully parasitize pea aphids (Henter 1995),

suggesting also that virulence, at least toward symbiont-mediated resistance, may evolve rapidly (Dion *et al.* 2011). Although we have not observed substantial variation in wasp virulence (Oliver, personal observation), we designed our parasitism assays to minimize such potential effects. In short, utilized wasps were collected haphazardly from our large laboratory culture (see above), which was maintained on a highly susceptible clone, and numerous female wasps were used to singly parasitize each line. After nine days, we counted the number of live aphids, dead aphids, and aphid mummies (dried aphids containing a wasp pupa) to determine the proportion of each measured as: survival (live aphids/20), mortality (dead aphids/20), and mummification (aphid mummies/20). A large majority of adult wasps eclose from mummies making them a suitable proxy for determining levels of successful parasitism (Oliver *et al.* 2012). To determine background rates of mortality for each line, we placed five replicates of twenty *unparasitized* 2nd to 3rd instar aphids from each line on fresh plants and mortality was recorded from the control lines after nine days.

Aphid fitness assays

We conducted cumulative fecundity assays under permissive conditions (no aphid or plant stresses) to investigate potential tradeoffs between parasitoid resistance and aphid fecundity. Six aphid lines, those with the most (WA4-AB, ZA17-AB, CJ1-13) and least resistance (G15, AS3-AB, LSR01) to *A. ervi* (Fig. 2.1), were selected for this assay. Prior to the experiment, each aphid line was reared on multiple plants, chosen haphazardly, from a cohort of healthy plants of similar age and size for several generations in 16L:8D intervals at 20° C in a Percival I-41LLVL environmental incubator to reduce effects resulting from variation in prior culturing (i.e. maternal and

grandmaternal effects). From these cultures, approximately twenty adults from each line were placed on a fresh plant and allowed to reproduce for 17±1 h before removal. The resulting offspring were left to mature until they were 48 ± 8.5 h-old and then six nymphs (x8 replicates = 48 aphids per line) were placed in a cup cage containing a single *Vicia faba* plant. Cages were examined at 3-day intervals. At these times, the numbers of live and dead aphids of the original cohort were recorded to measure longevity, while the numbers of offspring produced were recorded to measure fecundity. Offspring were discarded at the time of counting to prevent their growth to maturity and subsequent offspring production as in (Oliver *et al.* 2006).

Statistical analyses

Aphid survival, mortality, and mummification (see above) were determined for each replicate of each parasitized aphid line. These values were used to compare differences among aphid lines using a Generalized Linear Model (GzLM), with a binomial distribution and logit link function. Survival, mortality, and mummification data were mildly overdispersed and so final test values are reported with a quasibinomial adjustment. A Post hoc Tukey's HSD test on aphid survival, mortality, and mummification was performed using an ANOVA of arcsine transformed proportional data for pairwise comparisons among aphid lines. GzLM was also used to compare mortality of parasitized and control (unparasitized) aphids, both within and across lines (see Supplemental Figure 2.1). As aphid mortality after parasitism may be tied to differences among aphid lines, linear regression was performed on mean mortality between unparasitized and parasitized aphids among all lines used (see Supplemental

Figure 2.1). Mean mortality was natural log transformed to satisfy normality assumptions of the linear regression.

Several analyses were employed to compare fitness parameters (fecundity and longevity) between aphids with high and low resistance to parasitism. Linear mixed models with heterogeneous first order auto regressive (ARH1) covariance structure (to account for repeated measures) were used to examine the effect of aphid line and resistance on cumulative aphid fecundity through several time points. Multivariate analysis of variance (MANOVA) with repeated measures design was used to examine the effect of aphid line and resistance on aphid longevity. All analyses comparing the effect of resistance on aphid fecundity or longevity were done by nesting 'aphid genotype' (six aphid lines) within 'resistance' (high or low).

2.3 Results

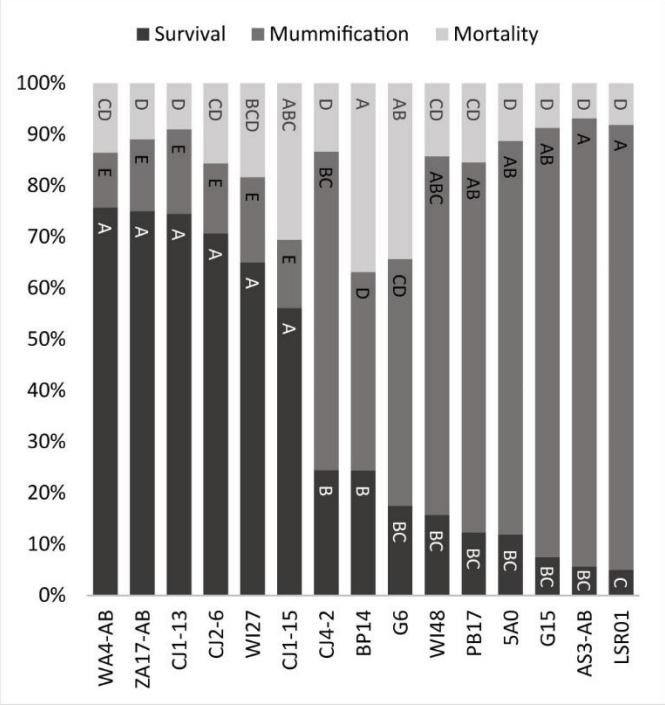


Figure 2.1 Survival, mummification, and mortality rates of *A. pisum* counted nine days after parasitism by *A. ervi.* Significant differences are indicated by letters (Tukey's HSD $\alpha = 0.05$). GzLM, df = 14, $p < 0.0001$ for all comparisons.

Parasitism assays

Parasitism by *A. ervi* results in three possible outcomes: wasps may complete development through pupation (i.e. aphid dies and is converted into a wasp "mummy"), aphids may survive parasitism and grow to adulthood, or both aphid and wasp may perish following parasitism. Among all fifteen pea aphid lines that were uninfected with facultative symbionts, we found significant variability in all three outcomes. From the aphid perspective, we find significant variation in their susceptibility to this important natural enemy (Survival: GzLM, $\chi^2 = 488.2$, df = 14, $p < 0.0001$) with survival rates ranging from 5 – 76%. Mortality (to both aphid and wasp) also varied significantly among lines (Mortality: 7 – 37%; GzLM, $\chi^2 = 100.8$, df = 14, $p < 0.0001$); however, in general, aphids that were not successfully parasitized (i.e. mummified) survived to adulthood (Fig. 2.1). Mummification (successful parasitism) also varied among lines (Mummification: 11 – 88%; GzLM, $\chi^2 = 424.6$, df = 14, $p < 0.0001$).

The majority of our *A. pisum* clones (12/15) were collected from alfalfa. To determine if there is significant variation in susceptibility among clones of this host race we conducted a restricted analysis and found similar variation in survival, mortality, and mummification (GzLM df = 11; $\chi^2 = 389.2$, 38.24, 344.82; $p < 0.0001$, < 0.0001, and < 0.0001; respectively).

Mortality also varied significantly among control lines not exposed to wasps (2 – 15%; GzLM, df = 14, $\chi^2 = 24.45$, $p = 0.0404$), and parasitism often resulted in significant increases in mortality relative to controls of the same line (Supplemental Figure 2.1). A linear regression analysis found no correlation between mortality of unparasitized controls and parasitized treatments (Linear Regression, $F_{1, 13} = 2.02$, $p = 0.1784$)

indicating that parasitism-induced mortality affects clonal lines differently than their background mortality.

Aphid fitness assays

Total aphid fecundity, per replicate cup cage, measured over a twenty-four-day period revealed significant variation among the six (three high and low resistance) aphid lines tested (Fig. 2.2A; Table 2.2A), but we found no inverse correlation (i.e. tradeoff) between resistance and fecundity (Fig. 2.2B; Table 2.2B)). We also estimated daily

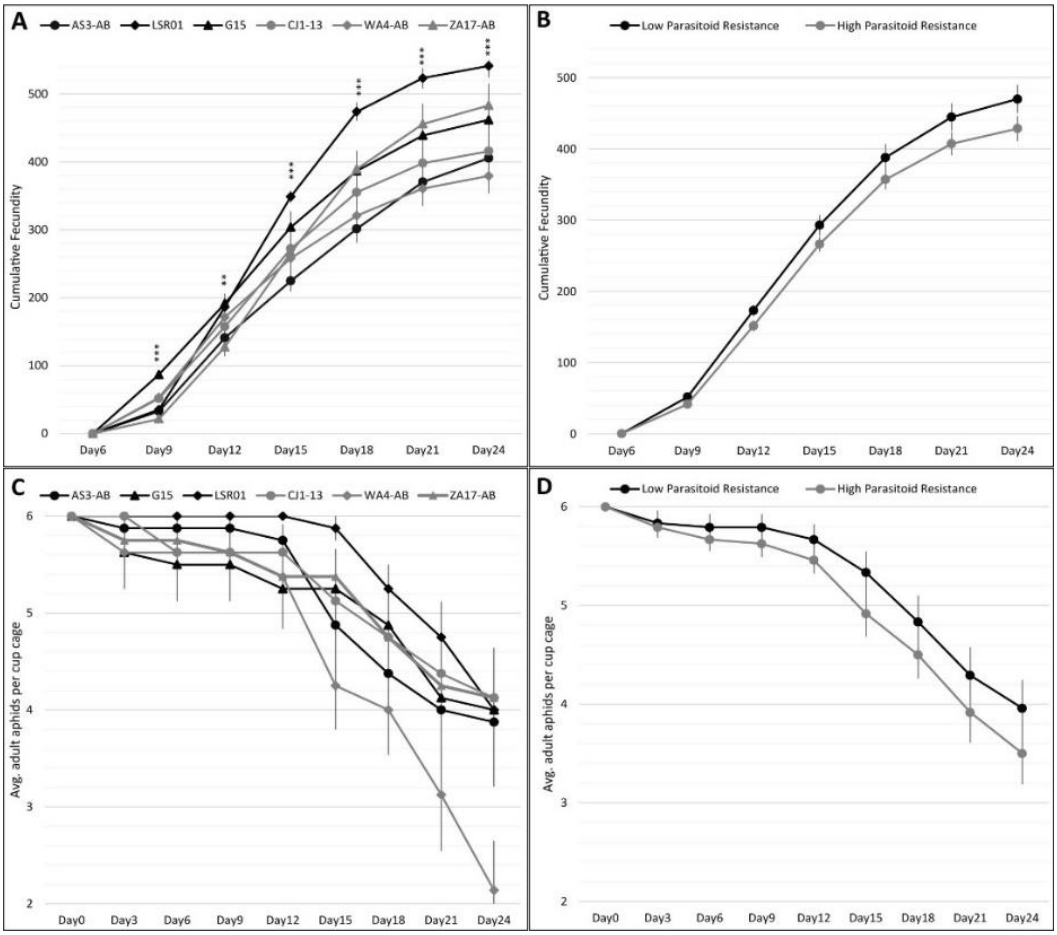


Figure 2.2 Aphid fecundity and longevity among lines showing low (black) and high (grey) resistance to parasitism. A) Cumulative fecundity for each aphid line. B) Cumulative fecundity between high and low parasitoid resistance groups. C) Longevity of each aphid line. D) Longevity between high and low parasitoid resistance groups. Black and grey lines correspond to aphids showing low and high resistance to parasitism, respectively. See Table 2.2 for tests and significance values. **p < 0.01, ***p < 0.001

Table 2.2 Effect of aphid genotype and resistance phenotype on fecundity and longevity

	Aphid Fecundity (Cumulative)						Aphid Longevity					
	(A) Aphid Line			(B) Resistance			(C) Aphid Line			(D) Resistance		
	Df	F-Value	Significance	Df	F-Value	Significance	Df	F-Value	Significance	Df	F-Value	Significance
Day 3	-	-	-	-	-	-	5,42	0.71	0.622	1,42	0.06	0.8047
Day 6	-	-	-	-	-	-	5,42	0.71	0.6226	1,42	0.49	0.4885
Day 9	5,39.1	10.38	<0.0001*	1,4.27	0.11	0.7593	5,42	0.64	0.668	1,42	0.76	0.3893
Day 12	5,40.5	3.7	0.0075*	1,5.75	0.43	0.5384	5,42	1.3	0.283	1,42	1.06	0.309
Day 15	5,41.9	5.68	0.0004*	1,8.82	0.57	0.4691	5,42	2.17	0.0758	1,42	1.92	0.1728
Day 18	5,44.4	8.6	<0.0001*	1,11.9	0.69	0.4214	5,42	0.95	0.4562	1,42	0.86	0.3595
Day 21	5,47.5	7.43	<0.0001*	1,11.9	1.03	0.331	5,42	1.15	0.3498	1,42	0.82	0.3709
Day 24	5,49.7	6.21	0.0002*	1,12.2	1.25	0.2859	5,42	2.24	0.068	1,42	1.32	0.2568

Analysis of (A) the effect of aphid line on cumulative fecundity (linear mixed model), (B) the effect of resistance to parasitism on cumulative fecundity (linear mixed model), (C) the effect of aphid line on aphid longevity (repeated measures MANOVA), (D) the effect of aphid resistance on longevity (repeated measures MANOVA). Six aphid genotypes were analyzed, three weakly and three highly resistant to parasitism. Aphid offspring were not present until day nine. Significant values are indicated with an asterisk. See Figure 2.2 for graphs of analyses.

fecundity per live adult aphid (Supplemental Figure 2.2) and, again found significant variation among lines, but we did not find a significant association with the resistance phenotype. We also measured longevity of the original cohort of aphids for each line (Fig. 2.2C; Table 2.2C) and between high and low resistance phenotypes (Fig. 2.2D; Table 2.2D), but found no significant differences in either. Overall, in the absence of parasitism, resistant and susceptible lines exhibited similar fitness profiles, with no significant impact on longevity or fecundity owed to resistance phenotype.

2.4 Discussion

Substantial variability in aphid-based resistance to parasitism

Pea aphids have been previously shown to exhibit substantial clonal variation in susceptibility to the parasitic wasp *A. ervi* (e.g. (Ferrari *et al.* 2001; Henter & Via 1995), with more recent studies showing that infection with the heritable protective symbiont *H. defensa* contributes to much of the observed variation (Martinez *et al.* 2013; Oliver *et al.*

2009; Oliver *et al.* 2005). In these latter studies, a total of eleven uninfected clones were all highly susceptible to attack, while infection with *H. defensa* resulted in varying levels of protection correlating with symbiont strain and associated bacteriophage haplotype (Degnan & Moran 2008; Martinez *et al.* 2013; Oliver *et al.* 2009; Oliver *et al.* 2005). Due to the limited number of aphid clones used in these studies, however, it remained unclear whether there was also appreciable variation in resistance encoded by the aphid genotype. Using fifteen clonal pea aphid lines free of *H. defensa* and other facultative symbionts, we report here that there is indeed extensive aphid-encoded variation for resistance to *A. ervi* (Fig. 2.1). In fact, the six most highly resistant clones (Fig. 2.1) exhibit levels of defense (~55 – 75% survival) comparable to those contributed by defensive symbionts (~35 – 100% survival) (Martinez *et al.* 2013; Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2012).

These findings indicate that pea aphids employ both aphid- and symbiont-based strategies to aid in their interactions with this prevalent natural enemy. In addition to aphid- and *H. defensa* encoded protection, other common aphid symbionts, including *S. symbiotica* (Oliver *et al.* 2003), or combinations of symbionts (Guay *et al.* 2009; Nyabuga *et al.* 2010; Oliver *et al.* 2006) show promise in influencing interactions with wasps. To date, however, the majority of uninfected clones (including those in this study: 21/27) examined in laboratory studies were found to be >65% susceptible to attack by this wasp (Martinez *et al.* 2013; Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2012; Oliver *et al.* 2003) suggesting that symbiont-based protection may be the most frequently used line of defense. However, infection frequencies of *H. defensa* are quite variable (10 to 58%) in N. American field populations (Russell *et al.* 2013), and there may be

dynamic temporal and spatial variation in the relative proportions of each mode of defense. This, of course, depends on the efficacy of symbiont- and aphid-based defenses under natural conditions, which is largely unknown (Bilodeau *et al.* 2013; Oliver *et al.* 2013). Temperature, for example, is known to affect *A. pisum* clonal resistance to parasitism and appears to be due primarily to losses in *H. defensa*-mediated protection at higher temperatures (Bensadia *et al.* 2006), but these assays were not conducted while controlling for aphid genotype and hence it is possible that temperature also impacts aphid-encoded resistance. If higher temperature indeed impacts symbiont-based resistance to a greater degree, then we would expect fewer *H. defensa*-bearing aphids and more aphid-encoded resistance in warmer regions and seasons.

The majority of clones we examined were collected from alfalfa (Table 2.1), and an analysis restricted to this host race also recovered substantial variation in susceptibility to parasitism (Fig. 2.1). Additional studies are required to determine if there is substantial aphid-encoded variation in susceptibility *within* populations or whether there is geographical variation among collection sites. We did not detect significant variation among sites in this study, but the sampling was very limited. Facultative symbiont infection frequencies are known to vary among pea aphid host races, and infection with *H. defensa* occurs more frequently on alfalfa than on other host plants (Ferrari *et al.* 2012; Russell *et al.* 2013). We might predict that aphid-encoded defenses against *A. ervi* are more common in other host races, such as clover, with lower *H. defensa* infection frequencies. One study, however, reported that variation in aphid resistance (due to any mechanism) was much lower on clover (~60 - 95% susceptible), compared to alfalfa (~5 – 90% susceptible), and that clover clones were generally more susceptible than alfalfa

clones to parasitism by *A. ervi* (Hufbauer & Via 1999). One possible explanation for the presence of both higher *H. defensa* infection frequencies and more aphid-encoded protection on alfalfa is that clover-derived *A. pisum* suffer reduced rates of attack under field conditions resulting in less selection pressure for the evolution and maintenance of resistance. However, we caution that further work is needed to evaluate the ranges of aphid-encoded resistance on clover (and other host races) and the importance of the various resistance components under field conditions.

The mechanisms underlying this aphid-based resistance to parasitism are unknown. The pea aphid lacks a strong encapsulation response, the innate cellular immune response used by many insects to encapsulate and asphyxiate invading parasitoid eggs (Carver & Sullivan 1988; Laughton *et al.* 2011; Strand & Pech 1995). Sequencing of the pea aphid genome revealed several pathways (e.g. IMD) involved in innate immunity against pathogenic microbes were missing, yet this aphid retains important pathways associated with encapsulation (Gerardo *et al.* 2010) and are able to melanize foreign objects (Laughton *et al.* 2011). We are currently investigating the phenology and mechanisms underlying both symbiont- and aphid-based immunity to this wasp.

Pea aphids also show clonal variation in susceptibility to the aphid-specific fungal entomopathogen *Pandora neoaphidis* (e.g. (Ferrari *et al.* 2001)). The heritable symbiont *Regiella insecticola* and other symbionts have been shown to confer protection against this and other specific fungal pathogens and thus contribute to variation in resistance (Lukasik *et al.* 2013; Parker *et al.* 2013; Scarborough *et al.* 2005). More recent work indicates that pea aphids also show aphid-based clonal variation in their susceptibility to *Pandora* (Parker *et al.* 2014), indicating both aphid- and symbiont-based

defensive strategies are utilized against diverse natural enemies. It will be interesting to determine if there is a negative correlation in resistance to parasitoids and fungal pathogens, providing a potential explanation for the persistence of susceptible genotypes. In addition, we are seeking to determine whether resistant genotypes are less likely to carry protective symbionts, as services are duplicated. Recently, two *H. defensa* strains were found not to confer any additional protection beyond that of their resistant host aphid genotype (Martinez *et al.* 2013) suggesting this may be the case, although it is also possible these strains would confer protection in a susceptible background, but that benefits are not additive. Duplication in defense, though, could partially explain why the beneficial symbiont *H. defensa* is not more prevalent in field populations (Martinez *et al.* 2013; Oliver *et al.* 2008; Oliver *et al.* 2013).

The black bean aphid, *Aphis fabae*, also shows variation in resistance to its common parasitoid *Lysiphlebus fabarum*, with some variation encoded by the defensive symbiont *H. defensa* (Schmid *et al.* 2012) and some likely encoded by the host genome (Sandrock *et al.* 2010). Other aphids, including *Aphis craccivora* and *Myzus persicae* show clonal variation in susceptibility to parasitoids, including evidence for both symbiont- and host-encoded resistance (Desneux *et al.* 2009; von Burg *et al.* 2008; Vorburger *et al.* 2010) indicating that aphids generally use a variety of mechanisms to aid in their defense.

Populations of the parasitoid *A. ervi* have also been shown to exhibit variation in the ability to successfully parasitize pea aphids (Henter 1995), but further work in this system is needed to determine whether there is variation in counter-resistance toward particular components of aphid defense. Such specificity in genotype by genotype

interactions may be directed toward either aphid- or symbiont-based components of resistance, and while duplicated services may not provide an advantage against the average wasp genotype, it may provide protection against a wider range of enemy genotypes. As mentioned above, the wasp *A. ervi* appears capable of evolving virulence toward symbiont-based protection (Dion *et al.* 2011), but it is unclear if it can do so against aphid-based defenses. Such genotype by genotype interactions have been best studied in the black bean aphid-*H. defensa*-*L. fabarum* interaction, where they occur between parasitoid genotypes and defensive symbiont strains, but have not been found between parasitoid and uninfected host genotypes (Cayetano & Vorburger 2013; Rouchet & Vorburger 2012; Sandrock *et al.* 2010). If wasps more readily evolve counter-resistance to symbiont-encoded resistance, this may lead to an increase in the frequency of *H. defensa*-free resistant clones in natural populations, or vice versa.

No apparent trade-offs between parasitoid resistance and fitness

The maintenance of clonal variation in pea aphid susceptibility to the parasitoid *A. ervi* could be explained by tradeoffs in other functions given limited resources. Aphids, including *A. pisum*, have evolved a number of life history traits associated with increasing reproductive output, including cyclical parthenogenesis, wing polyphenisms, and telescoping generations (Moran 1992). Thus, if aphid-based resistance to parasitism carries constitutive costs, then we might expect to see a negative correlation between resistance and fecundity or longevity. While we did find significant clonal variation in fecundity, we did not find a positive correlation between susceptibility and fecundity or longevity among the most and least resistant lines (Fig. 2.2; Table 2.2). Tradeoffs between resistance and aspects of host fitness, including development time, survival, and

fecundity have been observed in other systems (Armitage *et al.* 2003; Boots & Begon 1993; Webster & Woolhouse 1999; Yan *et al.* 1997), but they are often difficult to detect in aphid systems (Ferrari *et al.* 2001; von Burg *et al.* 2008). One study (Gwynn *et al.* 2005) did find a tradeoff between resistance and fecundity among ten clonal pea aphid lines, but it is unclear if resistance was symbiont or aphid-based.

One possible reason we did not find the expected trade-off is that costs are induced rather than constitutive, such that costs are only manifested upon attack. We are currently investigating fecundity among parasitized clonal lines and other sub-lethal effects of parasitism, but preliminary trials indicate parasitized aphids that survive have similar fecundity to unparasitized controls (AJM unpublished). It is also possible that tradeoffs may only become apparent under more stressful conditions or when clones are in direct competition for resources, as our lab assays were conducted using lines held separately and reared under very permissive conditions (Oliver *et al.* 2008). For example, Kraaijeveld and Godfray (Kraaijeveld & Godfray 1997) found trade-offs resulting from increased parasitoid resistance in *Drosophila melanogaster*, but these were only observable under high intraspecific competition for food resources. Costs associated with *H. defensa*-mediated resistance have also been difficult to detect in component fitness assays. Only when *H. defensa*-infected and uninfected lines sharing the same genotypes were reared together in population cages were costs to infection identified (Oliver *et al.* 2008). Thus, costs may become apparent under more realistic conditions, with varying biotic (e.g. plant quality) and abiotic factors (e.g. water stress, temperature), and when intra- and interspecific competition is present.

2.5 Conclusions

Here we show that pea aphid genomes maintain variation in susceptibility to a common natural enemy, the parasitoid *A. ervi*. Together, with prior work showing that infection with the heritable symbiont *H. defensa* confers varying levels of protection, depending on strain and phage type, it is clear that this aphid employs multiple strategies to thwart attack from parasitoids. It remains unclear whether resistant aphid genotypes and protective symbionts like *H. defensa* interact, or whether effects are additive or redundant, as this would be an important factor influencing the spread of symbiont- and aphid-based resistance in natural populations. It is important to understand the sources and amount of variation in resistance to common natural enemies, and how each is impacted by biotic and abiotic interactions. Temperature, for example, may differentially influence wasp and aphid behavioral responses and also affect the performance of aphid- and symbiont-encoded resistance (Oliver *et al.* 2012) depending on presence and type of defense. Multiple sources of resistance may limit the evolution of resistance (when, for e.g., both types are employed in same host) or generate complex genotype by genotype interactions where some wasp genotypes specialize on particular aphid clone-symbiont strain combinations. Understanding the sources and dynamics of resistance is also important for the effective management of pest populations. If resistance is due primarily to symbionts, for example, then a quick diagnostic screen may inform whether biological control applications are likely to be effective.

Availability of supporting data

The data sets supporting the results of this article are available in the Dryad repository (Martinez *et al.*), http://dx.doi.org/10.5061/dryad.6b5f0.

Competing interests

The authors declare that they have no competing interests.

Authors' Contributions

A.M. and K.O. designed the experiments. A.M., S.R., and M.D. performed the experiments. A.M. performed the microsatellite analyses, all statistical analyses, and created all figures and tables. A.M., K.O., and J.R. wrote the manuscript.

Acknowledgements

We would like to thank Kyungsun Kim for her technical support. We thank Edward Evans, Brandon Barton, Anthony Ives, Andrew Smith, and Benjamin Parker for help collecting aphid samples. We also thank Dr. Kim Love-Myers, Hsien-Lin Hsieh, and Xijue Tan for their statistical advice. This project was supported by National Science Foundation awards 1050128 and 1240892.

2.6 References

Angalet G, Fuester R (1977) The *Aphidius* parasites of the pea aphid *Acyrthosiphon pisum* in the eastern half of the United States. *Annals of the Entomological Society of America* **70**, 87-96.

Armitage SAO, Thompson JJW, Rolff J, Siva-Jothy MT (2003) Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. *Journal of Evolutionary Biology* **16**, 1038-1044.

Barribeau SM, Sok D, Gerardo NM (2010) Aphid reproductive investment in response to mortality risks. *Bmc Evolutionary Biology* **10**, 11.

Bensadia F, Boudreault S, Guay JF, Michaud D, Cloutier C (2006) Aphid clonal resistance to a parasitoid fails under heat stress. *Journal of Insect Physiology* **52**, 146-157.

Bilodeau E, Simon JC, Guay JF, Turgeon J, Cloutier C (2013) Does variation in host plant association and symbiont infection of pea aphid populations induce genetic and behaviour differentiation of its main parasitoid, *Aphidius ervi*? *Evolutionary Ecology* **27**, 165-184.

Boots M, Begon M (1993) Trade-offs with resistance to a granulosis virus in the Indian meal moth, examined by a laboratory evolution experiment. *Functional Ecology*, 528-534.

Boulinier T, Sorci G, Monnat JY, Danchin E (1997) Parent-offspring regression suggests heritable susceptibility to ectoparasites in a natural population of kittiwake *Rissa tridactyla*. *Journal of Evolutionary Biology* **10**, 77-85.

Brinkhof MW, Heeb P, Kölliker M, Richner H (1999) Immunocompetence of nestling great tits in relation to rearing environment and parentage. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 2315-2322.

Brisson JA, Nuzhdin SV, Stern DL (2009) Similar patterns of linkage disequilibrium and nucleotide diversity in native and introduced populations of the pea aphid, *Acyrthosiphon pisum*. *BMC Genetics* **10**.

Bryan-Walker K, Leung TL, Poulin R (2007) Local adaptation of immunity against a trematode parasite in marine amphipod populations. *Marine Biology* **152**, 687-695.

Caillaud M, Mondor-Genson G, Levine-Wilkinson S, *et al.* (2004) Microsatellite DNA markers for the pea aphid *Acyrthosiphon pisum*. *Molecular Ecology Notes* **4**, 446-448.

Caillaud MC, Via S (2000) Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. *American Naturalist* **156**, 606-621.

Carius HJ, Little TJ, Ebert D (2001) Genetic variation in a host-parasite association: potential for coevolution and frequency-dependent selection. *Evolution* **55**, 1136-1145.

Carver M, Sullivan DJ (1988) *Encapsulative defense reactions of aphids (Hemiptera: Aphididae) to insect parasitoids (Hymenoptera: Aphidiidae and Aphelinidae) (minireview).*

Cayetano L, Vorburger C (2013) Genotype-by-genotype specificity remains robust to average temperature variation in an aphid/endosymbiont/parasitoid system. *Journal of Evolutionary Biology* **26**, 1603-1610.

Chaboudez P, Burdon J (1995) Frequency-dependent selection in a wild plant-pathogen system. *Oecologia* **102**, 490-493.

Degnan PH, Moran NA (2008) Diverse Phage-Encoded Toxins in a Protective Insect Endosymbiont. *Applied and Environmental Microbiology* **74**, 6782-6791.

Desneux N, Barta RJ, Hoelmer KA, Hopper KR, Heimpel GE (2009) Multifaceted determinants of host specificity in an aphid parasitoid. *Oecologia* **160**, 387-398.

Dion E, Zele F, Simon JC, Outreman Y (2011) Rapid evolution of parasitoids when faced with the symbiont-mediated resistance of their hosts. *Journal of Evolutionary Biology* **24**, 741-750.

Eastop VF (1966) A Taxonomic Study of Australian Aphidoidea (Homoptera). *Australian Journal of Zoology* **14**, 399-592.

Ebert D, Zschokke-Rohringer CD, Carius HJ (1998) Within–and between–population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265**, 2127-2134.

Evison SEF, Fazio G, Chappell P, *et al.* (2013) Host-parasite genotypic interactions in the honey bee: the dynamics of diversity. *Ecology and Evolution* **3**, 2214-2222.

Fellowes MDE, Kraaijeveld AR, Godfray HCJ (1999) Association between Feeding Rate and Parasitoid Resistance in *Drosophila melanogaster*. *Evolution* **53**, 1302-1305.

Ferrari J, Darby AC, Daniell TJ, Godfray HCJ, Douglas AE (2004) Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology* **29**, 60-65.

Ferrari J, Godfray HCJ, Faulconbridge AS, Prior K, Via S (2006) Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. *Evolution* **60**, 1574-1584.

Ferrari J, Muller CB, Kraaijeveld AR, Godfray HCJ (2001) Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. *Evolution* **55**, 1805-1814.

Ferrari J, Scarborough CL, Godfray HCJ (2007) Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. *Oecologia* **153**, 323-329.

Ferrari J, Via S, Godfray HCJ (2008) Population differentiation and genetic variation in performance on eight hosts in the pea aphid complex. *Evolution* **62**, 2508-2524.

Ferrari J, West JA, Via S, Godfray HCJ (2012) Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. *Evolution* **66**, 375-390.

Foster SP, Tomiczek M, Thompson R, *et al.* (2007) Behavioural side-effects of insecticide resistance in aphids increase their vulnerability to parasitoid attack. *Animal Behaviour* **74**, 621-632.

Frantz A, Plantegenest M, Mieuzet L, Simon JC (2006) Ecological specialization correlates with genotypic differentiation in sympatric host-populations of the pea aphid. *Journal of Evolutionary Biology* **19**, 392-401.

Gerardo NM, Altincicek B, Anselme C, *et al.* (2010) Immunity and other defenses in pea aphids, Acyrthosiphon pisum. *Genome Biology* **11**.

Godfray HCJ (1994) *Parasitoids: behavioral and evolutionary ecology* Princeton University Press.

Grosholz ED (1994) The effects of host genotype and spatial distribution on trematode parasitism in a bivalve population. *Evolution*, 1514-1524.

Guay JF, Boudreault S, Michaud D, Cloutier C (2009) Impact of environmental stress on aphid clonal resistance to parasitoids: Role of *Hamiltonella defensa* bacterial symbiosis in association with a new facultative symbiont of the pea aphid. *Journal of Insect Physiology* **55**, 919-926.

Gwynn DM, Callaghan A, Gorham J, Walters KFA, Fellowes MDE (2005) Resistance is costly: trade-offs between immunity, fecundity and survival in the pea aphid. *Proceedings of the Royal Society B-Biological Sciences* **272**, 1803-1808.

Henter HJ (1995) The potential for coevolution in a host-parasitoid system. II. Genetic variation within a population of wasps in the ability to parasitize an aphid host. *Evolution*, 439-445.

Henter HJ, Via S (1995) The potential for coevolution in a host-parasitoid system 1. Genetic variation within a an aphid population in susceptibility to a parasitic wasp. *Evolution* **49**, 427-438.

Hufbauer RA, Via S (1999) Evolution of an aphid-parasitoid interaction: Variation in resistance to parasitism among aphid populations specialized on different plants. *Evolution* **53**, 1435-1445.

Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ (2010) Adaptation via Symbiosis: Recent Spread of a *Drosophila* Defensive Symbiont. *Science* **329**, 212-215.

Koskella B, Lively CM (2009) Evidence for negative frequency-dependent selection during experimental coevolution of a freshwater snail and sterilizing trematode. *Evolution* **63**, 2213-2221.

Kraaijeveld A, Godfray H (1997) Trade-off between parasitoid resistance and larval competitive ability in Drosophila melanogaster. *Nature* **389**, 278-280.

Kraaijeveld AR, Godfray HCJ (2009) Evolution of Host Resistance and Parasitoid Counter-Resistance. In: *Advances in Parasitology, Vol 70: Parasitoids of Drosophila* (ed. Prevost G), pp. 257-280.

Kurokawa T, Yao I, Akimoto Si, Hasegawa E (2004) Isolation of six microsatellite markers from the pea aphid, *Acyrthosiphon pisum* (Homoptera, Aphididae). *Molecular Ecology Notes* **4**, 523-524.

Lamb R, Pointing P (1972) Sexual morph determination in the aphid, *Acyrthosiphon pisum*. *Journal of insect physiology* **18**, 2029-2042.

Laughton AM, Garcia JR, Altincicek B, Strand MR, Gerardo NM (2011) Characterisation of immune responses in the pea aphid, Acyrthosiphon pisum. *Journal of insect physiology* **57**, 830-839.

Le Ralec A, Anselme C, Outreman Y, *et al.* (2010) Evolutionary ecology of the interactions between aphids and their parasitoids. *Comptes Rendus Biologies* **333**, 554-565.

Lukasik P, van Asch M, Guo HF, Ferrari J, Godfray HCJ (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters* **16**, 214-218.

Luong L, Polak M (2007) Environment-dependent trade-offs between ectoparasite resistance and larval competitive ability in the *Drosophila–Macrocheles* system. *Heredity* **99**, 632-640.

Martinez AJ, Ritter SG, Doremus MR, Russell JA, Oliver KM (2014) Data from: Aphid-encoded variability in susceptibility to a parasitoid. *Dryad Data Repository*.

Martinez AJ, Weldon SR, Oliver KM (2013) Effects of parasitism on aphid nutritional and protective symbioses. *Molecular Ecology*, n/a-n/a.

Moller AP (1990) Effects of a haematophagous mite on the barn swallow (*Hirundo rustica*): a test of the Hamilton and Zuk hypothesis. *Evolution*, 771-784.

Moran NA (1992) The evolution of aphid life-cycles. *Annual Review of Entomology* **37**, 321-348.

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16919-16926.

Nyabuga FN, Outreman Y, Simon JC, Heckel DG, Weisser WW (2010) Effects of pea aphid secondary endosymbionts on aphid resistance and development of the aphid parasitoid Aphidius ervi: a correlative study. *Entomologia experimentalis et applicata* **136**, 243-253.

Oliver KM, Campos J, Moran NA, Hunter MS (2008) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B-Biological Sciences* **275**, 293-299.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325**, 992-994.

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences* **102**, 12795-12800.

Oliver KM, Moran NA, Hunter MS (2006) Costs and benefits of a superinfection of facultative symbionts in aphids. *Proceedings of the Royal Society B-Biological Sciences* **273**, 1273-1280.

Oliver KM, Noge K, Huang EM, *et al.* (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC Biology* **10**, 10.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1803-1807.

Oliver KM, Smith AH, Russell JA (2013) Defensive symbiosis in the real world – advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Functional Ecology*.

Parker BJ, Barribeau SM, Laughton AM, de Roode JC, Gerardo NM (2011) Non-immunological defense in an evolutionary framework. *Trends in Ecology & Evolution* **26**, 242-248.

Parker BJ, Garcia JR, Gerardo NM (2014) Genetic variation in resistance and fecundity tolerance in a natural host–pathogen interaction. *Evolution*.

Parker BJ, Spragg CJ, Altincicek B, Gerardo NM (2013) Symbiont-Mediated Protection against Fungal Pathogens in Pea Aphids: a Role for Pathogen Specificity? *Applied and Environmental Microbiology* **79**, 2455-2458.

Peccoud J, Simon JC, McLaughlin HJ, Moran NA (2009) Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 16315-16320.

Richards S, Gibbs RA, Gerardo NM, *et al.* (2010) Genome Sequence of the Pea Aphid Acyrthosiphon pisum. *Plos Biology* **8**, 24.

Rolff J, Siva-Jothy MT (2003) Invertebrate ecological immunology. *Science* **301**, 472-475.

Rouchet R, Vorburger C (2012) Strong specificity in the interaction between parasitoids and symbiont-protected hosts. *Journal of Evolutionary Biology* **25**, 2369-2375.

Russell JA, Weldon S, Smith AH, *et al.* (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. *Molecular Ecology*.

Sadd BM, Barribeau SM (2013) Heterogeneity in infection outcome: lessons from a bumblebee-trypanosome system. *Parasite Immunology* **35**, 339-349.

Sadd BM, Schmid-Hempel P (2009) Principles of ecological immunology. *Evolutionary Applications* **2**, 113-121.

Sanders AE, Scarborough C, Layen SJ, Kraaijeveld AR, Godfray HCJ (2005) Evolutionary change in parasitoid resistance under crowded conditions in *Drosophila melanogaster*. *Evolution* **59**, 1292-1299.

Sandrock C, Gouskov A, Vorburger C (2010) Ample genetic variation but no evidence for genotype specificity in an all-parthenogenetic host-parasitoid interaction. *Journal of Evolutionary Biology* **23**, 578-585.

Sandstrom JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. *Molecular Ecology* **10**, 217-228.

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. *Science* **310**, 1781-1781.

Schmid-Hempel P (2005) Evolutionary ecology of insect immune defenses. *Annual Review of Entomology* **50**, 529-551.

Schmid M, Sieber R, Zimmermann YS, Vorburger C (2012) Development, specificity and sublethal effects of symbiont-conferred resistance to parasitoids in aphids. *Functional Ecology* **26**, 207-215.

Smith JA, Wilson K, Pilkington JG, Pemberton JM (1999) Heritable variation in resistance to gastro-intestinal nematodes in an unmanaged mammal population. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 1283-1290.

Spitze K (1992) Predator-mediated plasticity of prey life history and morphology: *Chaoborus americanus* predation on *Daphnia pulex*. *American Naturalist*, 229-247.

Stacey DA, Fellowes MDE (2002) Influence of temperature on pea aphid *Acyrthosiphon pisum* (Hemiptera : Aphididae) resistance to natural enemy attack. *Bulletin of Entomological Research* **92**, 351-357.

Strand MR, Pech LL (1995) IMMUNOLOGICAL BASIS FOR COMPATIBILITY IN PARASITOID HOST RELATIONSHIPS. *Annual Review of Entomology* **40**, 31-56.

Sutter GR, Rothenbuhler WC, Raun ES (1968) Resistance to American foulbrood in honey bees: VII. Growth of resistant and susceptible larvae. *Journal of Invertebrate Pathology* **12**, 25-28.

Triggs A, Knell RJ (2012) Interactions between environmental variables determine immunity in the Indian meal moth *Plodia interpunctella*. *Journal of Animal Ecology* **81**, 386-394.

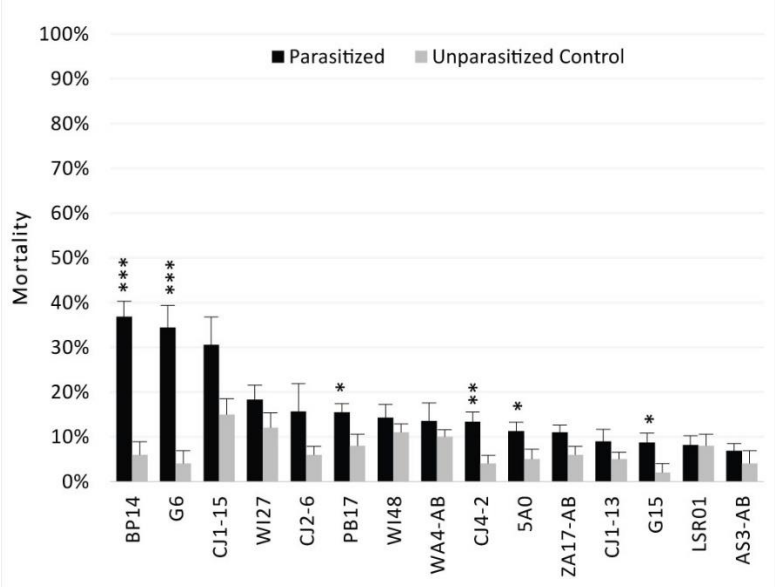
Via S, Bouck AC, Skillman S (2000) Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. *Evolution* **54**, 1626-1637.

von Burg S, Ferrari J, Müller CB, Vorburger C (2008) Genetic variation and covariation of susceptibility to parasitoids in the aphid *Myzus persicae*: no evidence for trade-offs. *Proceedings of the Royal Society B: Biological Sciences* **275**, 1089-1094.

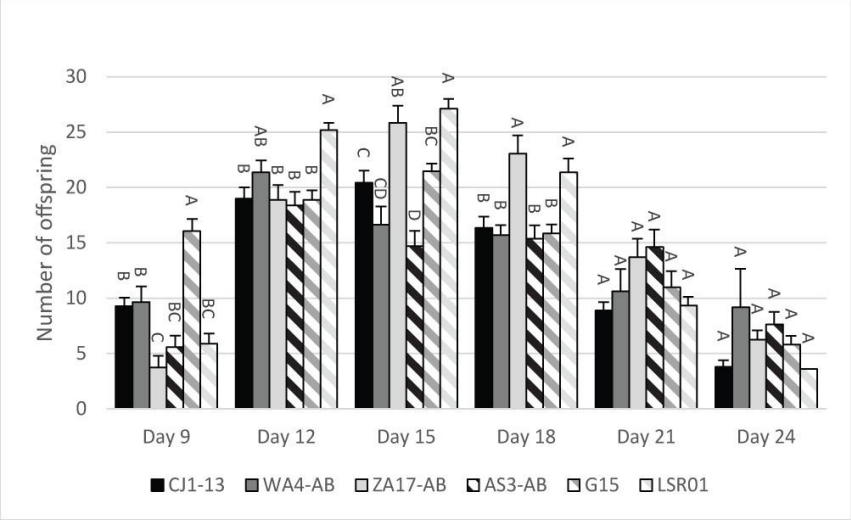
Vorburger C, Gehrer L, Rodriguez P (2010) A strain of the bacterial symbiont Regiella insecticola protects aphids against parasitoids. *Biology Letters* **6**, 109-111.

Webster J, Woolhouse M (1999) Cost of resistance: relationship between reduced fertility and increased resistance in a snail—schistosome host—parasite system. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 391-396.

Yan G, Severson DW, Christensen BM (1997) Costs and benefits of mosquito refractoriness to malaria parasites: implications for genetic variability of mosquitoes and genetic control of malaria. *Evolution*, 441-450.



Supplemental Figure 2.1 Mortality rates (excluding mummification) between parasitized and control (unparasitized) aphid lines, nine days after parasitism. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$



Supplemental Figure 2.2 Average fecundity per aphid per day, among lines showing high (solid bars) and low (striped bars) resistance to parasitism. Letters indicate significant differences within a single day (Tukey's HSD $\alpha = 0.05$). Linear mixed model, $F_{5,237}$, $p < 0.01$ for each day.

Supplemental Table 2.1 Allele sizes for four aphid microsatellite loci.

	Ap02		Ap03		Ap05		Aph10M	
	allele 1	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**
5A0	228	232	242		265		198	202
AS3-AB	228		242		265	271	198	202
BP14	228		240	242	265	267	194	
CJ1-13	228	232	240	242	267		196	198
CJ1-15	232		240	242	253	265	194	
CJ2-6	228	232	240	242	267		194	
CJ4-2	228	232	242		267		194	198
G6	228	232	242		265	267	194	
G15	228		236	240	267	271	194	202
LSR01	222	228	242	256	265		194	
PB17	232		242				194	198
WA4-AB	228	232	242	256	265	267	194	
WI27	232		240	242	265	267	194	198
WI48	232		242		265		194	198
ZA17-AB	228		240	256	253	257	194	

Blanks in the second column for each locus indicate homozygosity. Line PB17 did not amplify at the AP05 locus.

CHAPTER 3

CONDITIONAL BENEFITS OF INFECTION WITH A PROTECTIVE BACTERIAL SYMBIONT[2]

[2] Martinez AJ, Kraft L, Higashi C, and Oliver KM. To be submitted to *Proceedings of the Royal Society B.*

Abstract

Pea aphids, *Acyrthosiphon pisum,* are protected from parasitism by their most common parasitoid wasp, *Aphidius ervi*, through infection with a protective bacterial symbiont called *Hamiltonella defensa* or through endogenously encoded means and some individual aphids will occasionally maintain both types of defense. Here we sought to compare the strength and potential costs of both types of defenses, together and individually, to understand the selective pressures that maintain both mechanisms in pea aphid populations. We first performed parasitism assays on two susceptible and three resistant aphid genotypes that were experimentally infected with two symbiont strains, APSE2-*H. defensa* and APSE3-*H. defensa*, which confer moderate to high levels of protection against parasitism, respectively. We found that, even in resistant genotypes, infection typically resulted in increased aphid survival and/or reduced successful wasp development after parasitism. We then performed fecundity and longevity analyses on a subset of these aphids and found that, in the absence of parasitism, *H. defensa*-infected aphids produced significantly less offspring than control uninfected aphids, regardless of aphid genotype, and that APSE3 reduced aphid longevity. In the presence of parasitism, however, one resistant genotype (CJ113) did not suffer significantly reduced fecundity or longevity and therefore did not benefit from infection with either *H. defensa* strain, another resistant genotype (WI27) did suffer some fitness costs and benefitted from infection with APSE2 but not APSE3, and the susceptible genotype (AS3) suffered significant fecundity costs that were partially rescued by infection with APSE3 but not APSE2. Overall, it appears that benefits of infection with *H. defensa* are conditional on both parasitism pressure and host genotype. These results will help to describe infection

rates of defensive symbionts, which are only found at intermediate frequencies in insect populations.

3.1 Introduction

Virtually all multicellular eukaryotic organisms are attacked by natural enemies, including pathogens, parasites, and predators. Individuals may employ multiple modes of defense against one or more specific enemies, which may function independently or in concert to thwart attackers (Boman & Hultmark 1987; Lavine & Strand 2002; Lemaitre & Hoffmann 2007). However, depending on factors such as enemy abundance, resource allocation, and costs associated with defense, selective pressures may favor the maintenance of specific defenses over others. For example, selection on costly defense traits, like secondary chemical metabolites or immune factors (Ardia *et al.* 2012), may vary based on the potential for encountering natural enemies (Kraaijeveld & Godfray 1997; Pearson 1985; Poitrineau *et al.* 2003; Rantala *et al.* 2011; Smilanich *et al.* 2009) or forego resistance in favor of other traits such as nutrition and fecundity (Gerardo *et al.* 2010; Gwynn *et al.* 2005). Studying the potential benefits and trade-offs of different types of resistance is important understanding the selective pressures that insects and their natural enemies face.

Parasitoid wasps, which require a host for larval growth and development, are ubiquitous natural enemies of insects (Eggleton & Belshaw 1992; Pennacchio & Strand 2006). Given that the outcome of host-parasitoid interactions is typically death for either host or parasitoid, there is strong selection for resistance and counter-resistance. Interactions between pea aphids, *Acyrthosiphon pisum,* and their parasitoids, especially *Aphidius ervi*, are relatively well-studied (Cardinale *et al.* 2003; Chau & Mackauer 1997;

Hufbauer 2001; Hufbauer & Via 1999; Snyder & Ives 2003). Interestingly, (Henter & Via 1995) found that pea aphids maintained significant variability in their resistance to *A. ervi* that ranged from nearly 0 – 100% resistance, which, at the time, was assumed to be due to genotypically-encoded factors. However, pea aphids were later found to commonly carry a facultative symbiont, *Hamiltonella defensa,* which confers varying levels of protection to pea aphids against parasitism by *A. ervi,* depending on strain (Oliver *et al.* 2005; Oliver *et al.* 2003). Furthermore, *H. defensa* itself carries a bacteriophage called APSE, which encodes putative toxins hypothesized to function in defense against parasitism, and aphids carrying *H. defensa* that has lost APSE infection also lose their resistance against parasitism (Moran *et al.* 2005; Oliver *et al.* 2009). There are two APSE variants found commonly in *H. defensa*-infected pea aphids in North America, APSE2 and APSE3, which, respectively, contain *cdtB* and *YDp* putative toxin alleles in the virulence cassette region (Moran *et al.* 2005), cause late vs. early mortality to developing wasps (Martinez *et al.* 2014b), and confer moderate to high levels of protection against parasitism by *A. ervi* (Martinez *et al.* 2014b; Oliver *et al.* 2009; Oliver *et al.* 2012).

Because pea aphids appear to lack a strong encapsulation response (Laughton *et al.* 2011), the typical insect immune defense against parasitoids (Strand 2008), this new information led to the assumption that majority of variation in pea aphid resistance against parasitism was likely due to infection with protective bacterial symbionts instead of host genotype (Oliver *et al.* 2005). More recently, however, pea aphids *are* found to maintain significant variation in endogenous resistance without the aid of symbionts (mechanism currently unknown) against parasitism by *A. ervi* that is comparable to

variation conferred by *H. defensa* (Martinez *et al.* 2014a), and field-collected pea aphids with resistant genotypes sometimes also carry *H. defensa* infections (Martinez *et al.* 2014b). This has led to questions of whether maintaining multiple types of resistance, in this case symbiotic and genotypic, against a single natural enemy would be additive or redundant with respect to the protective phenotype, and whether maintaining multiple types of resistance is more costly than maintaining single sources of resistance. In aphids, fitness costs associated with infection are often manifested as reduced fecundity or longevity (Oliver *et al.* 2008b; Oliver *et al.* 2006; Russell & Moran 2006; Vorburger & Gouskov 2011; Weldon *et al.* 2013; White *et al.* 2011). Of course, costs associated with single or multiple sources of resistance may be offset by benefits accrued above a threshold of parasitism pressure.

Internally-developing parasitoids utilize several strategies to overcome their hosts, these often include host selection, suppression or evasion of immunity, degeneration of specific tissues, and manipulation of the host's metabolism (Beckage & Gelman 2004; Godfray 1994; Pennacchio & Strand 2006; Vinson & Iwantsch 1980). Aphidiine braconid parasitoids, like *A. ervi*, typically induce a slew of changes inside their host aphid to make the host environment more suitable for their own development, reviewed in (Pennacchio & Mancini 2012). In aphids that maintain resistance to *A. ervi*, however, developing wasps die prematurely depending on the type of resistance (genotypic or symbiont-conferred) as well as APSE-*H. defensa* strain (APSE2 or APSE3). For example, in aphids infected with APSE3-*H. defensa*, wasps rarely develop beyond the embryonic stage, whereas those infected with APSE2-*H. defensa* or with genotypic resistance are somewhat more variable, but wasps often die in the larval stage after 72

hours (Martinez *et al.* 2014b). Earlier wasp mortality may be more beneficial to aphids as the wasp does not consume as many resources, however, wasp maternal factors such as venom are still likely to be costly to aphid fecundity (Digilio *et al.* 2000; Falabella *et al.* 2007). Aphid genotypes that are resistant to *A. ervi* are also sometimes found to be naturally infected with *H. defensa* (so far only APSE2-*H. defensa*) (Martinez *et al.* 2014b), so it is possible that there are added selective benefits of maintaining both types of resistance. Perhaps each is more effective under different conditions or that the mechanisms of resistance do not overlap, so as to more completely eliminate deleterious costs of parasitism.

To determine the costs or benefits of individual aphids harboring both types of resistance against parasitism by *A. ervi*, we will first evaluate the resistance phenotype of fifteen experimental lines that vary in genotypic resistance and presence of the defensive symbiont, *H. defensa*, to determine if protective effects are additive or redundant. For a subset of these lines we will measure fecundity and longevity in the presence/absence of parasitism to assess costs and benefits of varying defense types (endogenous-only, symbiont-only, both). Our findings will inform on the selective pressures contributing to the maintenance of endogenous and symbiont-mediated resistance which both only occur at intermediate levels in natural populations.

3.2 Methods

Study organisms

The pea aphid, *Acyrthosiphon pisum*, is a cosmopolitan herbivorous pest of herbaceous legumes such as alfalfa and clover and was introduced from Eurasia in the late 1800s (Davis 1913, 1915; Thomas 1879). This aphid exhibits a cyclically

parthenogenetic life-cycle with females reproducing asexually in response to long-day lengths, until shorter days result in the production of sexual morphs which mate and the resulting eggs overwinter (Lamb & Pointing 1972). Clonally reproducing aphid lines can be maintained indefinitely in the lab by growing them in environmental incubators that mimic long-day lengths. We maintain our pea aphids on pre-flowering fava bean, *Vicia faba*, on a 16 hours light, 8 hours dark (16L: 8D) schedule at a constant 20±1˚C.

Several aphidiine braconid parasitoid species are known to attack the pea aphid, but *Aphidius ervi* (Hymenoptera: Braconidae) is found to be the dominant parasitic wasp of this aphid in North America (Angalet & Fuester 1977; Halfhill *et al.* 1972; Mackauer & Finlayso 1967; Start 1970). Female *A. ervi* wasps parasitize aphids, injecting a single egg and venom into the host aphid, where their larvae hatch and develop in the still-living aphid over an 8-9 day period before pupating, killing and 'mummifying' the host aphid; adult wasps emerge from aphid 'mummies' about 15 days after parasitism (He 2008). Our colony of *A. ervi* was obtained via field collected aphids from Wisconsin and North Dakota, as well as from a commercial source (Arbico Organics). All wasps are maintained in a single, large interbreeding colony, on a mixture of susceptible pea aphid lines that are uninfected with facultative symbionts. Adult wasps are kept at 20˚C in our environmental incubators and fed a diet of honey and water.

Creation of experimental aphid lines

Included in this study are pea aphids of five different genotypes (see Table 3.1) which are categorized as 'susceptible' or 'resistant' to parasitism by *A. ervi.* From each genotype we have created three sublines, one uninfected with facultative symbionts, one infected with APSE2-*H. defensa*, and one with APSE3-*H. defensa*, (Table 3.1). The

Table 3.1. Experimental aphid lines used in this study.

	Aphid genotype	Aphid Subline	Symbiont Infection	Symbiont-conferred resistance
Susceptible aphid genotypes	AS3	AS3ø	uninfected	-
		AS3+APSE2	*H. defensa*-APSE2	moderate
		AS3+APSE3	*H. defensa*-APSE3	high
	G15	G15ø	uninfected	-
		G15+APSE2	*H. defensa*-APSE2	moderate
		G15+APSE3	*H. defensa*-APSE3	high
Resistant aphid genotypes	CJ113	CJ113ø	uninfected	-
		CJ113+APSE2	*H. defensa*-APSE2	moderate
		CJ113+APSE3	*H. defensa*-APSE3	high
	ZA17	ZA17ø	uninfected	-
		ZA17+APSE2	*H. defensa*-APSE2	moderate
		ZA17+APSE3	*H. defensa*-APSE3	high
	WI27	WI27ø	uninfected	-
		WI27+APSE2	*H. defensa*-APSE2	moderate
		WI27+APSE3	*H. defensa*-APSE3	high

APSE2 and APSE3 bacterial strains were originally associated with the ZA17 and AS3 genotypes, respectively, thus the uninfected versions of these two genotypes (ZA17ø and AS3ø) were obtained through an antibiotic curing protocol (Martinez *et al.* 2014b), modified from (Douglas *et al.* 2006). Genotypes G15, CJ113, and WI27 were obtained uninfected from field collections; they were experimentally infected with both *H. defensa* strains via microinjections from infected aphids. Briefly, three to four *H. defensa*-infected aphid nymphs were squished into 40uL of 1X PBS buffer. Solid aphid exoskeletons were removed from the squish solution before centrifuging at 7000xg for fifteen seconds. Glass micropipettes were pulled from microcapillary tubes, using a needle puller and resulting micropipette needles were used to pipette a small amount of the squish solution supernatant and inject it into the body cavity of recipient 2-3rd instar aphids, which were then placed on fresh fava bean plants for 9-10 days. After 9-10 days, surviving adult aphids were placed, individually, in petri dishes with a fresh leaf and allowed to reproduce overnight. Offspring from each petri dish were screened for *H. defensa* using diagnostic quantitative PCR (qPCR); see (Martinez *et al.* 2014b) for primer sequences

and reaction conditions. Remaining offspring from petri dishes showing a positive qPCR result for *H. defensa* infection were placed individually on fresh fava bean plants and the resulting infected aphid sublines were screened for multiple generations to confirm stability of the infection. Experimentally created aphid sublines used in this study were produced at least six months prior to any experimentation.

Aphid parasitism resistance assays

160 aphids from each of the fifteen aphid sublines were individually parasitized by *A. ervi* and pooled before being split into eight replicates of twenty aphids. Briefly, aphids were placed in a petri dish with a single wasp and removed as they were parasitized; several wasps were selected haphazardly from our large wasp colony and were used to complete the parasitism assay. Each replicate was placed on a fresh, pre-flowering, broad bean plant for ten days before counting their total survival/20, dual-mortality/20 (both aphid and developing wasp die), and 'mummification'/20 (dried aphid containing wasp pupa). All surviving aphids were monitored for an additional five days to preclude the possibility that wasps develop more slowly in certain aphid lines.

Parasitized/Unparasitized aphid fitness assays

Three aphid genotypes were included in the aphid fitness analysis: AS3 (susceptible), CJ113 (resistant), and WI27 (resistant) and each of their three (uninfected, APSE2-*H. defensa*, APSE3-*H. defensa*) created sublines (Table 3.1) was used (total 9 aphid lines). For each aphid line, adults were removed and placed on fresh plants overnight (14 hours) and allowed to reproduce, before removing them the next day. The resulting offspring were grown for an additional three days and these 79±7 hour/old aphids (2^{nd} – 3^{rd} instars) were used in the experiment. For each aphid line, thirty aphids

were parasitized in a Petri dish and another thirty remained unparasitized as a control, however, both groups remained off plants for the same period of time (about 15 minutes) to standardize any potential stress from being off their food source. Afterwards, both groups of parasitized and unparasitized aphids were placed on fresh plants in five replicates of six aphids. Their survival, mortality, and total offspring were recorded every three days for the duration of the experiment. Any aphid 'mummies' containing wasp pupae were recorded and removed from the parasitized group to prevent their emergence and subsequent parasitization of remaining aphids

Statistical analyses

A generalized linear model (GzLM) with binomial distribution and logit link function was used to compare survival, mummification, and dual-mortality after parasitism. All data were mildly overdispersed and are reported with a quasibinomial adjustment. Pairwise comparisons were performed using a post-hoc Tukey's honestly significant difference (HSD) test, using an ANOVA of arcsine transformed proportional data. We used general linear models (GLM) analyses to compare effects of parasitism, *H. defensa* infection, and aphid genotype on aphid survival after 18 days and total aphid fecundity after 24 days. ANOVAs and post-hoc Tukey's HSD were performed to compare fitness within and among groups.

3.3 Results

Effects of symbiont strain and aphid genotype on resistance against parasitism

Table 3.2. GzLM showing effects of aphid genotype and *H. defensa* infection on aphid survival, mummification, and mortality after parasitism.

Variable	df	Survival	Mummification	Mortality
Aphid Genotype	4	$\chi2 = 236.35$ **p < .0001**	$\chi2 = 66.87$ **p < .0001**	$\chi2 = 10.09$ **p = .0390**
H. defensa infection	2	$\chi2 = 215.35$ **p < .0001**	$\chi2 = 251.72$ **p < .0001**	$\chi2 = 21.75$ **p < .0001**
Genotype X Infection	8	$\chi2 = 119.84$ **p < .0001**	$\chi2 = 82.28$ **p < .0001**	$\chi2 = 7.68$ p = .4651
Model	14	$\chi2 = 991.86$ **p < .0001**	$\chi2 = 1356.53$ **p < .0001**	$\chi2 = 44.80$ **p < .0001**

There are three potential outcomes for aphids after they have been parasitized by *A. ervi:* resistant aphids survive parasitism; susceptible aphids die and their tissues are consumed and converted into a wasp 'mummy'; or both aphid and developing wasp die resulting in dual-mortality. Using a GzLM we found significant effects of aphid genotype (AS3, G15, ZA17, CJ113, WI27), *H. defensa* infection (uninfected, APSE2-*H. defensa*, APSE3), and their interaction on all three potential outcomes except for mortality resulting from the interaction of genotype and infection (Table 3.2).

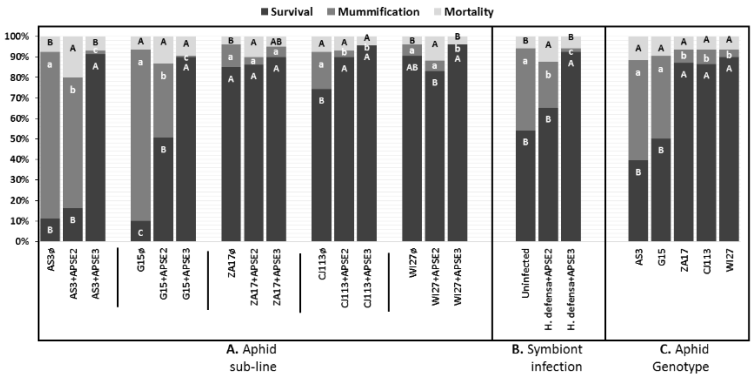


Figure 3.1. Survival, mummification, and mortality of pea aphids counted ten days after parasitism by *A. ervi*. A) Results arranged by experimental aphid sub-lines (GzLM, df = 14, $p < 0.0001$ for all comparisons), B) all sub-lines, averaged by infection status (GzLM, df = 2, $p < 0.0001$ for all comparisons), and C) averaged by aphid genotype (GzLM, df = 4, $p < 0.0001$ survival and mummification, $p = 0.0315$ mortality). Letters in 'A' indicate pairwise significant differences within each group of sub-lines (Arcsine transformed Tukey's HSD $\alpha = 0.05$).

Overall, there are general patterns of aphid susceptibility to parasitism that occur across all aphid sub-lines. Consistent with previous studies, aphids infected with *H. defensa* are less susceptible to parasitism than uninfected aphids (Fig. 3.1 A and B) and genotypes AS3 and G15 are more susceptible than ZA17, CJ113, and WI27 (Fig. 3.1 A and C). While past studies show that aphids infected with APSE2-*H. defensa* generally see moderate increases in aphid survival and decreases in mummification compared to

uninfected, susceptible aphid genotypes, we found that this was the case in one susceptible aphid genotype (G15), but not the other (AS3) (Fig. 3.1A). Across all aphid lines (susceptible and resistant) infection with APSE2-*H. defensa* was associated with fewer mummies, but not increased aphid survival (Fig. 3.1B). Significant increases in dual-mortality were most often associated with APSE2-*H. defensa* and this is consistent with wasp mortality occurring later in development (Fig. 3.1 A and B). In both susceptible aphid genotypes (AS3 and G15), APSE3-*H. defensa* was associated with substantial increases in survival and decreases in mummification relative to uninfected controls of same genotype (Fig. 3.1A) and overall APSE3-*H. defensa* increased survival and reduced mummification relative to uninfected genotypes and those with APSE2-*H. defensa* (Fig. 3.1B). Effects were more variable in resistant aphid genotypes. For example, infection with APSE2-*H. defensa* or APSE3 had no impact on the aphid survival or mummification of genotype ZA17 (Fig. 3.1A) although APSE2 did result in significantly more dual mortality. In contrast, infection with both APSE2-*H. defensa* and APSE3 increased aphid survival and reduced mummification in resistant genotype CJ113. See figure legend (Fig. 3.1) for statistical analyses.

Aphid fitness: Fecundity

Table 3.3. Factors influencing aphid fecundity (GLM).

A.	Effect	df	f-value	p-value
Unparasitized	Aphid genotype	2	0.11	0.8984
	H. defensa infection	2	78.18	**<0.0001**
	Genotype*infection	4	3.94	**0.0094**
	Whole model	8	21.54	**<0.0001**
Parasitized	Aphid genotype	2	79.39	**<0.0001**
	H. defensa infection	2	4.98	**0.0127**
	Genotype*infection	4	29.12	**<0.0001**
	Whole model	8	35.70	**<0.0001**

B.	Variable	df	f-value	p-value
uninfected	Parasitism	1	106.1913	**<0.0001**
	Aphid genotype	2	43.9567	**<0.0001**
	Parasitism X genotype	2	23.8444	**<0.0001**
	Whole model	5	48.36	**<0.0001**
H. defensa-APSE2	Parasitism	1	99.34	**<0.0001**
	Aphid genotype	2	33.18	**<0.0001**
	Parasitism X genotype	2	14.62	**<0.0001**
	Whole model	5	38.99	**<0.0001**
H. defensa-APSE3	Parasitism	1	11.15	**0.0030**
	Aphid genotype	2	8.31	**0.0021**
	Parasitism X genotype	2	2.26	0.1278
	Whole model	5	6.18	**0.0010**

C.	Variable	df	f-value	p-value
AS3	Parasitism	1	208.67	**<0.0001**
	H. defensa infection	2	15.29	**<0.0001**
	Parasitism X infection	2	34.33	**<0.0001**
	Whole model	5	61.58	**<0.0001**
CJ113	Parasitism	1	6.94	**0.0145**
	H. defensa infection	2	53.12	**<0.0001**
	Parasitism X infection	2	4.5	**0.0220**
	Whole model	5	24.43	**<0.0001**
WI27	Parasitism	1	58.26	**<0.0001**
	H. defensa infection	2	29.16	**<0.0001**
	Parasitism X infection	2	8.63	**0.0017**
	Whole model	5	27.12	**<0.0001**

See figure 3.2 for visualization of effects. A) effects of aphid genotype and *H. defensa* infection on fecundity of unparasitized and parasitized aphids, B) effects of parasitism and aphid genotype on fecundity of *H. defensa* infected and uninfected aphids, and C) effects of parasitism and *H. defensa* infection on fecundity of three aphid genotypes.

To compare potential effects on aphid fecundity we used a GLM to examine the influences of parasitism, *H. defensa* infection, aphid genotype, and their interactions on aphid fecundity (Table 3.3), we find significant interactions between all variables except genotype in unparasitized aphids (Table 3.3A) and parasitism by genotype in aphids infected with APSE3-*H. defensa* (Table 3.3B). Not surprisingly, parasitism by *A. ervi* generally reduced aphid fecundity (Fig. 3.2). In most cases, both APSE2 and APSE3 *H. defensa* strains resulted in clear infection costs in unparasitized aphids relative to uninfected, unparasitized controls, although APSE3 –the stronger protector – is more costly than APSE2 (Fig. 3.2A and B); fecundity of different aphid genotypes with the same infection status are were also mostly similar. Interestingly, in *parasitized* aphids, *H. defensa* did increase fecundity relative to parasitized, uninfected controls, but the effect was surprisingly variable. For example, despite the strong levels of protection conferred to genotype AS3 by APSE3-*H. defensa* (Fig. 3.1a), this only translated to modest (non-significant) increases in its fecundity relative to parasitized, uninfected, controls (Fig. 3.2a); the resistant genotype WI27 gained no additional protection from APSE2-*H. defensa* (Fig. 3.1a), but was able to reproduce significantly more after parasitism when carrying this strain than control, uninfected aphids (Fig. 3.2a). The fecundity of parasitized genotypes AS3 and WI27, however, did not benefit from infection with APSE2-*H. defensa* and APSE3, respectively, and genotype CJ113 suffered no significant reduction in fecundity when uninfected and did not benefit from infection with either strain. Finally, we did not find significant differences in fecundity among aphid genotypes, except when parasitized (Fig. 3.1a and c).

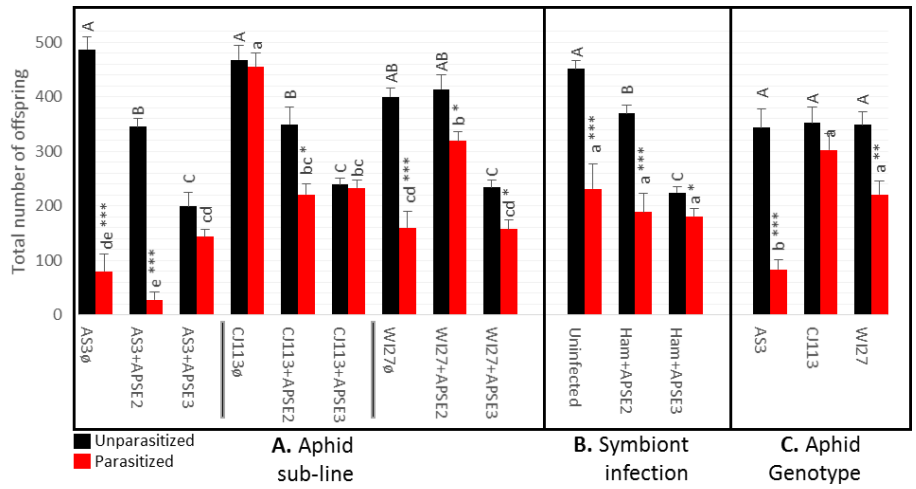


Figure 3.2. Average <u>total fecundity</u> of unparasitized and parasitized pea aphids after 24 days. A) Results arranged by experimental aphid sub-lines (ANOVA, df = 8, $p < 0.0001$ parasitized and unparasitized), B) all sub-lines, averaged by infection status (ANOVA, df = 2, $p < 0.0001$ unparasitized, $p = 0.5523$ parasitized), and C) averaged by aphid genotype (ANOVA, df = 2, $p = 0.9786$ unparasitized, $p < 0.0001$ parasitized). Capital and lowercase letters indicate pairwise significant differences within unparasitized and parasitized groups, respectively (Tukey's HSD $\alpha = 0.05$). Asterisks indicate significant differences between each pair of unparasitized and parasitized treatments (* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001). See Table 3.3 for all analyses.

Aphid fitness: Longevity

Table 3.4. Factors influencing aphid <u>longevity</u> (GzLM).

A.	Variable	Df	χ2	P-val
Unparasitized	Aphid genotype	2	15.49	**0.0004**
	H. defensa infection	2	35.62	**<0.0001**
	Genotype*infection	4	22.63	**0.0002**
	Whole model	8	91.94	**<0.0001**
Parasitized	Aphid genotype	2	51.83	**<0.0001**
	H. defensa infection	2	1.54	0.4638
	Genotype*infection	4	51.93	**<0.0001**
	Whole model	8	100.68	**<0.0001**

B.	Variable	Df	χ2	P-val
uninfected	Parasitism	1	15.54	**<0.0001**
	Aphid genotype	2	11.03	**0.0040**
	Parasitism*genotype	2	20.05	**<0.0001**
	Whole model	5	82.46	**<0.0001**
H. defensa-APSE2	Parasitism	1	25.72	**<0.0001**
	Aphid genotype	2	5.87	0.0532
	Parasitism*genotype	2	23.59	**<0.0001**
	Whole model	5	66.91	**<0.0001**
H. defensa-APSE3	Parasitism	1	0.95	0.3304
	Aphid genotype	2	61.05	**<0.0001**
	Parasitism*genotype	2	4.49	0.1061
	Whole model	5	70.24	**<0.0001**

C.	Variable	Df	χ2	P-val
AS3	Parasitism	1	71.12	**<0.0001**
	H. defensa infection	2	3.62	0.1638
	Parasitism*infection	2	15.02	**0.0005**
	Whole model	5	80.29	**<0.0001**
CJ113	Parasitism	1	0.23	0.6310
	H. defensa infection	2	106.53	**<0.0001**
	Parasitism*infection	2	5.23	0.0731
	Whole model	5	113.00	**<0.0001**
WI27	Parasitism	1	2.51	0.1129
	H. defensa infection	2	5.68	0.0583
	Parasitism*infection	2	6.15	**0.0462**
	Whole model	5	20.17	**0.0012**

See figure 3.3 for visualization of effects. A) effects of aphid genotype and *H. defensa* infection on longevity of unparasitized and parasitized aphids, B) effects of parasitism and aphid genotype on longevity of *H. defensa*-infected and uninfected aphids, and C) effects of parasitism and *H. defensa* infection on longevity of three aphid genotypes.

To compare potential effects on aphid longevity, we used a GzLM to examine effects of parasitism, *H. defensa* infection, and aphid genotype, and the interactions of these variables on aphid longevity, summarized in (Table 3.4). Generally, parasitism

reduced aphid longevity, but this effect was variable based on aphid genotype and *H. defensa* strain. Among *unparasitized* controls, uninfected aphids from all three genotypes shared similar numbers of surviving aphids (Fig. 3.3a) and infection with APSE3-*H. defensa* resulted in significantly reduced longevity in genotypes AS3 and CJ113, but not WI27; infection with APSE2-*H. defensa* did not significantly reduce longevity (Fig. 3.3 a & b). Among *parasitized* aphids, genotype AS3 suffered reduced longevity that was partially rescued (non-significant) by infection with APSE3-*H. defensa*, but not APSE2; genotype CJ113 performed best when uninfected, with no significant differences in longevity when parasitized; genotype WI27 performed best when infected with either APSE2-*H. defensa* or APSE3 and suffered reduced longevity when uninfected (Fig. 3.3a).

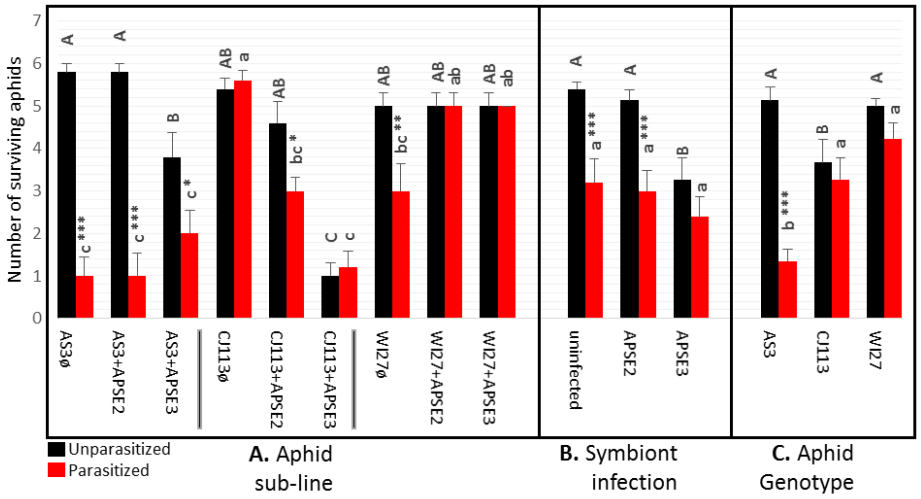


Figure 3.3. Average <u>longevity</u> of unparasitized and parasitized pea aphids after 18 days. A) Results arranged by experimental aphid sub-lines (GzLM, df = 8, $p < 0.0001$ parasitized and unparasitized), B) all sub-lines, averaged by infection status (GzLM, df = 2, $p < 0.0001$ unparasitized, $p = 0.5270$ parasitized), and C) averaged by aphid genotype (GzLM, df = 2, $p = 0.0103$ unparasitized, $p < 0.0001$ parasitized). Capital and lowercase letters indicate pairwise significant differences within unparasitized and parasitized groups, respectively (Arcsine transformed Tukey's HSD $\alpha = 0.05$). Asterisks indicate significant differences between each pair of unparasitized and parasitized treatments (* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001). See Table 3.4 for all analyses.

3.4 Discussion

Here we show that defensive symbiont infections in insects may only be conditionally beneficial, relying on compatibility between host and symbiont genotypes, natural enemy pressure, and the prevalence of other types of resistance present. Our results confirm that the pea aphid employs both symbiont- and aphid-based resistance against the parasitoid *A. ervi*, as in (Martinez *et al.* 2014a), but the costs and benefits of defense against parasitoids in this aphid are more complex than previously appreciated. Together, these results help explain why protective symbionts like *H. defensa* are only found infecting insects at intermediate levels in natural populations.

Consistent with previous results (Martinez *et al.* 2014b; Oliver *et al.* 2009; Oliver *et al.* 2012), we found that infection with APSE3-*H. defensa* conferred substantial resistance (i.e. adult survival through day 10 and not developing into mummy) in susceptible aphid genotypes (Fig. 3.1A). However, this only translated to modest increases in survival through day 18 and total average fecundity, relative to parasitized *H. defensa*-free controls, indicating that direct benefits to infections with this strain were smaller than expected. Infection with APSE2- *H. defensa* was more variable, conferring significant protection in only one of two susceptible genotypes (G15) and in one resistant genotype (CJ113) (Fig. 3.1A), indicating strong aphid X symbiont genotype interactions not reported before in pea aphids. We also found clear costs to infection with both APSE2 and APSE3 *H. defensa* strains (Figs 3.2 & 3.3), with the latter (the stronger protector) incurring greater costs to fecundity and longevity in the absence of parasitism. Previously, pea aphid population cage studies have indicated competitive costs to infection with *H. defensa* in the absence of parasitism (Oliver *et al.* 2008a), though

studies using component fitness assays have rarely reported specific costs and sometimes reported benefits, e.g.(Oliver *et al.* 2006; Russell & Moran 2006); longevity costs have been reported for *H. defensa* in black bean aphids(Cayetano *et al.* 2015; Vorburger & Gouskov 2011). We note that costs to infection with *H. defensa* were not found in all genotypes (see line WI27), again indicating aphid X symbiont genotype interactions. Costs associated with endogenous, non-symbiotic, types of insect defenses have also been reported previously (Kraaijeveld & Godfray 1997; Moret & Schmid-Hempel 2000), however, supporting previous results (Martinez *et al.* 2014a) we did not observe any costs associated with the maintenance of endogenously-encoded resistance to parasitism, which is surprising given that resistant genotypes are, anecdotally, much less common than susceptible genotypes.

Individual pea aphids sometimes have both resistant genotypes and protective symbionts (Martinez *et al.* 2014a; Martinez *et al.* 2014b). When examining the protective phenotype of aphids with both types, we found that only in one of three genotypes (CJ113) did having *H. defensa* significantly increase survival after 10 days, relative to parasitized, uninfected aphids. We expected this result as the high levels of innate resistance (80+ %) conferred in resistant genotypes left little phenotypic space to move within. We anticipated, however, that any benefits accruing to aphids with both types of resistance would be reflected in their ability to 'tolerate' parasitism –i.e. continued survival and reproduction. Surprisingly, though, we found that *H. defensa* only significantly increased fecundity in one (WI27) of two resistant genotypes tested, and in this case, it was only the APSE2-*H. defensa* strain (Fig. 3.2A), although both APSE2 and APSE3 significantly increased survival through day 18 in this background (Fig. 3.3A).

Another surprise is that the 'best' aphid line was the uninfected, innately-resistant, CJ113ø which performed equally well parasitized as unparasitized (Figs. 3.2A and 3.3A), and performed more poorly when infected with *H. defensa* in both the absence and presence of parasitism (Fig. 3.2A and 3.3A) –*despite* being the only resistant genotype where infection improved protection (Fig. 3.1A). For resistance against parasitism to be selectively advantageous, however, aphids must not only survive, but also reproduce more. Overall then, having both types of resistance was largely redundant; benefits, if any, were modest in the presence of parasitism, while costs in the absence of parasitism were often large. As a caveat, we note that our fitness and parasitism assays were performed under ideal conditions for pea aphids, and removed aphid offspring as they were counted, so it is possible that costs and benefits associated with both *H. defensa*-conferred and endogenously-encoded resistance are dependent on environmental conditions, intraspecific competition, and host-plant quality, which would be much more variable under natural conditions.

Field studies report that *H. defensa* is always found at intermediate frequencies (25 – 60% on alfalfa) across North American populations (Weldon et. al. unpublished). Given the clear benefits (and modest costs) owing to infection identified in previous lab assays and population cage studies (Martinez *et al.* 2014b; Oliver *et al.* 2008a; Oliver *et al.* 2009) it has seemed surprising that more aphids do not carry this symbiont. This work identifies a number of factors that likely prevent the spread of *H. defensa,* including, clear costs to infection in the absence of wasps, protective aphid genotypes that are just as fecund when parasitized, and host X symbiont genotype interactions that make

predictions more difficult. Clearly there is still much that is yet to be understood about these complex interactions.

3.5 Conclusions

Defenses against natural enemies can be particularly costly to maintain, so their maintenance is likely a function of balancing selection between enemy pressure and energetic investment into defenses. Both symbiotic and endogenous mechanisms of defense against parasites and parasitoids have now been found in two major insect study systems, aphids (Martinez *et al.* 2014a; Parker *et al.* 2014) and *Drosophila* (Poirie *et al.* 2000; Xie *et al.* 2010). However, another recent study also found that aphid species that maintain mutualistic associations with ants that protect them from natural enemies, including parasitoids, are less likely to maintain infections with facultative symbionts like *H. defensa* that provide redundant benefits (Henry *et al.* 2015), suggesting that differential costs of multiple types of resistance may promote one over the other. Other insects and animals may commonly rely on both symbiotic mutualisms and existing endogenous traits to protect them from natural enemies, but our findings and others (Kondo *et al.* 2005; Mouton *et al.* 2007; Spor *et al.* 2011) suggest that the outcome of these interactions may depend on the compatibility between host and symbiont genotypes, with selection acting against redundant mechanisms or genotype X genotype pairings that are more costly overall.

3.6 References

Angalet GW, Fuester R (1977) The *Aphidius* parasites of the pea aphid *Acyrthosiphon pisum* in the eastern half of the United States. *Annals of the Entomological Society of America* **70**, 87-96.

Ardia DR, Gantz JE, Schneider BC, Strebel S (2012) Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Functional Ecology* **26**, 732-739.

Beckage NE, Gelman DB (2004) Wasp parasitoid disruption of host development: Implications for New Biologically Based Strategies for Insect Control. *Annual Reviews in Entomology* **49**, 299-330.

Boman HG, Hultmark D (1987) Cell-free immunity in insects. *Annual Reviews in Microbiology* **41**, 103-126.

Cardinale BJ, Harvey CT, Gross K, Ives AR (2003) Biodiversity and biocontrol: emergent impacts of a multi-enemy assemblage on pest suppression and crop yield in an agroecosystem. *Ecology Letters* **6**, 857-865.

Cayetano L, Rothacher L, Simon J-C, Vorburger C (2015) Cheaper is not always worse: strongly protective isolates of a defensive symbiont are less costly to the aphid host. *Proceedings of the Royal Society of London B: Biological Sciences* **282**, 20142333.

Chau A, Mackauer M (1997) Dropping of pea aphids from feeding site: a consequence of parasitism by the wasp, *Monoctonus paulensis*. *Entomologia Experimentalis et Applicata* **83**, 247-252.

Davis JJ (1913) The Cyrus Thomas Collection of Aphididae and a Tabulation of Species Mentioned and Described in His Publications. *Bulletin of the Illinois state laboratory of natural history*.

Davis JJ (1915) *The pea aphis with relation to forage crops* US Department of Agriculture.

Digilio MC, Isidoro N, Tremblay E, Pennacchio F (2000) Host castration by *Aphidius ervi* venom proteins. *Journal of Insect Physiology* **46**, 1041-1050.

Douglas A, Francois C, Minto L (2006) Facultative 'secondary'bacterial symbionts and the nutrition of the pea aphid, *Acyrthosiphon pisum*. *Physiological Entomology* **31**, 262-269.

Eggleton P, Belshaw R (1992) Insect parasitoids: an evolutionary overview. *Philosophical Transactions of the Royal Society B: Biological Sciences* **337**, 1-20.

Falabella P, Riviello L, Caccialupi P, *et al.* (2007) A γ-glutamyl transpeptidase of *Aphidius ervi* venom induces apoptosis in the ovaries of host aphids. *Insect Biochemistry and Molecular Biology* **37**, 453-465.

Gerardo NM, Altincicek B, Anselme C, *et al.* (2010) Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. *Genome Biology* **11**.

Godfray HCJ (1994) *Parasitoids: behavioral and evolutionary ecology* Princeton University Press.

Gwynn DM, Callaghan A, Gorham J, Walters KFA, Fellowes MDE (2005) Resistance is costly: trade-offs between immunity, fecundity and survival in the pea aphid. *Proceedings of the Royal Society B-Biological Sciences* **272**, 1803-1808.

Halfhill JE, Featherston PE, Dickie AG (1972) History of the *Praon* and *Aphidius* parasites of the pea aphid in the Pacific Northwest. *Environmental Entomology* **1**, 402-405.

He XZ (2008) *Reproductive behaviour of Aphidius ervi Haliday (Hymenoptera: Aphidiidae)*, Massey University.

Henry LM, Maiden MC, Ferrari J, Godfray HCJ (2015) Insect life history and the evolution of bacterial mutualism. *Ecology Letters* **18**, 516-525.

Henter HJ, Via S (1995) The potential for coevolution in a host-parasitoid system. I. Genetic variation within an aphid population in susceptibility to a parasitic wasp. *Evolution*, 427-438.

Hufbauer RA (2001) Pea aphid-parasitoid interactions: Have parasitoids adapted to differential resistance? *Ecology* **82**, 717-725.

Hufbauer RA, Via S (1999) Evolution of an aphid-parasitoid interaction: Variation in resistance to parasitism among aphid populations specialized on different plants. *Evolution* **53**, 1435-1445.

Kondo N, Shimada M, Fukatsu T (2005) Infection density of Wolbachia endosymbiont affected by co-infection and host genotype. *Biology letters* **1**, 488-491.

Kraaijeveld A, Godfray H (1997) Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Nature* **389**, 278-280.

Lamb R, Pointing P (1972) Sexual morph determination in the aphid, *Acyrthosiphon pisum*. *Journal of Insect Physiology* **18**, 2029-2042.

Laughton AM, Garcia JR, Altincicek B, Strand MR, Gerardo NM (2011) Characterisation of immune responses in the pea aphid, Acyrthosiphon pisum. *Journal of Insect Physiology* **57**, 830-839.

Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology* **32**, 1295-1309.

Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. *Annual Review of Immunology* **25**, 697-743.

Mackauer M, Finlayso T (1967) Hymenopterous parasites (Hymenoptera - Aphidiidae et Aphelinidae) of pea aphid in Eastern North America. *Canadian Entomologist* **99**, 1051-&.

Martinez AJ, Ritter SG, Doremus MR, Russell JA, Oliver KM (2014a) Aphid-encoded variability in susceptibility to a parasitoid. *BMC Evol Biol* **14**, 127.

Martinez AJ, Weldon SR, Oliver KM (2014b) Effects of parasitism on aphid nutritional and protective symbioses. *Molecular ecology* **23**, 1594-1607.

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16919-16926.

Moret Y, Schmid-Hempel P (2000) Survival for immunity: The price of immune system activation for bumblebee workers. *Science* **290**, 1166-1168.

Mouton L, Henri H, Charif D, Boulétreau M, Vavre F (2007) Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. *Biology letters* **3**, 210-213.

Oliver KM, Campos J, Moran NA, Hunter MS (2008a) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B: Biological Sciences* **275**, 293-299.

Oliver KM, Campos J, Moran NA, Hunter MS (2008b) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society of London B: Biological Sciences* **275**, 293-299.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325**, 992-994.

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12795-12800.

Oliver KM, Moran NA, Hunter MS (2006) Costs and benefits of a superinfection of facultative symbionts in aphids. *Proceedings of the Royal Society of London B: Biological Sciences* **273**, 1273-1280.

Oliver KM, Noge K, Huang EM, *et al.* (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC biology* **10**, 11.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1803-1807.

Parker BJ, Garcia JR, Gerardo NM (2014) Genetic variation in resistance and fecundity tolerance in a natural host–pathogen interaction. *Evolution* **68**, 2421-2429.

PEARSON DL (1985) The function of multiple anti-predator mechanisms in adult tiger beetles (Coleoptera: Cicindelidae). *Ecological Entomology* **10**, 65-72.

Pennacchio F, Mancini D (2012) Aphid Parasitoid Venom and its Role in Host Regulation. *Parasitoid Viruses: Symbionts and Pathogens*, 247-254.

Pennacchio F, Strand MR (2006) Evolution of developmental strategies in parasitic Hymenoptera. *Annu. Rev. Entomol.* **51**, 233-258.

Poirie M, Frey F, Hita M, *et al.* (2000) Drosophila resistance genes to parasitoids: chromosomal location and linkage analysis. *Proceedings of the Royal Society of London B: Biological Sciences* **267**, 1417-1421.

Poitrineau K, Brown SP, Hochberg ME (2003) Defence against multiple enemies. *Journal of evolutionary biology* **16**, 1319-1327.

Rantala MJ, Honkavaara J, Dunn DW, Suhonen J (2011) Predation selects for increased immune function in male damselflies, *Calopteryx splendens*. *Proceedings of the Royal Society B-Biological Sciences* **278**, 1231-1238.

Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society of London B: Biological Sciences* **273**, 603-610.

Smilanich AM, Dyer LA, Gentry GL (2009) The insect immune response and other putative defenses as effective predictors of parasitism. *Ecology* **90**, 1434-1440.

Snyder WE, Ives AR (2003) Interactions between specialist and generalist natural enemies: parasitoids, predators, and pea aphid biocontrol. *Ecology* **84**, 91-107.

Spor A, Koren O, Ley R (2011) Unravelling the effects of the environment and host genotype on the gut microbiome. *Nature Reviews Microbiology* **9**, 279-290.

Start P (1970) Biology of aphid parasites (Hymenoptera: Aphidiidae) with respect to integrated control. *Series Ent.* **6**, 1-643.

Strand MR (2008) The insect cellular immune response. *Insect Science* **15**, 1-14.

Thomas C (1879) Noxious and beneficial insects of the State of Illinois. *Report of the State Entomologist (Illinois)* **8**, 1-212.

Vinson SB, Iwantsch G (1980) Host regulation by insect parasitoids. *Quarterly Review of Biology*, 143-165.

Vorburger C, Gouskov A (2011) Only helpful when required: a longevity cost of harbouring defensive symbionts. *Journal of evolutionary biology* **24**, 1611-1617.

Weldon S, Strand M, Oliver K (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. *Proceedings of the Royal Society B: Biological Sciences* **280**, 20122103.

White J, Kelly S, Cockburn S, Perlman S, Hunter M (2011) Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. *Heredity* **106**, 585-591.

Xie J, Vilchez I, Mateos M (2010) *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. *PLoS One* **5**.

Supplemental table 3.1 General linear model examining effects of parasitism, aphid genotype, and *H. defensa* infection on aphid fecundity.

Variable	df	f-value	p-value
Parasitism	1	193.91	**<.0001**
Aphid genotype	2	47.00	**<.0001**
H. defensa infection	2	47.21	**<.0001**
Parasitism X genotype	2	25.01	**<.0001**
Parasitism X infection	2	17.86	**<.0001**
Genotype X infection	4	23.34	**<.0001**
Parasitism X genotype X infection	4	9.81	**<.0001**
Whole model	17	35.30	**<.0001**

CHAPTER 4

EFFECTS OF PARASITISM ON APHID NUTRITIONAL AND PROTECTIVE SYMBIOSES [3]

[3] Martinez AJ, Weldon SR, and Oliver KM. 2014. *Molecular ecology.* 23:1594-1607.
Reprinted here with permission of the publisher.

Abstract

Insects often carry heritable symbionts that negotiate interactions with food plants or natural enemies. All pea aphids, *Acyrthosiphon pisum*, require infection with the nutritional symbiont *Buchnera*, and many are also infected with *Hamiltonella*, which protects against the parasitoid *Aphidius ervi*. *Hamiltonella*-based protection requires bacteriophages called APSEs with protection levels varying by strain and associated APSE. Endoparasitoids, including *A. ervi*, may benefit from protecting the nutritional symbiosis and suppressing the protective one, while the aphid and its heritable symbionts have aligned interests when attacked by the wasp. We investigated the effects of parasitism on the abundance of aphid nutritional and protective symbionts. First we determined strength of protection associated with multiple symbiont strains and aphid genotypes as these likely impact symbiont responses. Unexpectedly, some *A. pisum* genotypes cured of facultative symbionts were resistant to parasitism and resistant aphid lines carried *Hamiltonella* strains that conferred no additional protection. Susceptible aphid clones carried protective strains. qPCR estimates show that parasitism significantly influenced both *Buchnera* and *Hamiltonella* titers, with multiple factors contributing to variation. In susceptible lines, parasitism led to increases in *Buchnera* near the time of larval wasp emergence consistent with parasite manipulation, but effects were variable in resistant lines. Parasitism also resulted in increases in APSE and subsequent decreases in *Hamiltonella* and we discuss how this response may relate to the protective phenotype. In summary, we show that parasitism alters the within host ecology of both nutritional and protective symbioses with effects likely significant for all players in this antagonistic interaction.

4.1 Introduction

Many herbivorous insects, including diverse hemipteran groups, feed exclusively on plant phloem or xylem and require microbial symbionts to supplement their nitrogen poor diets (Baumann 2005; Douglas 1989; Zientz *et al.* 2004). These nutritional-symbiont associated herbivores are frequently attacked by one or more endoparasitic wasps, which develop within living hosts, and employ a variety of strategies to create a host environment suitable for larval development (Godfray 1994; Vinson & Iwantsch 1980), including avoiding or suppressing host encapsulation responses, limiting damage to critical host tissues, and redirecting host resources for use in wasp development (Beckage & Gelman 2004; Pennacchio & Strand 2006). Wasps developing in insects with obligate nutritional symbionts may also need to protect or commandeer the nutritional symbiosis for successful development (Pennacchio *et al.* 1999).

Some sap-feeding insects, however, maintain infections with both nutritional and protective symbionts including those that defend against parasitic wasps (Oliver et al. 2010). Parasitic wasps potentially benefit from suppressing the abundance of protective symbionts, while their parasitized hosts may benefit from the accumulation of defensive elements or increased expression of virulence genes. Thus, parasitism may be expected to alter the within host dynamics of both nutritional and defensive symbionts.

The pea aphid, *Acyrthosiphon pisum*, is an ideal system for investigating effects of parasitism on symbiont abundance as particular facultative symbionts can be manipulated and studied in genetically controlled aphid backgrounds (Oliver *et al.* 2010). Like most aphids, *A. pisum* requires an obligate bacterial symbiont, *Buchnera aphidicola*, which resides in specialized host cells called bacteriocytes and provisions

hosts with nutrients lacking in their diet (Douglas 1998; Moran & Degnan 2006). This aphid can also harbor at least eight additional facultative symbionts (Ferrari *et al.* 2012; Russell *et al.* 2013), several of which have been shown to confer diverse benefits, including protection against common natural enemies such as fungal pathogens and parasitic wasps (Oliver *et al.* 2003; Parker *et al.* 2013; Scarborough *et al.* 2005). The facultative symbiont *Hamiltonella defensa*, for example, confers resistance against the parasitoid *A. ervi* by causing mortality to wasps as they develop within the aphid hemocoel (Oliver *et al.* 2005; Oliver *et al.* 2003). *H. defensa* persists intracellularly in bacteriocytes and sheath cells, but also lives extracellularly in the host hemolymph (Fukatsu *et al.* 2000; Moran *et al.* 2005; Sandstrom *et al.* 2001). This bacterial symbiont is typically associated with toxin-encoding bacteriophages called APSEs (*Acyrthosiphon pisum* secondary endosymbiont), which are required to produce the defensive phenotype (Degnan & Moran 2008; Moran *et al.* 2005; Oliver *et al.* 2009; van der Wilk *et al.* 1999). Two variants, APSE2 and APSE3, have been characterized in North American *A. pisum* (Degnan & Moran 2008; Moran *et al.* 2005; Oliver *et al.* 2009) and contain homologs of cytolethal distending toxin (*cdtB)* and YD-repeat protein (*YDp*), respectively; both are putative toxins hypothesized to contribute to the mortality of developing parasitoids (Degnan & Moran 2008). No studies, however, have functionally demonstrated these toxins cause mortality to developing wasps.

Several studies have investigated how parasitism influences the aphid nutritional symbiosis. In non-resistant aphids, *A. ervi* uses maternal and embryonic factors to modulate the aphid host environment. At oviposition, *A. ervi* injects venom, which specifically targets the apical germaria of the ovarioles, disrupting oogenesis in *A. pisum*

(Digilio *et al.* 2000; Falabella *et al.* 2007). Later, when the parasitoid emerges from its serosal membrane enclosure, specialized wasp cells called teratocytes dissociate from the extra-embryonic membrane and are often observed localized around aphid bacteriocytes and developing aphid embryos. Teratocytes are hypothesized to perform a nutritional role by redirecting bacteriocyte-produced resources, originally destined to reach aphid nymphs, toward the developing parasitoid (Falabella *et al.* 2005; Falabella *et al.* 2000; Pennacchio *et al.* 1999). In general, *Buchnera* abundance and the number of bacteriocytes in *A. pisum* typically decline after aphids reach adulthood (Douglas 1998; Komaki & Ishikawa 2000; Nishikori *et al.* 2009; Wilkinson & Douglas 1998). One study, however, observed a greater number and biomass of bacteriocytes in parasitized *A. pisum* 4.5 days after parasitism compared to unparasitized controls (Cloutier & Douglas 2003), suggesting that parasitism may slow natural bacteriocyte decay and *Buchnera* loss. Other work has shown that parasitism results in significant increases in particular free amino acids, especially tyrosine (Rahbe *et al.* 2002). Together these results are consistent with wasps' protection of the aphid nutritional symbiosis, which is further supported by longer development times and reduced adult mass for wasps reared in *Buchnera*-free *A. pisum* compared to infected controls (Pennacchio *et al.* 1999). How *Buchnera* responds to parasitism, however, may depend on whether the aphid is infected with *H. defensa* or other facultative symbionts. Manipulation of *Buchnera* by the parasitoid may be thwarted by infection with protective symbionts if, for example, parasitoid mortality occurs prior to the emergence of teratocytes, thus preventing their interactions with bacteriocytes.

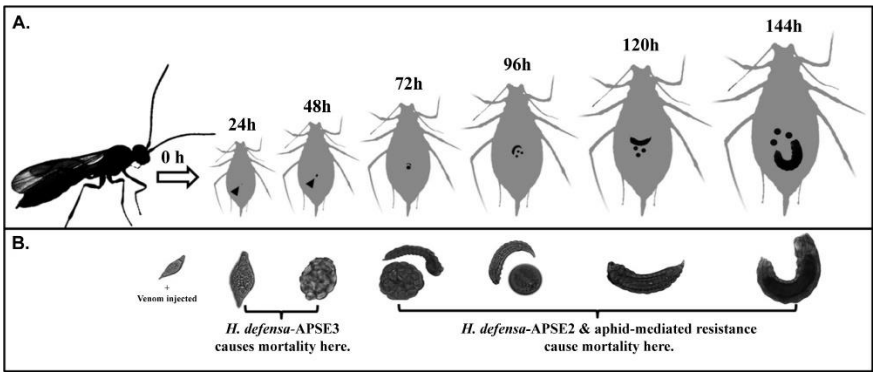


Figure 4.1 (A) Relative sizes of a free-living, adult female *A. ervi* wasp and her internally developing progeny (egg, morula and larva) compared to a parasitized 72h old (2nd instar). (B) Enlargement of wasp developmental stages over time and timing of mortality observed in APSE2- and APSE3- *H. defensa* infected aphids. 0h: egg and venom injected by female wasp. 24 - 48h: enlargement of egg and development of morula. 72h: wasp larva emerges from morula and dissociation of teratocytes. 96 – 144h: larval and teratocyte growth (> 20 teratocytes). Egg and morula are indicated by arrowheads at 24 – 48h. Aphid growth in diagram does not correspond to nymphal instars. Relative size and development timing were determined through observation using stereomicroscopy; timing of wasp development was comparable to that found in (He 2008).

Compared to this work on the primary nutritional symbiont, little is known about how parasitism affects the protective symbiosis. Parasitism potentially induces changes in symbiont and phage abundance directly or indirectly through effects on the aphid immune system. Symbiont abundance may also be influenced by parasitism-induced effects on APSE-*H. defensa* dynamics. Host stress resulting from parasitism, for example, may result in phage-mediated bacterial lysis. Previous work also indicates that the putative toxins encoded by *H. defensa*-associated APSEs are constitutively expressed (Moran *et al.* 2005; Oliver *et al.* 2009), but it is not known if parasitism results in changes in toxin transcript number. The timing of changes in the abundance of symbionts or defensive products following enemy challenge, may vary depending on *H. defensa* strain and associated APSE variant, which cause wasp mortality at different stages of development. In aphids carrying *H. defensa*-APSE2 strains, which typically confer moderate levels of protection (Oliver *et al.* 2005), mortality occurs after the emergence of

larvae (> 72h after parasitism), while wasp mortality occurs much earlier (< 48h after parasitism) (see results) in aphids infected with highly-protective *H. defensa*-APSE3 strains (Fig. 4.1) (Oliver *et al.* 2009; Oliver *et al.* 2005). Thus, the titers and responses of defensive elements (*H. defensa* and APSE) are potentially mobilized only at key time-points in the parasitoid lifecycle, including the parasitism event itself (oviposition and venom injection), rupturing of the chorion (~ 24h), and the emergence of larvae from the embryonic morulae and concomitant dissociation of teratocytes (~ 72h) (Fig. 4.1) (He 2008).

To address these important unknowns in the pea aphid-wasp-symbiont system, we characterize the protective phenotype of multiple *H. defensa* strains and aphid genotypes and use quantitative PCR (qPCR) and reverse transcription qPCR (RT-qPCR) to estimate the effects of parasitism on *Buchnera*, *H. defensa*, and APSE abundances, as well as APSE toxin gene expression, at key points in wasp development. We find that parasitism alters the within-host ecology of nutritional and protective symbionts associated with *A. pisum*. Microbial symbionts are widespread in insects, and enemy challenge potentially influences the dynamics of host-symbiont interactions in many systems.

4.2 Materials and methods

Study organisms, creation of experimental lines, and rearing

The pea aphid, *Acyrthosiphon pisum*, which feeds on a variety of herbaceous legumes, was introduced to North America from Europe in the late 1800s (Eastop 1966). This aphid is cyclically parthenogenetic and reproduction is asexual and viviparous for the majority of the year, but sexual morphs occur in the fall in response to shorter day

lengths (Lamb & Pointing 1972). Clonal lines were maintained in the laboratory by rearing them under long day conditions.

Table 4.1 Experimental aphid lines including symbiont infection status and levels of resistance to parasitism by *A. ervi*.

Aphid Clone	Collection locale	*H. defensa* infection	Phage Variant	APSE toxin gene	Resistance phenotype	Parasitoid Resistance timing
AS3	Utah, USA 2007	yes	APSE-3	*YDp*	very high	24 - 48h
A1A-5A	A1A, Utah, USA 2003; 5A, Wisconsin, USA 1999	yes	APSE-3	*YDp*	very high	24 - 48h
ZA17	Pennsylvania, USA 2010	yes	APSE-2	*cdtB2*	high	> 48h
WA4	Pennsylvania, USA 2010	yes	APSE-2	*cdtB2*	high	> 48h
82B	New York, USA 2000	yes	APSE-2	*cdtB1*	medium	> 48h
ZA17-AB	see ZA17	no	-	-	high	> 48h
WA4-AB	see WA4	no	-	-	high	> 48h
AS3-AB	see AS3	no	-	-	low	n/a
ZA29	Pennsylvania, USA 2010	No/*Rickettsiella*	-	-	low	n/a

'AB' in clone name signifies that this line was antibiotic-treated to eliminate *H. defensa*.

Each clonal aphid line used in this study (Table 4.1) was initiated from a single parthenogenetic female placed onto a caged broad bean plant, *Vicia faba,* and reared at 20±1˚C with a 16L: 8D photoperiod. Symbiont status was verified using diagnostic PCR and Denaturing Gradient Gel Electrophoresis (DGGE) with universal 16S rRNA bacterial primers to ensure that only expected symbionts were present in experimental lines using protocols described in (Russell *et al.* 2013). Lines AS3-AB, ZA17-AB, and WA4-AB were selectively cured of their *H. defensa* symbiont with an antibiotic cocktail comprised of cefotaxamine, gentamycin, and ampicillin as in (Douglas *et al.* 2006) so the effects of parasitism on their primary symbiont, *Buchnera,* could be assessed in the presence and absence of *H. defensa* in the same clonal background. Experiments were conducted at least six months (> 12 generations) after antibiotic treatment.

The solitary endoparasitoid, *Aphidius ervi* (Hymenoptera: Braconidae), is a common natural enemy of *A. pisum* in North America. This wasp was introduced from Europe as a means of controlling several aphid species (Angalet & Fuester 1977). The

wasps used in this study were commercially produced (Syngenta Bioline Ltd.) and reared continuously on a non-resistant aphid line (AS3-AB); adults were provided honey and water.

Aphid parasitism resistance assays to determine protective phenotype

Parasitism assays to determine the resistance phenotype were carried out on all aphid lines used in this study (Table 4.1) as in (Oliver *et al.* 2009). Briefly, twenty 2nd to 3rd instar aphids were singly parasitized (each aphid is removed as it is parasitized) for each replicate (indicated in Fig. 4.2) and placed on a fresh *V. faba* plant in a cup cage and held at 20±1˚C with a 16L: 8D photoperiod. After nine days, we counted the number of live aphids and aphid mummies (dried aphids containing a wasp pupa) to determine percent resistance measured as [total live adult aphids/(total live + total mummified)].

Serial aphid dissections and timing of aphid resistance

The approximate timing of aphid resistance was estimated by conducting timed serial dissections of parasitized aphids to monitor the development of *A. ervi.* Twenty parasitized aphids from each line were individually dissected in 60uL of 1X PBS at 24, 48, 72, 96, and 120h intervals after parasitism and observed using a Leica M80 stereoscope. Presence, stage, and status (healthy, moribund, or dead) of each developing wasp were recorded at each time-point.

Estimating aphid symbiont copy numbers after parasitism

Adult aphids of each experimental line were placed in a cage with a fresh *V. faba* plant for 12±1h and allowed to reproduce; afterwards all adults were removed and discarded while offspring continued to grow for 72h. Aphids aged 78±6h then were divided evenly into two groups: a parasitized treatment and an unparasitized control. In

the parasitized treatment, aphids were singly parasitized by *A. ervi* in a Petri dish, while unparasitized controls were moved to a Petri dish free of wasps. Parasitism was completed over the course of about thirty minutes for each aphid line. Parasitized and unparasitized aphids were then placed on separate, but same-aged plants with identical rearing histories until removal at specific time points for qPCR assays.

To estimate symbiont and phage titers we conducted "absolute" real-time qPCR of singly parasitized and unparasitized *A. pisum* at 24, 48, 72, 96, 120, and 144h intervals after parasitism. For the protective symbiont *H. defensa* and phage APSE we amplified fragments of single-copy genes, as one copy approximates one bacterial cell or phage genomic copy. The nutritional symbiont, *Buchnera,* however is polyploid (Komaki & Ishikawa 1999), so qPCR estimates provide genome abundance, but not the number of *Buchnera* cells. We performed whole single aphid DNA extractions on eight individual aphids for each aphid line and treatment (parasitized and unparasitized) at each time point. Samples were homogenized in 50µL of lysis buffer containing 0.5µl of 20 mg/mL proteinase K and incubated using the following protocol: 38˚ C for 35 minutes, 95˚ C for 2.5 minutes, and held at 4˚ C or on ice for immediate use (Engels *et al.* 1990; Oliver *et al.* 2006). Target genes and oligonucleotide primers for each organism (bacterial symbionts, virus, and aphid) are listed in Supplemental Table 4.1. All 10µl qPCR reactions were performed on a Roche LightCycler 480 II using Roche LightCycler 480 SYBR Green I Master chemistry and 0.5 µM of each primer. PCR cycling conditions for all primer sets was 95˚ C for 5 min; 45 cycles of 95˚ C for 10 sec, 68-56˚ C touchdown for 13 cycles, then 55˚ C for 32 cycles, each cycle for 10 sec; 72˚ C for 10 sec. Amplifications were analyzed with an external standard curve for each respective gene, produced with serial

dilutions from $1x10^2$ to $1x10^9$ (Oliver *et al.* 2006). The aphid gene *Ef*-1 α was used to correct for differences in extraction efficiency for all time points between 24-120h. To calibrate extraction efficiency, the highest *Ef-1* α copy number was divided by the *Ef-1* α copy number of each sample per comparison and symbiont copy numbers were then multiplied by the resulting 'correction' factor. However, because parasitism in susceptible aphids can cause deterioration of aphid tissue at the latest time point we sampled (144h after parasitism), *Ef-1* α values can vary significantly between equal-aged parasitized and unparasitized aphids. Thus, in a few noted instances we did not use *Ef-1 α* to calibrate symbiont abundances. Instead, comparison of parasitized and unparasitized aphids at 144h were made using un-calibrated data. In all cases, symbiont abundances were adjusted to reflect copy number per aphid.

Estimating APSE toxin expression after parasitism

To compare APSE toxin expression between parasitized and unparasitized aphids, we conducted reverse transcriptase qPCR (RT-qPCR) relative to three *H. defensa* reference genes (Moran *et al.* 2005) and one aphid reference gene. Aphids were reared and singly-parasitized as described above. RNA extractions were performed on whole aphids at 6, 30, 70, and 96h intervals following parasitism with the Omega Bio-Tek E.Z.N.A. Mollusc RNA kit, including DNase treatment, following manufacturer protocols. RNA extractions were performed on four samples for each treatment at each time-point, with 4 aphids per sample to increase yields, and were eluted with 50μL of PCR-grade water. All extractions were converted to cDNA using Invitrogen SuperScript III, following the *First-Strand cDNA Synthesis* protocol.

APSE toxin expression (*cdtB* for APSE2 or *YDp* for APSE3) was measured using toxin-specific primers (Supplemental Table 4.1) and compared to the *H. defensa gyrB, dnaK, proC* and *A. pisum Ef-1 α* reference genes. Results are presented as the ratio of APSE toxin (*cdtB* or *YDp*) to each reference gene and were considered significant only when consistent differences occurred relative to multiple reference genes (and not when significant differences also occurred between treatments in the reference gene). The PCR protocol and cycling conditions are as described in (Moran *et al.* 2005; Oliver *et al.* 2009).

Statistical Analyses

General linear models (GLM) were employed to investigate effects of parasitism on aphid symbioses. Explanatory variables analyzed in the GLM included: parasitism, time elapsed after treatment, *H. defensa* infection status, and the clone-strain-APSE combination as we cannot isolate effects of APSEs independent of the *H. defensa* strains in which they occur. Explanatory variables were analyzed factorially to determine if they contributed to observed symbiont patterns either independently or in concert with other factors. Response variables in the GLM included: copy numbers for *Buchnera, H. defensa,* and APSE. For GLMs, symbiont copy numbers and APSE/*H. defensa* ratio were natural log transformed to satisfy normality assumptions and normality was checked with a goodness-of-fit test for each aphid line at each time-point.

Analyses of variance (ANOVA) were performed to compare means of symbiont copy numbers, toxin expression, and APSE integration rate, obtained through qPCR, for parasitized and unparasitized treatments at particular time points. Normality assumptions were checked with the goodness-of-fit test. If normality was not met, values were natural

log transformed for analysis, then back transformed for reporting. Parasitism resistance assays were compared among all aphid lines using ANOVA and a Tukey's Honestly Significant Different (HSD) test.

Sequencing of the *cdtB* toxin gene

The *cdtB* toxin gene was sequenced in all three APSE2-*H. defensa* lines (82B, ZA17, and WA4). Primers used in *cdtB* sequencing are found in Supplementary Table 4.1. Reactions were carried out in 20μL volumes using GoTaq HotStart polymerase (Promega): sequencing reactions were heated to 94°C for 2 min, then underwent 35 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 30s, and then a final 5 min extension at 72°C before being held at 4°C. All amplicons were sequenced by Eurofins MWG Operon (Huntsville, Alabama) in the forward and reverse directions. Sequences were aligned in Geneious version 6.1 (Biomatters) using the MUSCLE algorithm (Edgar 2004) and manual inspection.

4.3 Results

Parasitism resistance assays

In previously published work, *A. pisum* infected with APSE2 and APSE3 *H. defensa* received moderate and high levels of protection, respectively, while those uninfected with *H. defensa* or infected with APSE-free *H. defensa* were highly susceptible to parasitism (Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2003). We conducted parasitism assays to confirm that each of our experimental lines exhibited the expected defensive phenotype (Fig. 4.2). As expected, aphid lines infected with APSE3 *H. defensa,* AS3 and A1A-5A, received high levels of protection (Oliver *et al.* 2009; Oliver *et al.* 2005). An

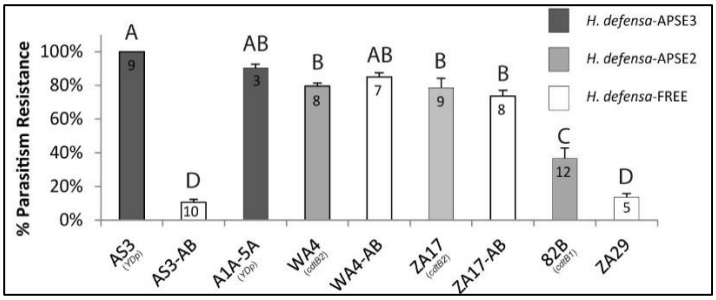


Figure 4.2 Percent resistance to parasitism by *Aphidius ervi* for each aphid line. Letters indicate significance (Tukey's HSD), numbers indicate replicate assays (each with 20 parasitized aphids) included in the analysis. ***p < .0001

antibiotic cured line (AS3-AB) sharing the same genetic background as AS3 was highly susceptible to parasitism (Fig. 4.2), while line 5A (the uninfected counterpart to A1A-5A) has been shown to be highly susceptible (10 – 20% resistant) to parasitism in several prior studies (Oliver et al. 2003; 2005; 2009). One line (ZA29) naturally uninfected with *H. defensa* was also highly susceptible to parasitism. Unexpectedly, two APSE2 *H. defensa* infected aphid lines (WA4 and ZA17) were not significantly more resistant (Fig. 4.2) than their genetically identical, but symbiont free counterparts (WA4-AB, ZA17-AB). Interestingly, these two symbiont-free lines were themselves very resistant to parasitism by *A. ervi*. After this discovery, we rescreened these resistant aphid lines with diagnostic PCR specific to known *A. pisum* symbionts and 'universal' 16S-based DGGE (see methods) capable of identifying unexpected symbionts and confirmed they were uninfected with facultative symbionts. Another APSE2 line, 82B, exhibited parasitism rates consistent with previous assays suggesting this strain confers moderate protection (Oliver *et al.* 2005). Results are summarized in Table 4.1.

Distinct *cdtB* allele in ZA17 and WA4 *H. defensa* strains

Interestingly, the potentially non-protective *H. defensa* strains found in WA4 and ZA17 harbor APSE2s that encode a novel *cdtB* toxin allele. The new allele, called *cdtB2*, is

distinct at seven nucleotide residues, with six non-synonymous changes, relative to the *cdtB1* allele found in the protective 82B APSE2 strain (GenBank KF551594; Supplemental Fig. 4.3). However, according to published annotations (ABA29376) (Moran *et al.* 2005), none of the non-synonymous variation is predicted to interfere with toxin protein functionality.

Timing of aphid resistance to parasitism varies by APSE type

We conducted serial dissections to determine if the timing of parasitoid mortality varied with *H. defensa* strain phage type (Supplemental Fig. 4.1). In the *H. defensa*-APSE3 lines (lines AS3 and A1A-5A) eggs were found prior to 24h and *A. ervi* morulae were found in most (15/20 in AS3 and 14/20 in A1A-5A) aphids at 24h, but only a single developing wasp was found at 48h (A1A-5A), and none in either line thereafter. In the *H. defensa*-APSE2 lines (lines 82B, WA4, ZA17), however, *A. ervi* typically developed to the larval stage and were present through the last dissection time-point at 120h after parasitism. So in conjunction with greater protection conferred by APSE3 *H. defensa*, mortality of developing parasitoids occurs earlier than seen for aphids with APSE2 *H. defensa*. Results are summarized in Figure 4.1 and Table 4.1.

Effects of parasitism on the abundance of the nutritional symbiont *Buchnera*

Overall, we found substantial variation in *Buchnera* abundance, with aphid line and age contributing the bulk of the variation (Table 4.2, Fig. 4.3). Considering only unparasitized lines (Table 4.2a), *Buchnera* titers were influenced by co-infection with *H. defensa*, although effects of infection varied among aphid lines. *H. defensa* infection correlated with decreased *Buchnera* copy number in AS3 and ZA17 (Fig. 4.3 A and B), and increased *Buchnera* copy number in WA4 (Fig. 4.3C).

Table 4.2 GLM analyzing variables affecting *Buchnera* abundance.

Aphid Lines	Explanatory Variable	DF	F-ratio	Significance
a. ALL LINES (unparasitized)	Time point	5	15.1741	**<.0001**
	H. defensa infection	1	5.8485	**0.0161**
	Aphid line	7	189.7857	**<.0001**
b. ALL LINES (parasitized and unparasitized)	Clone-strain-APSE combination	3	161.5593	**<.0001**
	Parasitism	1	0.1879	0.665
	Time point*parasitism	5	7.6176	**<.0001**
	Clone-strain-APSE*parasitism	3	3.7223	**0.0118**
	Clone-strain-APSE*time point*parasitism	15	5.6417	**<.0001**
c. AS3 & AS3-AB	Parasitism	1	0.3627	0.5472
	H. Defensa infection	1	15.2398	**0.0001**
	Parasitism**H. defensa* infection	1	0.3026	0.5824
d. WA4 & WA4-AB	Parasitism	1	1.4452	0.231
	H. defensa infection	1	1040.415	**<.0001**
	Parasitism**H. defensa* infection	1	0.4015	0.5272
e. ZA17 & ZA17-AB	Parasitism	1	16.1864	**<.0001**
	H. defensa infection	1	70.6055	**<.0001**
	Parasitism**H. defensa* infection	1	0.1592	0.6592
f. 82B	Parasitism	1	5.4592	**0.0207**
g. ZA29	Parasitism	1	6.7133	**0.0113**

Significant values are bolded. 'Aphid line' values are from a separate model. All models p<.0001. (Full models available in Supplemental table 4.2)

Across all lines and time-points (Table 4.2b) parasitism did not result in overall consistent significant effects on *Buchnera* titers. When examined by individual aphid line (i.e. same line parasitized vs. non-parasitized), parasitism significantly influenced *Buchnera* abundance in three genotypes (Table 4.2 e, f, g). The observed effects of parasitism on *Buchnera*, however, did not depend on *H. defensa* infection status (Parasitism**H. defensa* infection) (Table 4.2 c, d, e). At a finer scale, all lines exhibited some time points when parasitism resulted in significant changes in *Buchnera*, even if effects were not significant overall. This may be expected if *Buchnera* modulation occurs only at specific points. Again, however, net effects were variable with parasitism increasing *Buchnera* at some time points and decreasing it at others (Fig. 4.3 A, B, C).

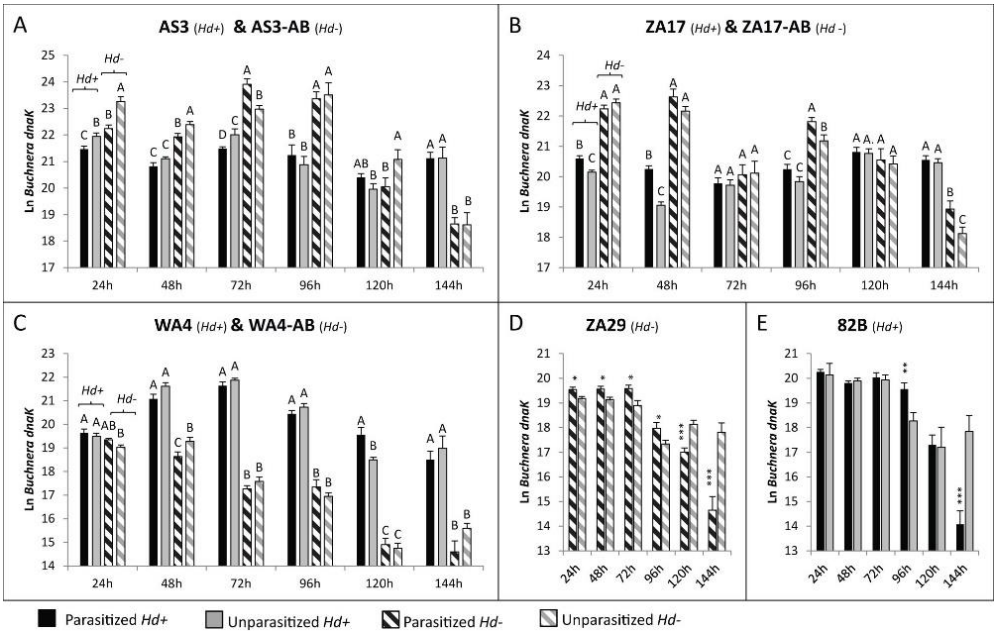


Figure 4.3 Effects on parasitism on *Buchnera*. Y-axis represents Ln *Buchnera* copy number calibrated by the aphid gene *Ef-1 α* (Lines AS3-AB, WA4-AB, ZA29, and 82B were not adjusted with *Ef-1 α* at 144h –see methods for details).

Successful manipulation of *Buchnera* abundance by the parasite would perhaps be most obvious in susceptible aphid lines where wasps are able to complete development. The aphid lines with lowest resistance to parasitism (Fig. 4.2: AS3-AB and ZA29) both showed significantly higher *Buchnera* titers in parasitized aphids at 72h, followed by significantly lower titers at 120h and/or 144h (Fig. 4.3A and D). A moderately resistant line (Fig. 4.2: 82B), in which many wasps still complete development, showed a similar pattern with significantly higher *Buchnera* at 96h and lower at 144h (Fig. 4.3E). The increases in nutritional symbiont abundance may correlate with the emergence of wasp larvae (though line ZA29 shows increases earlier than 72h), and the declines may be associated with wasps consuming bacteriocytes later in development (Fig. 4.1).

In highly resistant aphid lines (Fig. 4.2: AS3, WA4, WA4-AB, ZA17, and ZA17-AB) where effects of parasitism on *Buchnera* might be diminished due to the death of

most wasps, we observed variable effects of parasitism on *Buchnera* gene copy number. For all of these lines, except ZA17 at 48h, we saw no significant increases in *Buchnera* in parasitized aphids (but several significant decreases) prior to 120h, and no significant decreases after this time point as seen in susceptible lines (Fig. 4.3A-C).

Table 4.3 GLM analyzing variables influencing defensive symbiont abundance.

Response Variable	Explanatory Variable	DF	F-ratio	Significance
a. *H. defensa* copy #	Clone-strain-APSE combination	3	648.6097	**<.0001**
	Time point	5	47.0438	**<.0001**
	Parasitism	1	40.1922	**<.0001**
	Clone-strain-APSE*Parasitism	3	19.1278	**<.0001**
b. APSE copy #	Clone-strain-APSE combination	3	563.3855	**<.0001**
	Time point	5	53.3787	**<.0001**
	Parasitism	1	0.5835	0.4455
	Clone-strain-APSE*Parasitism	3	16.8615	**<.0001**
c. APSE/*H. defensa* ratio	Clone-strain-APSE combination	3	81.6996	**<.0001**
	Time point	5	18.2991	**<.0001**
	Parasitism	1	91.0196	**<.0001**
	Clone-strain-APSE*Parasitism	2	1.2574	0.2857

Statistically significant p-values are bolded. Individual models are separated by solid lines. All models p<.0001 (Full models available in Supplemental table 4.3)

Effects of parasitism on *H. defensa* abundance and APSE genomic copy number

Overall, we found that *H. defensa* and APSE abundance increased over time and that genomic copy numbers of APSE exceeded those of *H. defensa*. Across all lines, *H. defensa* abundance was influenced by parasitism and clone-strain-APSE combination (and the interaction between the two), while APSE copy number was largely influenced by clone-strain-APSE combination (Table 4.3). We also found significant effects of parasitism on the copy number of defensive elements at particular time points following parasitism. Given the many effects that parasitism is likely to exert on the host environment (e.g. host immune responses, effects on aphid development, fecundity) it is perhaps not surprising that its effects on defensive symbionts were not consistent across all time points. However, in general, parasitism leads to increases in APSE abundance,

which are frequently followed by decreases in *H. defensa* titers at later time points (Fig. 4.4). The ratio of APSE/*H. defensa* (Fig. 4.4 bar graphs) is higher in parasitized aphids in almost all instances (significantly so in many). Despite some exceptions at particular time points, this general pattern was observed in all four *H. defensa*-infected lines (Fig. 4.4).

Linear regressions (Supplemental Fig. 4.2) used to describe the relationship of *H. defensa* and APSE revealed that increases in the APSE/*H. defensa* ratio corresponded to significant increases in APSE for all lines except AS3 (Supplemental Fig. 4.2 column A) and significant decreases in *H. defensa* for all lines (Supplemental Fig. 4.2 column B).

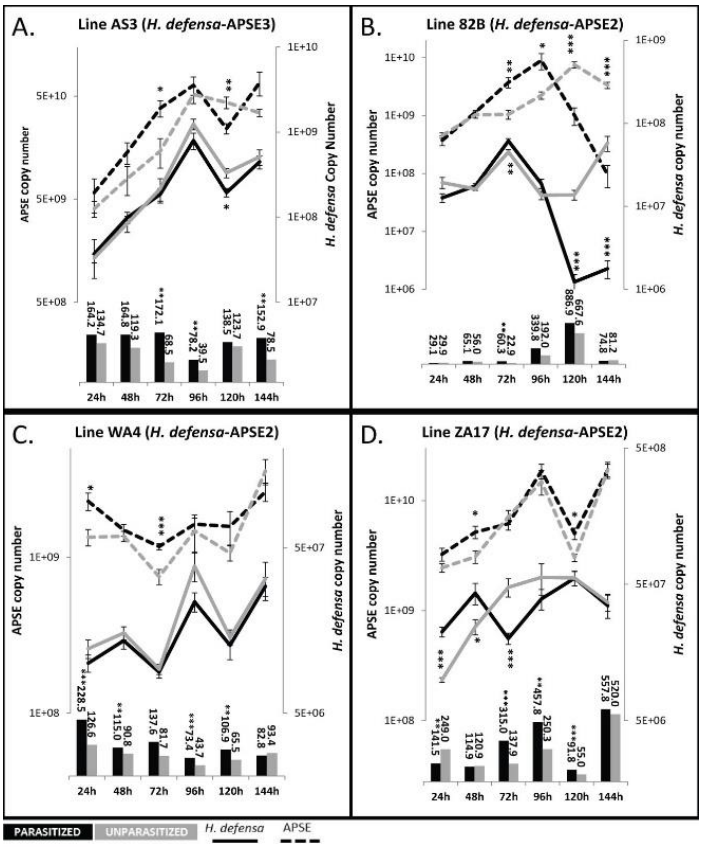


Figure 4.4 *H. defensa* and APSE copy numbers between parasitized and unparasitized aphids over time. APSE/*H. defensa* ratio is represented by bar graphs. Ratio means and significance levels (for parasitized vs. unparasitized treatments at each time point) are placed above each bar. *≤.05, **<.01, ***<.001

Line AS3 (*H. defensa*-APSE3) showed complete resistance in our parasitism assays (Fig. 4.2) and early mortality (between 24-48h) (Fig. 4.1, Supplemental Fig. 4.1) to developing wasps in our serial dissections, so we expected to see the largest effects of parasitism on the protective symbionts prior to, or concomitant with, these time-points. However, significant increases in APSE copy number were not seen until 72h after parasitism (Fig. 4.4A), after the parasitoid has perished in this line.

Line 82B (*H. defensa*-APSE2-*cdtB1*) was moderately resistant to parasitism (Fig. 4.2), and parasitoids survived at least five days after oviposition. We thus expected effects of parasitism to persist throughout our measurement period. In this line, *H. defensa* and APSE copy numbers both significantly increased at 72h, but the rate of APSE increase exceeded that of *H. defensa* as seen by the significantly higher ratio of APSE/*H. defensa* (Fig. 4.4B). APSE copy number continues to increase at 96h likely leading to the crash in *H. defensa* and subsequent crash in APSE.

Lines WA4 and ZA17 (*H. defensa*-APSE2-*cdtB2*) exhibited high resistance to parasitism (Fig. 4.2) and late wasp mortality (Fig. 4.1, Supplemental Fig. 4.1) in parasitized aphids. Here we found significantly higher APSE/*H. defensa* in parasitized aphids at 24, 48, 96, and 120h for line WA4 and 72, 96, and 120h in line ZA17 (Fig. 4.4 C&D), despite substantial resistance contributed by the host genotype and not the APSE2 *H. defensa* in these lines.

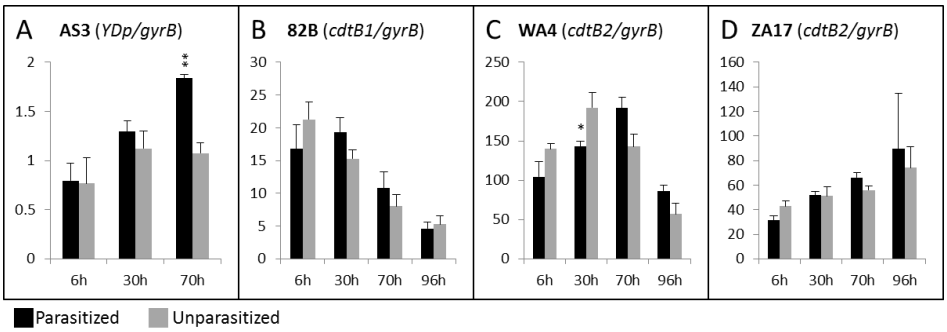


Figure 4.5 APSE toxin expression relative to *H. defensa gyrB* in parasitized versus unparasitized aphids at several time-points after parasitism.

Effect of parasitism on toxin expression

RT-qPCR assays of phage-associated toxins in parasitized versus non-parasitized *A. pisum* revealed that toxin expression levels are not generally elevated in response to parasitism. In the *H. defensa*-APSE2 lines (82B, WA4, and ZA17), for example, we found no significant increases in *cdtB* expression (Fig. 4.5 B, C, D). The highly resistant aphid line AS3, on the other hand, did show a significant increase in *YDp* expression in parasitized aphids at 70h (Fig. 4.5A), but again this increase occurs after the mortality of the developing wasp and hence is unlikely to contribute to the protective phenotype. All toxin expression comparisons are available in Supplemental Figure 4.4.

4.4 Discussion

There is increasing awareness of the roles of heritable symbionts in influencing interactions with their hosts' natural enemies (Brownlie & Johnson 2009; Jaenike 2012; Oliver & Moran 2009). In *A. pisum*, the obligate nutritional symbiont *Buchnera* and the facultative protective symbiont *H. defensa* are both likely important in determining whether parasitism by the endoparasitic wasp, *A. ervi*, is successful. Here we show that parasitism, in turn, affects these aphid symbioses, albeit often in unpredicted ways.

Aphid-encoded parasitoid resistance and potentially non-protective *H. defensa* strains

Our most surprising result was the identification of two symbiont-free (after antibiotic treatment) *A. pisum* genotypes (WA4 and ZA17) that are resistant to the parasitoid *A. ervi* (Fig. 4.2). Work prior to the discovery of protective heritable bacterial symbionts in aphids showed that *A. pisum* exhibited substantial variation in resistance to the wasp *A. ervi* (Henter & Via 1995). Later studies, however, found that the bulk of the variation in protection appeared due to *H. defensa* infection (with APSE) with numerous uninfected clones exhibiting high susceptibility to parasitism by *A. ervi* (Ferrari *et al.* 2004; Nyabuga *et al.* 2010; Oliver *et al.* 2009; Oliver *et al.* 2005). Aphids typically have a weak encapsulation response, (Bensadia *et al.* 2006; Carver & Sullivan 1988; Laughton *et al.* 2011) which is the cellular innate response insects employ to deal with large invading entities, including parasitoids. Our finding of highly resistant aphid genotypes suggests encapsulation strength or other innate mechanisms of defense vary substantially among aphid clones and impact resistance to natural enemies. We are currently investigating how symbiont-free, but resistant aphids, successfully deter parasitism. The presence of innate defense in aphids may also partially explain why the protective symbiont, *H. defensa,* remains at intermediate frequencies in natural populations (Weldon *et al.* 2013) as these aphids would benefit less from infection, while potentially incurring infection costs (Oliver et al. 2008). Based on aforementioned published reports, it appears that symbiont-based protection is more common than host-based protection in *A. pisum*, however further work is needed to confirm this. If both are common, then correlative field studies, such as those attempting to link symbiont infection frequencies and

parasitism rates will be difficult without markers that also readily identify host-based resistance.

We also discovered two strains of APSE2 *H. defensa* that conferred no additional protection against the wasp *A. ervi* beyond the innate protection attributable to the aphid genotypes WA4 and ZA17. Other than APSE-free strains, all *H. defensa* examined to date in *A. pisum* conferred at least some protection to parasitism (Oliver et al. 2003, 2005, 2009). We caution, however, that it is possible that these strains do confer protection in more susceptible aphid clones and assays are underway to determine the protective phenotype of these strains. We sequenced the phage-associated toxin (*cdtB*), which has been hypothesized to be at least partially responsible for causing mortality to wasps (e.g. Moran et al. 2005) and discovered a distinct allele present in the putatively non-resistant strains WA4 and ZA17, relative to the protective wildtype (Supplemental Fig. 4.3). If these strains are non-protective, then these changes in the *cdtB* allele may underlie the loss of protection, or may simply correlate with other changes on the phage and bacterial chromosomes responsible for the phenotype. Non-protective *H. defensa* strains have been reported in other aphid hosts, for example, APSE-carrying *H. defensa* conferred no resistance to parasitism in the grain aphid *Sitobion avenae* (Łukasik *et al.* 2013a). Non-protective *H. defensa* strains are presumably maintained in natural populations by conferring different benefits, such as thermal protection (Russell & Moran 2006) or defense against other enemies. Some strains of the aphid symbiont *Regiella*, for example, confer protection against fungal pathogens (Parker *et al.* 2013; Scarborough *et al.* 2005), while others protect against parasitoids (Vorburger *et al.* 2010). It is also interesting that these two distinctive *H. defensa* strains were found in resistant aphid genotypes. It is

possible that relaxed selection to maintain defensive properties could result in the loss of phenotype. These findings illustrate the importance of experimental studies capable of isolating effects owing to host genotype versus symbiont strain as, for example, resistance in *H. defensa*-infected aphid lines cannot be automatically attributed to infection.

Effect of parasitism on *H. defensa*, APSE, and toxin expression

We found that parasitism significantly affects abundances of the defensive elements *H. defensa* and its bacteriophage APSE. Our qPCR-based estimates show that parasitism generally led to an increase in APSE copy number and a decrease in *H. defensa* abundance. The APSE/*H. defensa* ratio, for example, was almost always higher in parasitized aphids and was significantly higher at several time points in all four lines examined (Fig. 4.4). Linear regression models (Supplemental Fig. 4.2) showed that increases in this ratio are due to both relative increases in APSE and decreases in *H. defensa* abundance in parasitized aphids. While little is currently known about the specific mechanisms underlying *H. defensa*-conferred protection, these observed decreases in *H. defensa* are consistent with the hypothesis (Moran *et al.* 2005) that toxin production may be regulated by APSEs, and delivery achieved by bacteriophage-mediated lysis of a sub-set of *H. defensa* cells – similar to what occurs with some shiga toxin-producing pathogenic strains of *Escherichia coli* (e.g. 0157:H7) (Wagner *et al.* 2001; Wagner & Waldor 2002). Given that phage are known to undergo lysis in response to hostile host environments (Little 1996; Lwoff 1953; Roberts 1983), parasitism could conceivably induce this cycle. In line 82B, for example, increases in APSE over the first 96h after parasitism, led to a dramatic decline in *H. defensa* and subsequent crash in

APSE (Fig. 4.4B), consistent with lytic activity and prior work showing that APSEs can influence symbiont abundance (Weldon *et al.* 2013).

Surprisingly, we observed increases in APSE/*H. defensa* in all strains at two or more time points > 72h post parasitism –times which correspond with the emergence and development of wasp larvae and teratocytes – even though some strains appear to confer no additional protection (Fig. 4.4). However, if protective symbionts were specifically mobilized to battle emerging larvae, then we would only expect this response for the moderately protective line 82B, which confers moderate protection with late mortality. Wasps are present at these times in lines WA4 and ZA17, but given that these strains of *H. defensa* offer no additional protection in their native, innately protective aphid hosts, we would not expect increases unless these strains offer redundant protection or the response is vestigial. Finally, wasp mortality occurs prior to 72h in line AS3, yet we only see significant increases in APSE/*H. defensa* at 72h and beyond (Fig. 4.4A). We did detect non-significant increases at 24 and 48h and there may be a lag between biological events occurring in vivo and our qPCR-based estimates, which measure genomic copy including DNA from dead bacterial cells.

We also investigated whether parasitism would result in increased levels of APSE-associated toxin expression, which could influence the protective phenotype. While prior work found that both the *cdtB* toxin homolog (APSE2) and *YDp* (APSE3) are constitutively expressed at high levels (Degnan & Moran 2008; Oliver *et al.* 2009), we found that parasitism generally had little effect on toxin expression (Fig. 4.5). We identified a significant increase in *YDp* expression in line AS3 only at 70h after parasitism (Fig. 4.5A), yet serial dissections indicated that the parasitoid is deceased at

this time point (Supplemental Fig. 4.1). We also observed greater APSE3 abundance in parasitized aphids at this time point, but increased expression likely does not simply correlate with phage genomic copy as similar increases in APSE2 genomic copy in parasitized aphids did not result in increased *cdtB* expression for any of the APSE2 *H. defensa* lines. A caveat about our symbiont abundance and toxin expression assays is that they are based on whole-aphid extractions. However, symbiont responses to parasitism may not be systemic, as the wasp and its teratocytes are localized in the aphid abdomen (Falabella *et al.* 2000) and our global estimates may mask stronger local effects.

Effect of parasitism on the primary symbiont *Buchnera*

Overall, we found aphid genotype and age were the most important determinants of *Buchnera* genomic copy (Table 4.2a; Fig. 4.3), consistent with findings from prior work (Vogel & Moran 2011). We found parasitism also influences *Buchnera* genomic abundance – although not always in consistent ways. In the two lines most susceptible to parasitism (ZA29 & AS3-AB) we found significantly higher *Buchnera* genomic copy number in parasitized aphids at the time-points coinciding with the emergence of the parasitoid larvae and teratocytes (72h). Teratocytes are thought to process *Buchnera*-supplied nutrients that are redirected to a rapidly growing larva (Falabella *et al.* 2005; Falabella *et al.* 2000), so an increase in *Buchnera* at this time point may benefit the wasp. A similar pattern was observed from line 82B, which contains a strain of APSE2 *H. defensa* that confers moderate, but delayed protection (relative to APSE3 *H. defensa*), and which showed significantly higher *Buchnera* titers in parasitized aphids at 96h. Wasps usually complete development in the most susceptible lines (ZA29 &AS3-AB) and each is associated with decreases in *Buchnera* by day five or six possibly associated

with the consumption of bacteriocytes prior to wasp pupation. Thus, in susceptible aphids, our results are generally consistent with prior studies showing that parasitoids may protect the nutritional symbiosis (Cloutier & Douglas 2003; Falabella *et al.* 2000). Aphid lines ZA29 and 82B were infected with *Rickettsiella* and *H. defensa*, respectively, which suggests these particular strains were not capable of preventing the proposed wasp manipulation of *Buchnera.* The role of this *Rickettsiella* strain is also unknown, but our parasitism assays indicate it is unlikely to confer protection against parasitoids as this line was highly susceptible to parasitism (Fig. 4.2). Other reports show that other *Rickettsiella* strains confer protection against fungal pathogens (Łukasik *et al.* 2013b) and modify aphid body color (Tsuchida *et al.* 2010).

In most symbiont- and aphid-based resistant lines (AS3, WA4 & WA4-AB), however, parasitism resulted in no differences, or occasional reductions, in *Buchnera* gene copy prior to 120h (Fig. 4.3). This suggests that *H. defensa* or aphid-based factors may inhibit the parasitoid's ability to influence *Buchnera*, which in turn, may influence host suitability for developing wasps.

4.5 Conclusions

Here we show that parasitism by the wasp *A. ervi* influences the within-host dynamics of both an obligate nutritional symbiont and facultative protective bacteria in the aphid *A. pisum*. The observed effects of parasitism on symbiont abundance were complex, but perhaps this is unsurprising given that parasitism likely triggers important changes in host immune function, development, and other processes that also affect host-symbiont interactions. Parasites are well-known for manipulating host biology to create an environment suitable for development (Godfray 1994; Vinson & Iwantsch 1980), and

this manipulation likely extends to the microbial partners of the hosts as well. In turn, hosts and heritable symbionts, whose interests are aligned when attacked, may work in concert to thwart common enemies.

A majority of insect species are likely infected with heritable bacteria, and there is growing awareness that many of these mediate interactions with host natural enemies (Weldon *et al.* 2013). Hence, we expect that enemy challenge will affect the abundance and performance of heritable bacteria in many systems. More broadly, interactions between invertebrate animals and the heritable bacteria that mediate important ecological interactions are likely highly dynamic, with changes occurring in responses to enemy challenge, food plant quality, and abiotic factors, including temperature.

Acknowledgements

We would like to thank Kyungsun Kim for her technical support and Dr. Kim Love-Meyers for her statistical advice. We'd also like to thank Dr. Jacob Russell and his lab members at Drexel University for their review and critique of an early version of this paper. This project was supported by a National Science Foundation (IOS) grant no. 1050128 to K.M.O.

4.6 References

Angalet G, Fuester R (1977) The Aphidius parasites of the pea aphid Acyrthosiphon pisum in the eastern half of the United States. *Annals of the Entomological Society of America* **70**, 87-96.

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. In: *Annual Review of Microbiology*, pp. 155-189.

Beckage NE, Gelman DB (2004) Wasp parasitoid disruption of host development: Implications for new biologically based strategies for insect control. *Annual review of entomology* **49**, 299-330.

Bensadia F, Boudreault S, Guay J-F, Michaud D, Cloutier C (2006) Aphid clonal resistance to a parasitoid fails under heat stress. *Journal of insect physiology* **52**, 146-157.

Brownlie JC, Johnson KN (2009) Symbiont-mediated protection in insect hosts. *Trends in Microbiology* **17**, 348-354.

Carver M, Sullivan DJ (1988) Encapsulative defence reactions of aphids (Hemiptera: Aphididae) to insect parasitoids (Hymenoptera: Aphidiidae and Aphelinidae). . In: *Ecology and Effectiveness of Aphidophaga* (ed. Niemczyk E DA), pp. 299-303. SPB Academic Publishing, The Hague.

Cloutier C, Douglas AE (2003) Impact of a parasitoid on the bacterial symbiosis of its aphid host. *Entomologia Experimentalis Et Applicata* **109**, 13-19.

Degnan PH, Moran NA (2008) Diverse Phage-Encoded Toxins in a Protective Insect Endosymbiont. *Applied and Environmental Microbiology* **74**, 6782-6791.

Digilio MC, Isidoro N, Tremblay E, Pennacchio F (2000) Host castration by< i> Aphidius ervi</i> venom proteins. *Journal of insect physiology* **46**, 1041-1050.

Douglas AE (1989) Mycetocyte Symbiosis in Insects. *Biological Reviews of the Cambridge Philosophical Society* **64**, 409-434.

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria Buchnera. *Annual Review of Entomology* **43**, 17-37.

Douglas AE, Francois CLMJ, Minto LB (2006) Facultative 'secondary' bacterial symbionts and the nutrition of the pea aphid, Acyrthosiphon pisum. *Physiological Entomology* **31**, 262-269.

Eastop VF (1966) A Taxonomic Study of Australian Aphidoidea (Homoptera). *Australian Journal of Zoology* **14**, 399-592.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792-1797.

Engels WR, Johnson-Schlitz DM, Eggleston WB, Sved J (1990) High-frequency P element loss in Drosophila is homolog dependent. *Cell* **62**, 515-525.

Falabella P, Perugino G, Caccialupi P, *et al.* (2005) A novel fatty acid binding protein produced by teratocytes of the aphid parasitoid Aphidius ervi. *Insect Molecular Biology* **14**, 195-205.

Falabella P, Riviello L, Caccialupi P, *et al.* (2007) A gamma-glutamyl transpeptidase of Aphidius ervi venom induces apoptosis in the ovaries of host aphids. *Insect Biochemistry and Molecular Biology* **37**, 453-465.

Falabella P, Tremblay E, Pennacchio F (2000) Host regulation by the aphid parasitoid Aphidius ervi: the role of teratocytes. *Entomologia Experimentalis Et Applicata* **97**, 1-9.

Ferrari J, Darby AC, Daniell TJ, Godfray HCJ, Douglas AE (2004) Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology* **29**, 60-65.

Ferrari J, West JA, Via S, Godfray HCJ (2012) Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex *Evolution* **66**, 375-390.

Fukatsu T, Nikoh N, Kawai R, Koga R (2000) The secondary endosymbiotic bacterium of the pea aphid Acyrthosiphon pisum (Insecta : Homoptera). *Applied and Environmental Microbiology* **66**, 2748-2758.

Godfray HCJ (1994) *Parasitoids: Behavioral and Evolutionary Ecology* Princeton University Press, Princeton.

He XZ (2008) Reproductive behaviour of *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae). *Massey University* **Dissertation**.

Henter HJ, Via S (1995) The potential for coevolution in a host-parasitoid system 1. Genetic variation within a an aphid population in susceptibility to a parasitic wasp. *Evolution* **49**, 427-438.

Jaenike J (2012) Population genetics of beneficial heritable symbionts. *Trends in Ecology & Evolution* **27**, 226-232.

Komaki K, Ishikawa H (1999) Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. *Journal of Molecular Evolution* **48**, 717-722.

Komaki K, Ishikawa H (2000) Genomic copy number of intracellular bacterial symbionts of aphids varies in response to developmental stage and morph of their host. *Insect Biochemistry and Molecular Biology* **30**, 253-258.

Lamb R, Pointing P (1972) Sexual morph determination in the aphid,< i> Acyrthosiphon pisum</i>. *Journal of Insect Physiology* **18**, 2029-2042.

Laughton AM, Garcia JR, Altincicek B, Strand MR, Gerardo NM (2011) Characterisation of immune responses in the pea aphid,< i> Acyrthosiphon pisum</i>. *Journal of Insect Physiology* **57**, 830-839.

Little JW (1996) *The SOS regulatory system* R. G. Landes Co. {a}, Austin, Texas, USA.

Łukasik P, Dawid MA, Ferrari J, Godfray HCJ (2013a) The diversity and fitness effects of infection with facultative endosymbionts in the grain aphid, *Sitobion avenae*. *Oecologia* **Accepted**.

Łukasik P, van Asch M, Guo HF, Ferrari J, Godfray HCJ (2013b) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters* **16**, 214-218.

Lwoff A (1953) LYSOGENY. *Bacteriological Reviews* **17**, 269-337.

Moran NA, Degnan PH (2006) Functional genomics of Buchnera and the ecology of aphid hosts. *Molecular Ecology* **15**, 1251-1261.

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16919-16926.

Nishikori K, Morioka K, Kubo T, Morioka M (2009) Age- and morph-dependent activation of the lysosomal system and Buchnera degradation in aphid endosymbiosis. *Journal of Insect Physiology* **55**, 351-357.

Nyabuga FN, Outreman Y, Simon JC, Heckel DG, Weisser WW (2010) Effects of pea aphid secondary endosymbionts on aphid resistance and development of the aphid parasitoid Aphidius ervi: a correlative study. *Entomologia Experimentalis Et Applicata* **136**, 243-253.

Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative Symbionts in Aphids and the Horizontal Transfer of Ecologically Important Traits. In: *Annual review of entomology*, pp. 247-266.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325**, 992-994.

Oliver KM, Moran NA (2009) Defensive symbionts in aphids and other insects In: *Defensive mutualism in microbial symbiosis* (eds. White JF, Torres MS). Taylor & Francis, London.

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences* **102**, 12795-12800.

Oliver KM, Moran NA, Hunter MS (2006) Costs and benefits of a superinfection of facultative symbionts in aphids. *Proc Biol Sci* **273**, 1273-1280.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1803-1807.

Parker BJ, Spragg CJ, Altincicek B, Gerardo NM (2013) Symbiont-mediated protection against fungal pathogens in pea aphids: a role for pathogen specificity? *Applied and Environmental Microbiology* **79**, 2455-2458.

Pennacchio F, Fanti P, Falabella P, *et al.* (1999) Development and nutrition of the braconid wasp, Aphidius ervi in aposymbiotic host aphids. *Archives of Insect Biochemistry and Physiology* **40**, 53-63.

Pennacchio F, Strand MR (2006) Evolution of developmental strategies in parasitic hymenoptera. In: *Annual Review of Entomology*, pp. 233-258.

Rahbe Y, Digilio MC, Febvay G, *et al.* (2002) Metabolic and symbiotic interactions in amino acid pools of the pea aphid, Acyrthosiphon pisum, parasitized by the braconid Aphidius ervi. *Journal of Insect Physiology* **48**, 507-516.

Roberts J (1983) Lysogenic Induction. *Lambda II*, 123-144.

Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society B-Biological Sciences* **273**, 603-610.

Russell JA, Weldon S, Smith AH, *et al.* (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. *Molecular Ecology*.

Sandstrom JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. *Molecular Ecology* **10**, 217-228.

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. *Science* **310**, 1781-1781.

Tsuchida T, Koga R, Horikawa M, *et al.* (2010) Symbiotic bacterium modifies aphid body color. *Science* **330**, 1102-1104.

van der Wilk F, Dullemans AM, Verbeek M, van den Heuvel J (1999) Isolation and characterization of APSE-1, a bacteriophage infecting the secondary endosymbiont of Acyrthosiphon pisum. *Virology* **262**, 104-113.

Vinson SB, Iwantsch G (1980) Host regulation by insect parasitoids. *Quarterly Review of Biology*, 143-165.

Vogel KJ, Moran NA (2011) Effect of Host Genotype on Symbiont Titer in the Aphid—Buchnera Symbiosis. *Insects* **2**, 423-434.

Vorburger C, Gehrer L, Rodriguez P (2010) A strain of the bacterial symbiont Regiella insecticola protects aphids against parasitoids. *Biology Letters* **6**, 109-111.

Wagner PL, Neely MN, Zhang X, *et al.* (2001) Role for a Phage Promoter in Shiga Toxin 2 Expression from a Pathogenic Escherichia coliStrain. *Journal of bacteriology* **183**, 2081-2085.

Wagner PL, Waldor MK (2002) Bacteriophage control of bacterial virulence. *Infection and Immunity* **70**, 3985-3993.

Weldon SR, Strand MR, Oliver KM (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. *Proceedings of the Royal Society B-Biological Sciences* **280**.

Wilkinson T, Douglas A (1998) Host cell allometry and regulation of the symbiosis between pea aphids,< i> Acyrthosiphon pisum</i>, and bacteria,< i> Buchnera</i>. *Journal of Insect Physiology* **44**, 629-635.

Zientz E, Dandekar T, Gross R (2004) Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiology and Molecular Biology Reviews* **68**, 745-+.

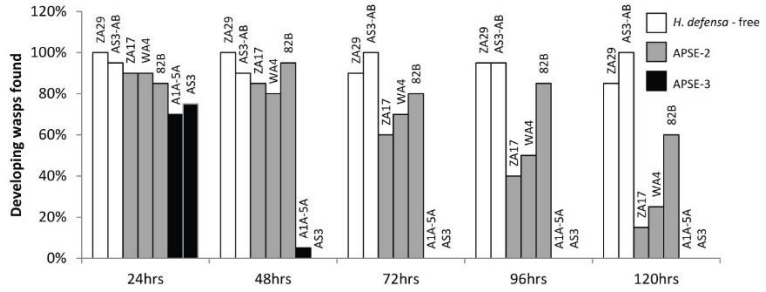
Data Accessibility

The CdtB2 allele sequence was deposited in GenBank under accession number KF551594.

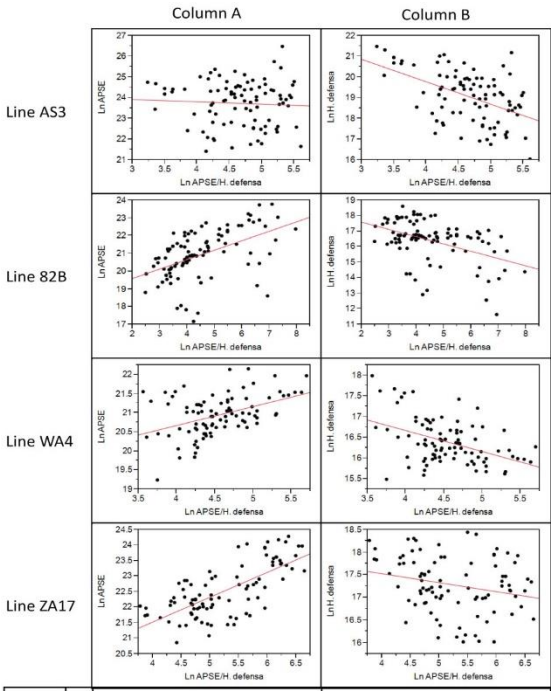
The CdtB sequence alignment, full General Linear Models, and gene expression data are available as supporting online information. The FASTA CdtB sequence alignment file, qPCR-based abundance, RT-qPCR expression and parasitism assay data are deposited in Dryad doi:10.5061/dryad.89c3r

Author Contributions

A.M. and K.O. designed the experiments and wrote the manuscript. A.M. performed the experiments and statistical analyses and created all figures and tables. S.W. performed the *cdtB* allele sequencing and alignments.

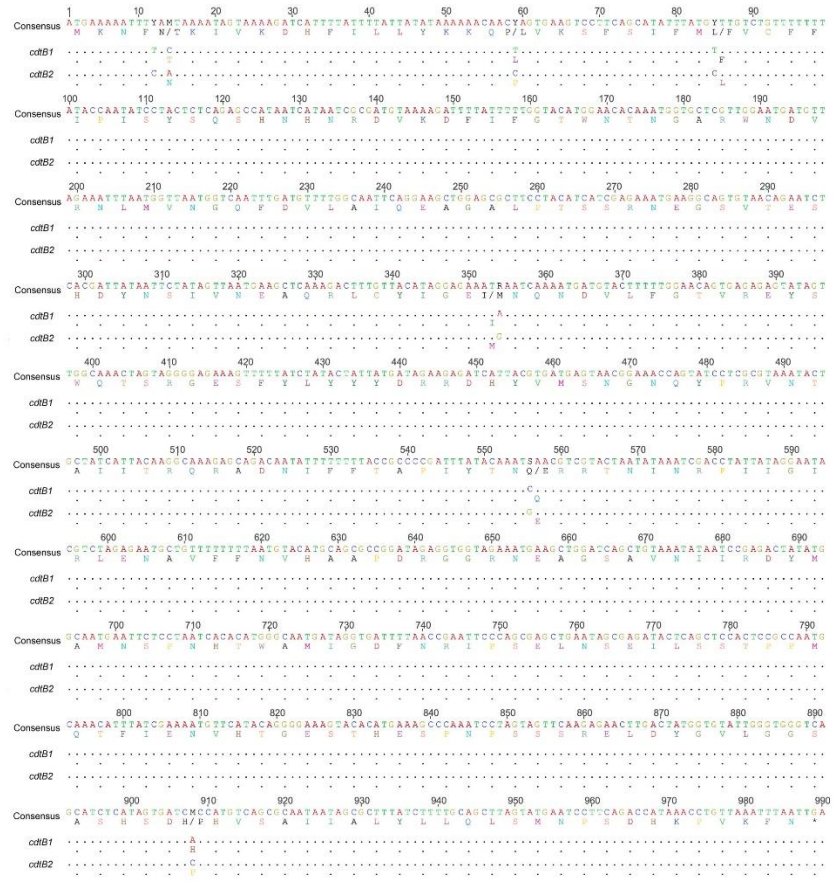


Supplemental Figure 4.1: Parasitized aphid dissections, showing percent aphids found to contain live-developing wasps.

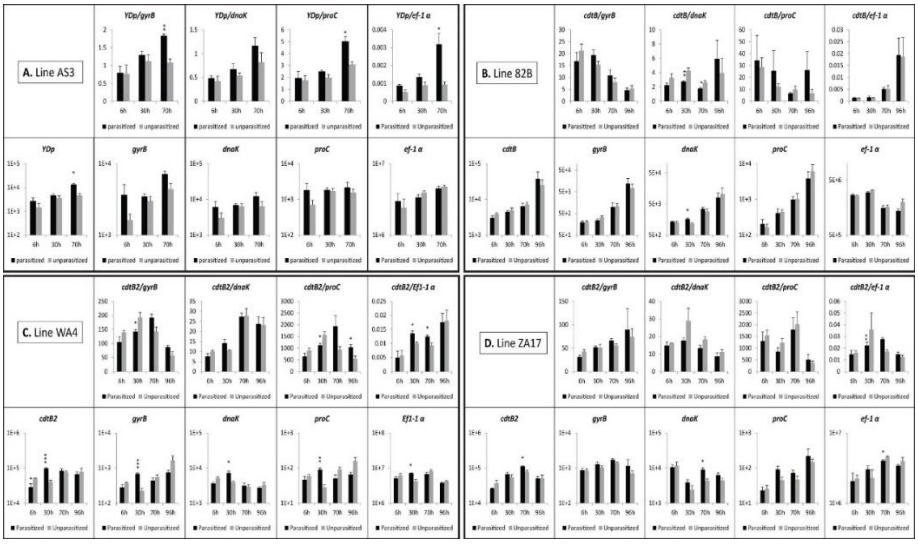


	DF	Column A F Ratio	Column A p-value	Column B F Ratio	Column B p-value
AS3	1	0.2884	0.5926	27.1373	<.0001*
82B	1	31.2362	<.0001*	24.2362	<.0001*
WA4	1	20.7801	<.0001*	21.763	<.0001*
ZA17	1	87.4907	<.0001*	5.4059	0.0223*

Supplemental Figure 4.2 Linear regression of Ln APSE by Ln APSE/*H. defensa* (Column A). Linear regression of Ln *H. defensa* by Ln APSE/*H. defensa* (Column B).



Supplemental Figure 4.3 Alignment of 82B's *cdtB1* (GenBank accession KF551594) to ZA17 and WA4's *cdtB2* allele. Identity is indicated by a dot.



Supplemental Figure 4.4 Expression of APSE putative toxins (YDp and cdtB) relative to four reference genes (top row). Absolute quantification of toxin expression and reference gene expression (bottom row). * < .05, ** < .01, *** < .001

Supplemental Table 4.1 Target genes and primer sequences for qPCR assays.

Target Gene : source organism	Primer sequence 5' - 3'	Primer & Reaction Citation
P2 : bacteriophage APSE	F: GTC CAG GCA TTA TTA GCG C	(Moran *et al.* 2005a)
	R: TTT TTC TAA GGC AAC CAT	
P28 : bacteriophage APSE	F: TGA TAA AAG CGG ATA ATG CC	(Moran et al. 2005a)
	R: GCG TTT GTC ATA CTG AAA AGG	
cdtB : APSE-2 putative toxin	F: ATA TTT TTT TTA CCG CCC CG	(Moran et al. 2005a)
	R: CCA GCT TCA TTT CTA CCA CCT C	
ydp : APSE-3 putative toxin	F: CGC CCA CGC CCT CAA CGA TT	(Weldon et al. 2013)
	R: CTG GCC GGC CTT TGA CCA GG	
dnaK : Hamiltonella defensa	F: GGT TCA GAA AAA AGT GGC AG	(Moran et al. 2005a)
	R: CGA GCG AAA GAG GAG TGA	
ef-1 α : Acyrthosiphon pisum	F: CTG ATT GTG CCG TGC TTA TTG	(Wilson et al. 2006)
	R: TAT GGT GGT TCA GTA GAG TCC	
gyrB : Hamiltonella defensa	F: TGG GAG AAA TGA ATC CGG GC	this study
	R: TAC CGC ATC TCC CAT CAA CG	
dnaK : Buchnera aphidicola	F: ACG TTC ACC CTG AAG CAC GTG TA	this study
	R: TGG AGC TGC AGT ACA GGG AGG A	
proC : Hamiltonella defensa	F: TGT CGG TAA GGT TTG CTG GT	this study
	R: TAA ACG GGC CCT TTC CTG AC	

Supplemental Table 4.2 General Linear Models (GLMs) analyzing variables affecting *Buchnera* symbiont densities. All models p < .0001

Aphid Lines	Explanatory Variable	DF	F-ratio	Significance
a. ALL LINES (unparasitized)	whole model	11	9.3538	**<.0001**
	time point	5	15.1741	**<0.0001**
	H. defensa infection	1	5.8485	**0.0161**
	time point*_H. defensa_ infection	5	3.9421	**0.0017**
a. ALL LINES (unparasitized)	whole model	47	47.2417	**<.0001**
	Aphid line	7	189.7857	**<.0001**
	Time point	5	84.6112	**<.0001**
	Aphid line*Time point	35	13.6526	**<.0001**
b. ALL LINES (parasitized and unparasitized)	whole model	47	27.5565	**<.0001**
	Clone-strain-APSE combination	3	161.5593	**<.0001**
	time point	5	50.5681	**<.0001**
	parasitism	1	0.1879	0.665
	Clone-strain-APSE*time point	15	27.9104	**<.0001**
	Clone-strain-APSE*parasitism	3	3.7223	**0.0118**
	time point*parasitism	5	7.6176	**<.0001**
	Clone-strain-APSE*time point*parasitism	15	5.6417	**<.0001**
c. AS3 & AS3-AB	whole model	23	11.374	**<.0001**
	time point	5	40.027	**<.0001**
	parasitism	1	0.3627	0.5472
	H. defensa infection	1	15.2398	**0.0001**
	time point*parasitism	5	1.9011	0.092
	time point*_H. defensa_ infection	5	6.9277	**<.0001**
	parsitism*_H.defensa_ infection	1	0.3026	0.5824
	time point*parasitism*_H. defensa_ infection	5	0.448	0.8148
d. WA4 & WA4-AB	whole model	23	36.7636	**<.0001**
	time point	5	143.6317	**<.0001**
	parasitism	1	1.4452	0.231
	H. defensa infection	1	1040.415	**<.0001**
	time point*parasitism	5	4.9066	**0.0003**
	time point*_H. defensa_ infection	5	40.9011	**<.0001**
	parsitism*_H.defensa_ infection	1	0.4015	0.5272
	time point*parasitism*_H. defensa_ infection	5	1.3877	0.2315
e. ZA17 & ZA17-AB	whole model	23	29.4836	**<.0001**
	time point	5	46.3745	**<.0001**
	parasitism	1	16.1864	**<.0001**
	H. defensa infection	1	70.6055	**<.0001**
	time point*parasitism	5	2.4935	**0.0334**
	time point*_H. defensa_ infection	5	70.7602	**<.0001**
	parsitism*_H.defensa_ infection	1	0.1592	0.6592
	time point*parasitism*_H. defensa_ infection	5	1.8004	0.1159
f. 82B	whole model	11	6.2569	**<.0001**
	time point	5	11.7091	**<.0001**
	parasitism	1	5.4592	**0.0207**
	time point*parasitism	5	0.769	0.5734
g. ZA29	whole model	11	35.7848	**<.0001**
	time point	5	57.8017	**<.0001**
	parasitism	1	6.7133	**0.0113**
	time point*parasitism	5	20.1029	**<.0001**

All models p < .0001

Supplemental Table 4.3 GLMs analyzing variables influencing defensive symbiont abundances.

Response Variable	Explanatory Variable	DF	F-ratio	Significance
a. *H. defensa* copy #	whole model	47	67.1512	**<.0001**
	Clone-strain-APSE combination	3	648.6097	**<.0001**
	Time point	5	47.0438	**<.0001**
	Parasitism	1	40.1922	**<.0001**
	Clone-strain-APSE*Time point	15	36.6285	**<.0001**
	Clone-strain-APSE*Parasitism	3	19.1278	**<.0001**
	Time point*Parasitism	5	13.7067	**<.0001**
	Clone-strain-APSE*Time point*Parasitism	15	12.8087	**<.0001**
b. APSE/*H. defensa* ratio	whole model	47	38.678	**<.0001**
	Clone-strain-APSE combination	3	81.6996	**<.0001**
	Time point	5	18.2991	**<.0001**
	Parasitism	1	91.0196	**<.0001**
	Clone-strain-APSE*Time point	15	86.2355	**<.0001**
	Clone-strain-APSE*Parasitism	3	2.3102	0.0762
	Time point*Parasitism	5	9.048	**<.0001**
	Clone-strain-APSE*Time point*Parasitism	15	2.6701	**0.0008**
c. APSE copy #	whole model	47	55.0967	**<.0001**
	Clone-strain-APSE combination	3	563.3855	**<.0001**
	Time point	5	53.3787	**<.0001**
	Parasitism	1	0.5835	0.4455
	Clone-strain-APSE*Time point	15	21.0745	**<.0001**
	Clone-strain-APSE*Parasitism	3	16.8615	**<.0001**
	Time point*Parasitism	5	15.7494	**<.0001**
	Clone-strain-APSE*Time point*Parasitism	15	11.5249	**<.0001**

Statistically significant p-values are bolded. Individual models are separated by solid lines. All models $p < .0001$

CHAPTER 5

SPECIALIZATION OF MULTI-MODAL APHID DEFENSES AGAINST TWO PARASITIC WASPS [4]

[4] Martinez AJ, Kim KL, Harmon JP, and Oliver KM. Submitted to *Ecology*, 8/10/2015.

Abstract

Insects are often attacked by multiple natural enemies, imposing dynamic selective pressures for the development and maintenance of enemy-specific resistance. Pea aphids (*Acyrthosiphon pisum*) have emerged as models for the study of variation in resistance to parasitoid wasps and resistance against their most common enemy, *Aphidius ervi*, is sourced through both intrinsic mechanisms and infection with the heritable bacterial symbiont, *Hamiltonella defensa*. In North America, introductions of *A. ervi* appear to have excluded other previously common parasitoid competitors, yet the related aphidiine braconid parasitoid, *Praon pequodorum*, continues to persist on this host. Previous studies have compared competitive traits between both parasitoids, but have not examined specialization of host resistance as a factor potentially influencing *P. pequodorum*'s persistence. Using an array of experimental aphid lines, we examined whether intrinsic- and symbiont-based resistance varied in effectiveness toward these two wasps. We find all aphid lines that are protected from *A. ervi* by either infection with *H. defensa* or aphid-based mechanisms are highly susceptible to attack by *P. pequodorum*. Moreover, we show that *P. pequodorum* suffers no sublethal fitness costs for developing in lines that are highly resistant to *A. ervi*. Together, these results indicate that aphids resistant to *A. ervi* likely serve as a reservoir of hosts available only to *P. pequodorum*, hence contributing to its persistence in field populations despite being a poor external competitor. Further comparison of both wasp species' early development within aphids reveals that *P. pequodorum*'s eggs are more thickly-chorionated and hatch about two days later than those of *A. ervi*. Given that mortality in resistant lines typically occurs prior to the hatching of the more protected *P. pequodorum* eggs suggests this wasp's

developmental trajectory may influence its ability to overcome host defenses. Our results suggest that defensive symbionts and host-encoded factors that target specific natural enemies likely influence the composition of natural enemies attacking a particular host. In turn, natural enemy compositions are also likely to influence aphid and symbiont diversity resulting overall in the maintenance of enhanced biological diversity.

5.1 Introduction

Host-parasitoid interactions are ubiquitous, consisting of a parasite which kills its host as a prerequisite for completing development (Godfray 1994), thus imposing strong selective pressures on both parties to survive the antagonistic interaction (Kraaijeveld *et al.* 1998). Insect hosts often deploy the cellular arm of their innate immune system to encapsulate and asphyxiate internally developing parasitoids (Lavine & Strand 2002; Strand 2008; Strand & Pech 1995) or engage in defensive mutualisms with microbial symbionts for protection (Oliver & Martinez 2014). Parasitoids, in turn, have evolved specific tactics (e.g. venom, teratocytes, polydnaviruses) to overcome host- or symbiont-derived defenses, and commandeer resources, in an effort to create a suitable environment for wasp development (Beckage & Gelman 2004; Cloutier & Douglas 2003; Martinez *et al.* 2014b; Oliver *et al.* 2012). The evolution of host resistance and wasp counter-resistance can result in the specialization of traits that mediate host-parasitoid interactions (Kraaijeveld & Godfray 2009). Given that insect hosts are often attacked by multiple parasitoid species, e.g. (Asplen *et al.* 2014; Fisher 1961; Price 1972; Smith 1929; Starý 2006), hosts may vary in resistance to particular natural enemies, e.g. (Asplen *et al.* 2014; Cayetano & Vorburger 2015). Such differences in resistance may occur locally or globally and, especially when multiple parasitoids are present, impact or

depend on the dynamics of competing parasitoids, ultimately influencing other factors such as composition of natural enemies, enemy and host abundance, and evolutionary history of interacting parasitoids and their respective host species.

Aphids have emerged as important models for the study of variation in resistance to parasitoids, including defensive symbiosis (Vorburger 2014). The pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae) - *Aphidius ervi* (Hymenoptera: Braconidae: Aphidiinae) interaction is particularly well-studied. The pea aphid is a polyphagous pest of herbaceous legumes such as alfalfa and clover and its dominant parasitoid in North America is *A. ervi* (Angalet & Fuester 1977; Danyk 1992; McBrien & Mackauer 1991; Thiboldeaux *et al.* 1987). Pea aphids, however, maintain near maximum variation (0 to nearly 100%) in their susceptibility to *A. ervi* (Henter & Via 1995; Martinez *et al.* 2014a; Oliver *et al.* 2009).

Given that pea aphids have a weak cellular encapsulation response to parasitoids (Bensadia *et al.* 2006; Carver *et al.* 1988; Laughton *et al.* 2011) it was recently assumed that the bulk of their variation in resistance was owed to infection with the defensive bacterial endosymbiont, *Hamiltonella defensa* (Oliver *et al.* 2009; Oliver *et al.* 2005; Oliver *et al.* 2003), however, more recent work indicates that resistance can also be derived from intrinsic aphid-encoded mechanisms (Martinez *et al.* 2014a). Little is known about how innate or symbiont-based aphid defenses harm wasps, however, toxin-encoding bacteriophages, called APSEs, are required to infect *H. defensa* to produce the defensive mutualism (Oliver *et al.* 2009). There are multiple strains of *H. defensa*-APSE, but *H. defensa* containing either APSE2 or APSE3 are found most commonly among North American pea aphids and are associated with moderate to high protection,

respectively, against parasitism by *A. ervi* (Martinez *et al.* 2014b; Oliver *et al.* 2012). Based on previous developmental assays, APSE3 strains appear to kill developing *A. ervi* shortly after egg hatching, while mortality caused by APSE2 strains is more variable, but generally later in wasp development (Martinez *et al.* 2014b).

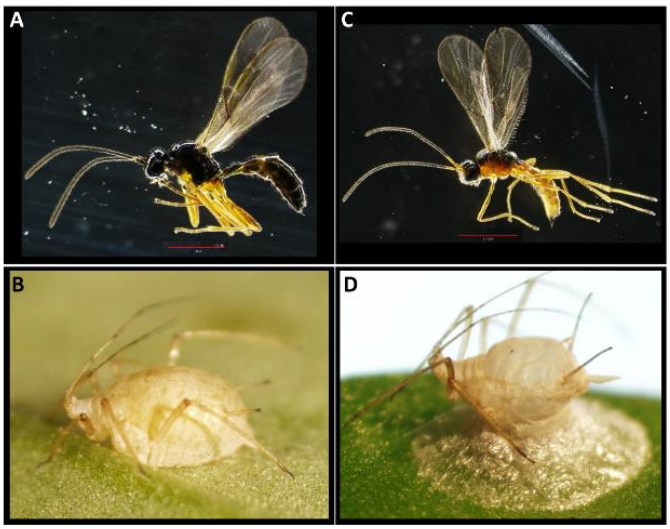


Figure 5.1 (A,B) Adult female *Aphidius ervi* and aphid mummy. (C,D) Adult female *Praon pequodorum* and aphid mummy.

Since its introduction, *A. ervi* has become the dominant parasitoid of NA pea aphids while the abundance of other, once common, parasitoids have been reduced or completely eliminated (Angalet & Fuester 1977; Danyk 1992; McBrien & Mackauer 1991; Thiboldeaux *et al.* 1987). For example, another introduced biological control agent, *Aphidius smithi* was largely, if not completely, displaced by *A. ervi* (Angalet & Fuester 1977; Halfhill *et al.* 1972), while a species native to NA, *Praon pequodorum* (Hymenoptera: Braconidae: Aphidiinae) (Fig. 5.1 c & d), a once abundant parasitoid of pea aphids, retains viable, although diminished populations (Schellhorn *et al.* 2002). The elimination of parasitoid species other than *A. ervi* attacking NA pea aphids is likely a function of competitive exclusion (Danyk 1992; Schellhorn *et al.* 2002) and several

studies have provided explanations for the low-level persistence of *P. pequodorum*. First, experimental bioassays indicated that *P. pequodorum* larvae internally outcompeted *A. ervi* in instances of multiparasitism within the same host (Danyk & Mackauer 1996; Danyk 1992), which may happen when aphid populations are low and hosts are scarce (Campbell 1974). And, second, while *A. ervi* may typically be the better external competitor, the presence of common non-target aphid species like the spotted alfalfa aphid may reduce the foraging efficiency of *A. ervi* more than *P. pequodorum* (Meisner *et al.* 2007).

Another factor potentially contributing to the persistence of *P. pequodorum* is that defensive symbionts, including *H. defensa*, or aphid-encoded resistance, may vary in effectiveness toward particular parasitoid species (Asplen *et al.* 2014; Cayetano & Vorburger 2015; Fellowes & Godfray 2000). Here, we performed parasitism assays on several experimental pea aphid lines to determine whether aphid-encoded and symbiont-mediated resistance vary in levels of protection conferred towards these two wasp species. We also performed serial dissections of parasitized aphids to determine whether there are differences in developmental trajectory of either wasp species that may account for any observed differences in aphid susceptibility after parasitism.

5.2 Methods

Pea aphids and creation of experimental lines

The pea aphid, *Acyrthosiphon pisum*, was introduced to North America from Europe in the late 1800s (Davis 1913, 1915; Thomas 1879). This aphid is cyclically parthenogenetic, reproducing asexually via viviparous production of clonal offspring during the Spring and Summer; sexual morphs arise in the Fall in response to shortening

day-lengths (Lamb & Pointing 1972). Clonal aphid lines were maintained in the laboratory by rearing them under long day conditions in environmental incubators. Aphid lines used in this study (Table 5.1) differ in genotype and/or infection status with *H. defensa*, and were collected from several different locations. Each line was initiated from a single parthenogenetic female placed onto a caged broad bean plant, *Vicia faba*, and reared at 20±1˚C with a 16L: 8D photoperiod. Lines AS3ø, AS3+APSE2, and

Table 5.1 Aphid clonal lines used in this study.

Aphid Clonal Line	Secondary symbiont	Collection Location	Reference
WA4ø	uninfected	Pennsylvania 2010	(Martinez *et al.* 2014b)
G15ø	uninfected	Georgia 2008	(Parker *et al.* 2014)
*CJ1-13ø (original)	uninfected	Utah 2012	(Martinez *et al.* 2014a)
*CJ113+APSE2	*H. defensa* + APSE2	Experimentally created	This paper
*CJ113+APSE3	*H. defensa* + APSE3	Experimentally created	This paper
*AS3ø	uninfected	Experimentally created	(Martinez *et al.* 2014b)
*AS3+APSE2	*H. defensa* + APSE2	Experimentally created	This paper
*AS3+APSE3 (original)	*H. defensa* + APSE3	Utah 2007	(Oliver *et al.* 2009)
WI301-33	*H. defensa* + APSE2	Wisconsin 2014	This paper
WI412-52	*H. defensa* + APSE3	Wisconsin 2014	This paper

Aphid lines sharing the same genotype, but different infection status are indicated with an asterisk; see methods for information on the creation of these experimental lines.

AS3+APSE3 all share the same aphid genotype but are uninfected with *H. defensa,* infected with *H. defensa*-APSE2, and infected with *H. defensa*-APSE3, respectively. Line AS3+APSE3 is the original aphid line, AS3ø was cured of its *H. defensa* infection, as in (Douglas *et al.* 2006; Martinez *et al.* 2014b), line AS3+APSE2 is infected with an *H. defensa* strain from aphid line ZA17 (Martinez *et al.* 2014b), and was created for use in this study. All experimentally infected/cured aphid lines were established more than 6 months prior to their use in this study. Diagnostic PCR and microsatellite analyses were used to determine symbiont infection status and clonal identity of each aphid line, as in (Martinez *et al.* 2014a).

Parasitoids: *Aphidius ervi* and *Praon Pequodorum*

The two most abundant parasitoids currently attacking the pea aphid in NA are *Aphidius ervi* and *Praon pequodorum* (Fig. 5.1). Like the pea aphid, *A. ervi* is also native to Eurasia, and is a generalist parasitoid of large Macrosiphinae aphids (Marsh 1977; Starý 1974; Starý *et al.* 1993), though pea aphids appear to be the preferred host (Takada & Tada 2000). To aid in control of pea aphids, multiple introductions of *A. ervi* to NA occurred between 1959 and 1968 and this wasp has now established throughout NA (Angalet & Fuester 1977; Halfhill *et al.* 1972; Mackauer & Finlayso 1967; Start 1970). Historically more abundant, *P. pequodorum* is native to NA and its populations on pea aphids saw declines after the introductions of *A. ervi* and *A. smithi* (Danyk 1992).

Aphidius ervi and *P. pequodorum* reside within the Aphidiinae subfamily of Braconidae, which is composed of parasitic wasps that attack aphids, but belong to the separate tribes Aphidiini and Praiini, respectively (Belshaw & Quicke 1997; Sanchis *et al.* 2000; Shi & Chen 2005; Smith *et al.* 1999). Both are solitary endoparasitoids, typically injecting a single egg into their aphid host at oviposition, which develops to adulthood in the still-living aphid. Mummification of aphids attacked by *A. ervi* (Fig. 5.1b), followed by the wasp's pupation, typically occurs 8-10 days after parasitism at 20°C (He 2008), whereas those attacked by *P. pequodorum* mummify (Fig. 5.1d) 6-8 days after parasitism (Chow & Sullivan 1984); timing of development was confirmed through personal observation (AJM). Adult wasps of both species eclose approximately five days after mummification. While better studied for *A. ervi*, both wasps employ a variety of tactics, including deployment of venom and teratocytes, to overcome aphid

defenses and create an environment suitable for wasp development (Digilio *et al.* 2000; Falabella *et al.* 2000; He *et al.* 2005).

The *A. ervi* wasps (Fig. 5.1a) used in this study were obtained from a single, large, interbreeding laboratory colony containing a mixture of wasps collected from Wisconsin and North Dakota, as well as commercially produced wasps (Arbico Organics). The *P. pequodorum* wasps (Fig. 5.1c) were obtained from a single, large, interbreeding laboratory colony containing a mixture of wasps collected from Wisconsin and North Dakota. Wasps were reared continuously on a mixture of the same susceptible aphid lines which were uninfected with facultative symbionts; adults were provided with constant access to honey and water**.**

Aphid parasitism resistance assays

We conducted parasitism assays, as in (Martinez *et al.* 2014a; Oliver *et al.* 2009), across numerous aphid lines (Table 5.1) to determine each line's resistance phenotype against *A. ervi* and *P. pequodorum*. Briefly, 2nd to 3rd instar aphids were singly parasitized (each aphid is removed as it is parasitized) in cohorts of 20 (one replicate) and placed on a fresh *V. faba* plant in a cup cage and held at 20±1˚C and 50% relative humidity with a 16L: 8D photoperiod; eight replicates were conducted for each line (= 160 total aphids). Wasps were selected haphazardly from our large laboratory culture and used to singly parasitize individual aphids from each line. Numerous individual female wasps were used per treatment and parasitized aphids were pooled before being split up into replicate cohorts of 20. Ten days after parasitism, we counted the number of live aphids, dead aphids, and aphid mummies (dried aphids containing a wasp pupa) to determine the proportion of each, measured as: aphid survival (live aphids/20), dual

mortality (dead aphids/20), and mummification (aphid mummies/20). Mummification rate is used as a suitable proxy for successful parasitism, as a large majority of wasps emerge after mummification (Oliver *et al.* 2012). Aphid mummies were collected from each replicate and were monitored for an additional week to confirm successful eclosion, confirming mummification as a suitable proxy for both *A. ervi* and *P. pequodorum*. Four replicates of twenty *unparasitized* 2nd to 3rd instar aphids of each line were also placed on fresh plants and used as a control for background mortality measures.

Aphid serial dissections and wasp development

The egg and larval development of *A. ervi* (He 2008; Martinez *et al.* 2014b; Pennacchio & Digilio 1989) and *P. pequodorum* (Chow & Sullivan 1984; Danyk & Mackauer 1996; Danyk 1992) in pea aphids has been described previously, showing some differences in early morphology of the two species. Here, we performed serial dissections on aphid line AS3ø, which is free of facultative symbionts and susceptible to both wasp species, to confirm reported differences in wasp development while controlling for symbiont status and aphid genotype. At least ten dissections of parasitized aphids were performed for each wasp species at each 24h-interval (from 1 to 144h after parasitism) and a representative image was made for each parasitoid species at every interval. All dissections were performed in 60uL of 1X PBS under an Olympus SZX16 stereo microscope. Photos of wasp egg-larval development stages were taken using an AMG EVOS digital inverted microscope.

An additional experiment examining sublethal effects of aphid resistance on *P. pequodorum.* These measurements were taken from approximately 50 wasps emerging from each of the following aphid lines AS3ø, CJ113ø, CJ113+APSE2, and

CJ113+APSE3, which represent aphids maintaining the least to most resistance against *A. ervi*, with the latter two lines having both symbiont- and aphid-based resistance. Wasps were allowed to complete development but they were killed at adult emergence by freezing at -20˚C for 6 hours and then desiccated by drying at 60˚C for 24 hours. We then measured wasp dry weight, right-hind tibia length, and sex ratio. Tibia length measurements were performed at 100x magnification and wasp dry weight was measured on a Mettler MT5 microbalance.

Statistical analyses

Aphid survival, mortality, and mummification (see above) were determined for each replicate of each parasitized aphid line. Using these data we performed a Generalized Linear Model (GzLM) with factorial design, using parasitoid species and aphid line to describe effects on aphid survival, mummification, and mortality. The same data were then used in another GzLM to compare differences in survival, mummification, and mortality among aphid lines exposed to either wasp species. Post hoc Tukey's honestly significant difference (HSD) tests on aphid survival, mortality, and mummification were performed using ANOVA of arcsine transformed proportional data for pairwise comparisons among aphid lines. GzLM was also used to compare mortality of parasitized and control (unparasitized) aphids, both within and across lines. Finally, a GzLM was used to compare effects of *H. defensa* infection and APSE strain on the outcome of parasitism by either parasitoid species; this analysis was restricted to a control genotype (AS3) of pea aphids, which had been split into three experimental lines (see Table 5.1). All generalized linear models were performed with a binomial distribution and logit link function; survival, mortality, and mummification data were

also mildly overdispersed and so final test values are reported with a quasibinomial adjustment. Because aphid mortality after parasitism may also be tied to differences among aphid lines, linear regression was performed on mean mortality between unparasitized controls and aphids parasitized by either parasitoid species. The mean mortality was natural log transformed to satisfy normality assumptions of the linear regression.

Fitness measures of *P. pequodorum* emerging from parasitized aphids included dry weight at emergence, right-hind tibia length, and sex ratio. Dry weight and tibia length were natural log transformed to satisfy normality assumptions. Right-hind tibia length and wasp weight typically correlate with each other as indicators of wasp fitness, so both were compared to each other via linear regression analysis. Analyses of variance (ANOVA) were used to compare dry weight and tibia length (separately for male and female): among four aphid lines, between lines infected/uninfected with *H. defensa*, and between *H. defensa*-infected lines with APSE2 or APSE3. Finally, we compared sex ratios of emergent wasps using Fisher's exact test (FET).

5.3 Results

There are three potential outcomes after an aphid is parasitized by either wasp species used in this study. 1) *Aphid Survival*: The aphid survives to adulthood and the developing wasp dies. 2) *Mummification*: The developing wasp survives and pupates, mummifies the aphid, and eventually emerges as an adult. 3) *Dual Mortality*: Both aphid and wasp die due to the stresses of this antagonistic interaction, which is a null outcome for both parties involved.

Table 5.2 Generalized linear model (GzLM) with factorial design.

	DF	Aphid Survival	Mummification	Dual Mortality
Wasp Species	1	$\chi2 = 123.9$, $p <$ **0.0001**	$\chi2 = 331.0$, $p <$ **0.0001**	$\chi2 = 0.01$, $p = 0.9111$
Aphid Line	7	$\chi2 = 171.1$, $p <$ **0.0001**	$\chi2 = 176.5$, $p <$ **0.0001**	$\chi2 = 26.0$, $p =$ **0.0005**
Wasp Species X Aphid Line	7	$\chi2 = 79.0$, $p <$ **0.0001**	$\chi2 = 156.3$, $p <$ **0.0001**	$\chi2 = 5.5$, $p = 0.5991$
Whole Model	15	$\chi2 = 834.0$, $p <$ **0.0001**	$\chi2 = 678.9$, $p <$ **0.0001**	$\chi2 = 31.3$, $p =$ **0.0080**

Effects of wasp species and aphid line on aphid susceptibility to parasitism. Significant values indicated in bold.

Using a GzLM to describe the interactions between wasp species and experimental aphid lines (Table 5.2) we see significant effects on aphid survival, mummification, and dual morality owing to differences between the two wasp species and among the eight experimental aphid lines.

Aphid susceptibility to *Aphidius ervi*

Overall, we found strongly significant variation in aphid survival and mummification, but no significant variation in dual mortality among the eight pea aphid clonal lines exposed to *A. ervi* (Fig. 5.2a). Significant variation in pea aphid susceptibility to this parasitoid wasp was present (Aphid Survival: GzLM, $\chi2 = 422.1$, $df = 7$, $p < 0.0001$) and aphid survival rates ranged from 4 – 86%. In general, there is an inverse relationship between aphid survival and mummification, and the latter also varied significantly (Mummification: GzLM, $\chi2 = 396.0$, $df = 7$, $p < 0.0001$) with rates ranging from 2 – 72%. We found no significant differences in mortality among parasitized aphid lines (Dual Mortality: GzLM, $\chi2 = 11.5$, $df = 7$, $p < 0.1170$), which ranged from 8 – 19%. Our results here with *A. ervi* were consistent with past studies demonstrating that lines WA4ø and CJ1-13ø have highly resistant genotypes in the absence of facultative symbionts, while uninfected lines G15ø and AS3ø have highly susceptible genotypes (Martinez *et al.* 2014a). Similarly, aphids carrying the *H. defensa*-APSE3 strain (line

AS3+APSE3) were highly resistant to *A. ervi*, while those with *H. defensa*-APSE2 (line AS3+APSE2) were only moderately resistant (Martinez *et al.* 2014b). All phage-carrying *H. defensa* strains characterized to date confer some protection (Oliver et al. 2014), but since the *H. defensa* strains and genotypes of aphid lines WI301-33 and WI412-52 (Table 5.1) have not been experimentally partitioned it is unclear how much protection is attributable to each potential source for these two lines.

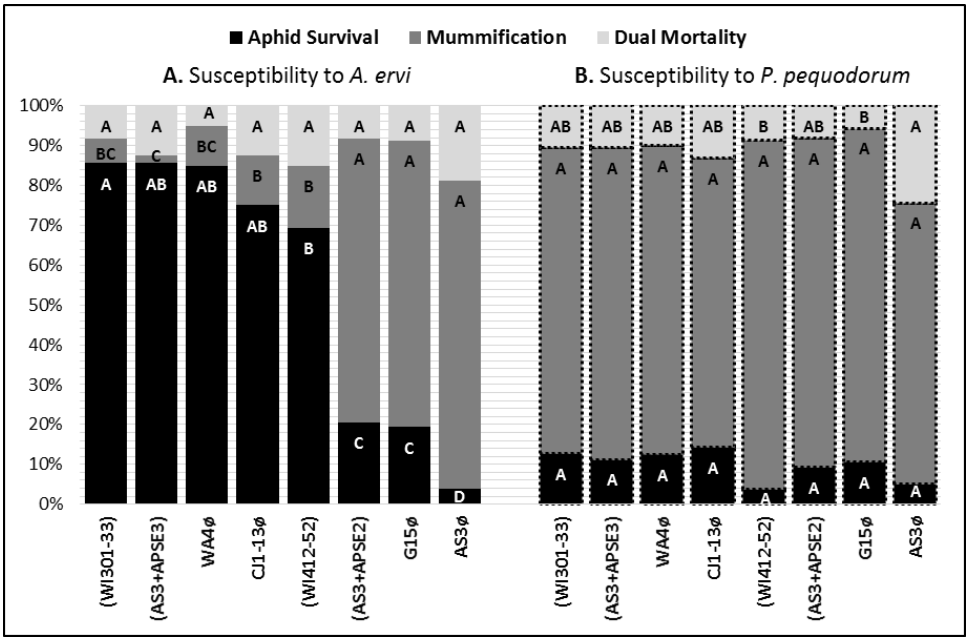


Figure 5.2 Pea aphid susceptibility to (A) *Aphidius ervi* and (B) *Praon pequodorum*, measured as mean aphid survival, mummification, and dual mortality. Aphid lines infected with *H. defensa* are indicated in parentheses. Statistical analyses were performed separately on aphids parasitized by either wasp species. Significant differences are indicated by letters (Tukey's HSD $\alpha = 0.05$). See 'Results' for GzLM analyses.

Aphid susceptibility to *Praon pequodorum*

In contrast to our results with *A. ervi*, we found no significant variation in aphid survival and mummification across our eight experimental aphid lines following exposure to *P. pequodorum* (Fig. 5.2b); aphid survival ranged from 4 – 14% (GzLM, $\chi 2 = 12.4$, $df = 7$, $p < 0.0892$) while mummification ranged from 71 – 88% (GzLM, $\chi 2 = 11.3$, $df = 7$,

$p < 0.1255$). Thus, despite the inclusion of a range of aphid genotypes and *H. defensa* strains that exhibit strong variation in resistance to *A. ervi,* we found that, surprisingly, all experimental aphid lines utilized in this study were uniformly highly susceptible to parasitism by *P. pequodorum.* We did detect significant variation in mortality among the eight pea aphid clonal lines exposed to *P. pequodorum* (GzLM, $\chi 2 = 20.9$, $df = 7$, $p < 0.0039$), which is largely attributable to a single line, AS3ø. However, the control unparasitized AS3ø line also shows higher mortality than other control lines (see Supplemental fig. 5.1) so it does not appear that this mortality is specifically due to parasitism by *P. pequodorum*.

Analysis of aphid mortality

Occasionally, neither parasitoid nor host aphid survive the parasitism event, resulting in dual mortality; here we compared the mortality of unparasitized control lines with the dual-mortality present in parasitized lines to establish whether parasitism resulted in mortality rates above background. We found aphid mortality to be significantly variable across control lines not exposed to wasps (GzLM, $\chi^2 = 15.2$, $df = 7$, $p = 0.0330$), but within lines, parasitism by either wasp species did not result in significant increases in aphid-wasp dual mortality relative to mortality of unparasitized control aphids of the same clonal line (Supplemental figure 5.1). Across lines, we also found no correlation between mortality of unparasitized control aphids and dual mortality of those parasitized by either *A. ervi* (Linear regression, $F_{1,7} = 1.73$, $p = 0.2368$) or *P. pequodorum* (Linear regression, $F_{1,7} = 0.64$, $p = 0.4551$), meaning that aphid-wasp dual mortality is affected differently by parasitism and does not trend with the background mortality levels of aphid lines.

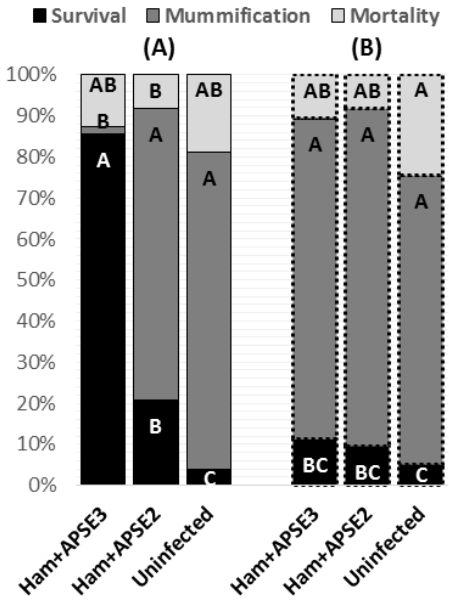


Figure 5.3 Effect of *H. defensa* and APSE strain on aphid susceptibility to both parasitoids. The three aphid clonal lines used here are of a single genotype (AS3) and are uninfected with *H. defensa* (line AS3ø), infected with *H. defensa*+APSE2 (line AS3+APSE2), and infected with *H. defensa*+APSE3 (AS3+APSE3). $P < 0.0001$, <0.0001, $= 0.0078$ for survival, mummification, and mortality, respectively (GzLM). Letters indicate significant differences (Tukey's HSD $\alpha = 0.05$). See 'Table 3' for GzLM effect tests of *H. defensa* infection and APSE strain on aphid susceptibility.

Restricted analysis to single aphid genotype

We also conducted analyses restricted to lines where the aphid genotype (AS3) was held constant, but varied in presence (+/-) and type of defensive symbiont (AS3+APSE2, or AS3+APSE3) to determine conclusively whether the two strains of *H. defensa* confer protection to *P. pequodorum*. As shown previously, we find significant effects of *H. defensa* infection on successful parasitism by *A. ervi* (infected aphids have higher survival, lower mummification) and APSE strain correlated with strength of protection (APSE3 provides stronger protection than APSE2; Fig. 5.3, Table 5.3) and no

Table 5.3 Generalized linear model showing effects of 'protective' symbiont infection and infecting bacteriophage APSE strain on aphid susceptibility to parasitism.

	Explanatory Variable	DF	Aphid Survival	Mummification	Dual Mortality
A. ervi	*H. defensa* infection	1	$\chi2 = 19.8$, $p <$ **.0001**	$\chi2 = 8.44$, $p =$ **.0037**	$\chi2 = 3.15$, $p = .0759$
	APSE Strain	1	$\chi2 = 86.9$, $p <$ **.0001**	$\chi2 = 217.3$, $p <$ **.0001**	$\chi2 = 0.95$, $p = .3305$
P. pequodorum	*H. defensa* infection	1	$\chi2 = 3.01$, $p = .0828$	$\chi2 = 3.54$, $p = .0598$	$\chi2 = 14.3$, $p =$ **.0002**
	APSE Strain	1	$\chi2 = 0.17$, $p = .6786$	$\chi2 = 0.77$, $p = .3788$	$\chi2 = 0.64$, $p = .4238$

Significant values indicated in bold.

significant effects were observed on aphid mortality. In contrast, *H. defensa* infection with either APSE type had no effects on aphid survival or mummification after parasitism by *P. pequodorum* compared to genetically identical, uninfected controls. Mortality was significantly affected by *H. defensa* infection due to the uninfected line, AS3ø, having higher mortality than either of the *H. defensa* infected lines. Thus, the protective effects of *H. defensa* appear specialized to the dominant parasitoid *A. ervi* and have no significant effects on *P. pequodorum*.

Variations in wasp development may underlie differences in aphid susceptibility

Serial dissections of parasitized aphids at 24h intervals confirmed that while the overall developmental trajectories of *A. ervi* and *P. pequodorum* are similar (Fig. 5.4) some striking differences exist through the 72h time-point, namely that the chorion of the *A. ervi* egg ruptures around 24h, potentially exposing the wasp embryo to aphid or symbiont defenses, whereas the *P. pequodorum* egg chorion does not rupture until after 72h. Mortality to *A. ervi* wasps in highly resistant aphids typically occurs prior to 72h (Martinez et al. 2014).

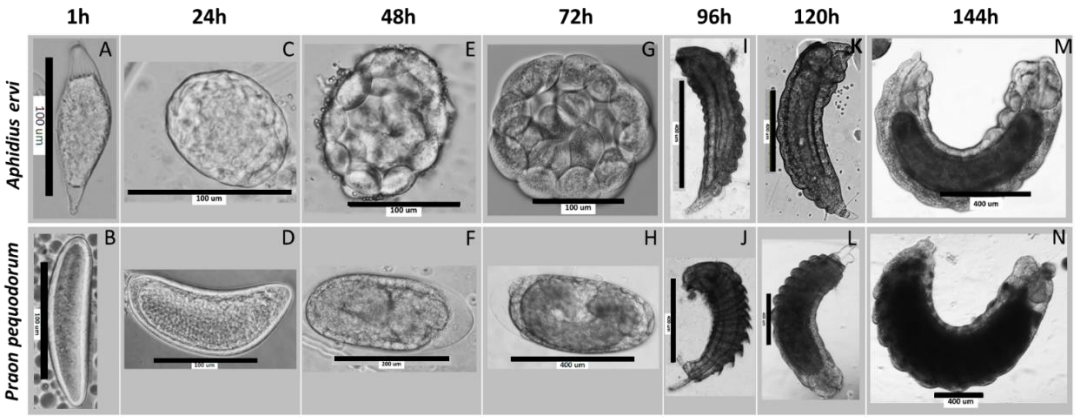


Figure 5.4 Serial dissections of parasitized pea aphids, revealing differences in early parasitoid wasp development. Development of *Aphidius ervi* (top) and *Praon pequodorum* (bottom). (A and B) Eggs of both parasitoids 1 hour after parasitism. (C) The morula of *A. ervi* shortly after the egg has hatched, revealing the developing embryo surrounded by serosal cells. (D) The egg of *P. pequodorum* remains intact with a thick chorion. (E) The *A. ervi* morula continues to grow. (F) The *P. pequodorum* egg has not yet hatched, but continues to grow. (G) First instar *A. ervi* larva (not shown) emerges from morula, serosal cells have not yet dissociated to form teratocyte cells. (H) The egg chorion, serosal cells, and developing embryo of *P. pequodorum* are clearly visible. The egg finally hatches between 72-96h. (I and J) Second instar larvae of both wasps. (K-N) Similar continued larval development of both wasps.

At 1h, we found that *A. ervi*'s egg is an elongate oval with tapered ends and each end is translucent (Fig. 5.4a); we find the chorion here to be thinner than that of *P. pequodorum* (Fig. 5.4b). The egg chorion then ruptures around 24h releasing a roughly spherical morula, composed of the growing serosal teratocyte cells which surround the developing wasp (Fig. 5.4c). At 48h the morula is roughly spherical (Fig. 5.4e). At 72h the morula has increased in size and is roughly spherical; shortly thereafter, the larval parasitoid emerges from the morula (Fig. 5.4g). The *A. ervi* larva continues its growth through the last measurement at 144h (Fig. 5.4m).

At 1h the *P. pequodorum* egg is an opaque elongate oval with rounded ends (Fig. 5.4b). At 24h the elongate egg and a distinct thickened chorion (compared to *A. ervi*) are still intact (Fig. 5.4d). At 48h the egg and chorion remain intact and some, but not all,

eggs have a distinct translucent portion of the chorion at one end (Fig. 5.4f). At 72h the egg and chorion still remain intact (Fig. 5.4h) and the first instar wasp larva emerges from the egg between 72h and 96h. The *P. pequodorum* larva continues growing through the last measurement at 144h (Fig. 5.4n).

Table 5.4 Fitness measures of *P. pequodorum* wasps emerging from aphid lines AS3ø, CJ113ø, CJ113+APSE2, and CJ113+APSE3.

a.			Male (♂)			Female (♀)		
Variable	**Fitness Measure**	**df**	**n**	**f-value**	**p-value**	**n**	**f-value**	**p-value**
Aphid line	Dry weight	3	77	2.47	0.0687	96	1.55	0.2061
	Tibial length	3	77	5.67	**0.0015**	96	1.85	0.1431
Aphid resistance	Dry weight	1	77	3.98	**0.0496**	96	1.35	0.2489
	Tibial length	1	77	0.77	0.3841	96	0.45	0.5039
H. defensa infection	Dry weight	1	77	1.20	0.2765	96	0.01	0.9222
	Tibial length	1	77	0.21	0.6468	96	0.35	0.5545
APSE strain	Dry weight	1	36	2.26	0.1422	51	2.85	0.0978
	Tibial length	1	36	10.52	**0.0026**	51	3.71	0.0599

b.		♂	♀	P-value (FET)
Aphid line	AS3ø	21	21	0.4975
	CJ113ø	20	24	
	CJ113+APSE2	19	20	
	CJ113+APSE3	17	31	
Aphid resistance	AS3 (low)	21	21	0.4765
	CJ113 (high)	56	75	
H. defensa infection	Infected	36	51	0.4461
	Uninfected	41	45	
APSE strain	APSE2	19	20	0.2746
	APSE3	17	31	

(A) Analysis of dry weight and right-hind tibia length of emergent wasps (ANOVA). (B) Sex ratios of emergent wasps (Fisher's exact test). See supplemental figure 5.2 for dry weight and tibia length values.

Sublethal fitness effects of resistance on *P. pequodorum*

Though *P. pequodorum* is able to consistently develop in aphid lines resistant to *A. ervi*, we tested whether there were sub-lethal fitness effects on *P. pequodorum* emerging from aphids maintaining intrinsic *and H. defensa*-mediated resistance against *A. ervi* relative to control lines susceptible to *A. ervi*. As indicators of *P. pequodorum* fitness we measured dry weight at emergence and right-hind tibia length for both males (♂) and females (♀). As expected, we found that dry weight and tibia length were strongly correlated with each other among both males and females (Linear regression, F = 180.6 ♂ / 312.8 ♀, $p < 0.0001$ ♂ and ♀). Among female wasps, *P. pequodorum* dry weight and tibia length did not vary significantly with respect to the resistance phenotype (i.e. between the susceptible AS3 and resistant CJ113 lines), *H. defensa* or APSE strain

types (Table 5.4a, Supplementary fig. 5.2). Male wasp dry weight varied significantly by resistance phenotype (Average: 0.131mg Low and 0.116mg High) and male tibia length varied significantly among aphid lines and between APSE types, but each's corresponding tibia length and dry weights, respectively, did not follow the same pattern (Table 5.4a, Supplementary fig. 5.2). We also measured sex ratio of *P. pequodorum* but found they did not vary by aphid line, aphid resistance phenotype, *H. defensa* infection, or APSE strain (Table 5.4b).

5.4 Discussion

As expected from previous studies, we found substantial variation in pea aphid- and *H.defensa*/APSE-sourced resistance to *A. ervi*, the dominant parasitoid of this aphid in NA (Fig 5.2a) (Martinez *et al.* 2014a; Oliver *et al.* 2009; Oliver *et al.* 2005). Using these same lines that varied in aphid- and symbiont-based resistance to *A. ervi*, however, we found no variation in pea aphid susceptibility to the related, and second most abundant parasitoid, *P. pequodorum*, (Fig. 5.2b). Further, we show that *H. defensa* strains carrying either APSE2 or APSE3 confer no resistance to *P. pequodorum* relative to uninfected controls sharing the same aphid genotype (Fig. 5.3, Table 5.3). Similarly, all pea aphid genotypes (uninfected with symbionts) that were resistant to *A. ervi* were highly susceptible to *P. pequodorum*. Not only were *P. pequodorum* able to develop in all aphid lines resistant to *A. ervi*, we found no consistent sub-lethal fitness costs to *P. pequodorum* developing in resistant lines.

Such specificity of *H. defensa* to particular natural enemies has now been shown in all three cases where this symbiont is known to confer protection (pea aphid, cowpea aphid, and black bean aphid) (Asplen *et al.* 2014; Cayetano & Vorburger 2015). *H.*

defensa in cowpea aphid, *Aphis craccivora*, conferred protection against *Binodoxys communis* and *B. koreanus*, but not against *Lysiphlebus orientalis* or *Aphidius colemani* (all Hymenoptera: Braconidae: Aphidiinae) (Asplen *et al.* 2014) while *H. defensa* in the black bean aphid, *Aphis fabae*, protected against *L. fabarum* and *A. colemani*, but not against *B. angelicae* or *Aphelinus chaonia* (Cayetano & Vorburger 2015). Most recently, *H. defensa* strains from several European pea aphid biotypes were shown to vary in levels of resistance conferred to *A. ervi* versus *Aphelinus abdominalis*, a distantly related parasitoid (Chalcidoidea; Aphelinidae) (McLean & Godfray 2015), although we note that this parasitoid is not commonly found in NA populations. Together these findings indicate that specificity of protective *H. defensa* to particular natural enemies is likely a general phenomenon.

In pea aphids, both the aphid-encoded and symbiont-based defenses tested provided specific and effective protection against *A. ervi*, but had no effect on *P. pequodorum*. It remains possible that uncommon, and hitherto undiscovered strains of *H. defensa* are protective against *P. pequodorum.* However, given that the *H. defensa* strains examined contained the dominant APSE types (2 & 3) present in NA pea aphid populations, this indicates that it is likely that *H. defensa* is not generally effective against *P. pequodorum.* Similarly, if we sampled additional aphid genotypes we may discover some that are resistant to this wasp.

Aphids that are resistant to *A. ervi* may serve as a reservoir of hosts available only to *P. pequodorum* and, at least partially, explain why this wasp has not been completely eliminated by competition, as is the case with *A. smithi* and other previously common parasitoids (Angalet & Fuester 1977; Danyk 1992; McBrien & Mackauer 1991;

Thiboldeaux *et al.* 1987). Before the introduction of *A. ervi*, *P. pequodorum* was relatively abundant, making up 42% of parasitized pea aphids on alfalfa in Wisconsin, USA (Fluke 1925) and 25% in British Columbia, Canada (Campbell & Mackauer 1973). Since *A. ervi*'s introduction, though, populations of *P. pequodorum* have steadily declined with more recent estimates ranging between 6% in British Columbia (Danyk 1992), 8% (or locally absent) in Wisconsin (Schellhorn *et al.* 2002), and 14% in NY and PA (Smith *et al.* 2015). In contrast, populations of *A. ervi* increased to 86-100% of parasitized aphids over the same time-period (Danyk 1992; McBrien 1992; Smith *et al.* 2015). Helping to explain *A. ervi*'s abundance, these studies found that *A. ervi* is more efficient at foraging for aphid hosts than other pea aphid parasitoids, an attribute which may further benefit recolonization of this parasitoid when faced with human agricultural practices such as cutting and harvesting of alfalfa (Schellhorn *et al.* 2002). The persistence of *P. pequodorum* in pea aphid populations, however, indicates that it may be able to successfully compete with *A. ervi* under certain conditions. Larval competition assays, for example, found that *P. pequodorum* consistently outcompeted *A. ervi* in instances of multiparasitism (Danyk 1992), which may occur when parasitism rates are high or when aphid populations are low (Campbell 1974). Further, *A. ervi*'s foraging efficiency may be more affected by non-target aphid species than *P. pequodorum*'s (Meisner *et al.* 2007). Our results indicate that resistance specialized to *A. ervi* is likely another factor contributing to the persistence of *P. pequodorum.* Thus, even though *A. ervi* is a more efficient at foraging for hosts, *P. pequodorum* may be able to persist in aphid populations with high *H. defensa* infection frequencies or high genotypic resistance to *A. ervi.*

More generally, given that many insect hosts are attacked by multiple parasitoids, the invasion of enemy-specific protective symbionts or resistant alleles (Benassi *et al.* 1998; Carton *et al.* 1992; Poirie *et al.* 2000), could alter the competition dynamics between parasitoid species, e.g. (Condon *et al.* 2014; Decaestecker *et al.* 2003; Morgan *et al.* 2009). Common insect symbionts with known roles in defense like *Wolbachia* and *Spiroplasma* can spread rapidly through insect populations (Himler *et al.* 2011; Jaenike *et al.* 2010; Xi *et al.* 2005; Xie *et al.* 2015) and are also shown to vary temporally and spatially, e.g. (Russell *et al.* 2013), potentially resulting in rapid changes in the composition of natural enemies. Future field or population cage studies could, for example, show that high levels of *H. defensa* or resistant genotypes result in increases in the abundance of *P. pequodorum* at the expense of *A. ervi.* On the other hand, differences in natural enemy ability to overcome symbiont-based defenses may, in turn, influence composition and frequency of symbiont infections in the field. Pea aphids infected with *H. defensa* only occur at intermediate frequencies throughout natural populations of pea aphids (Russell *et al.* 2013), but with such clear advantages to infection in the face of parasitism by *A. ervi* and near 100% vertical transmission efficiency in laboratory colonies, it may be surprising that a higher proportion of aphids are not infected. Selective factors that may limit its spread include infection costs in the absence of parasitism (Oliver *et al.* 2008), the presence of resistant uninfected genotypes (Martinez *et al.* 2014a), or ineffectiveness against other natural enemies, such as *P. pequodorum*.

Further work is needed to understand the basis for differential resistance to *A. ervi* vs. *P. pequodorum*. It is possible that aphid and symbiont encoded factors specifically

target *A. ervi* genotypes, or it may be that *P. pequodorum* exhibits features that counter or prevent effective aphid defenses. For example, because *A. ervi* and the pea aphid originated in Eurasia and share a long evolutionary history, pea aphid defense mechanisms may have evolved specifically to combat *A. ervi* and other common native Eurasian parasitoids. We did, however, find marked differences in early egg-larval development between these two wasp species (Fig. 5.4) that correlate with observed differences in aphid resistance. While the overall biology and development of both wasps is similar, we found that the thinly chorionated *A. ervi* egg hatches around 24 hours after parasitism while the thicker chorion of the *P. pequodorum* egg remains intact until after 72 hours. This is important because the highly-resistant *H. defensa*-APSE3 strain causes mortality to *A. ervi* between 24 – 48 hours after parasitism, after the chorion has ruptured, but before first instar larva develops. Hence, prolonged development in a chorionated egg may protect developing *P. pequodorum* during the stages which are most susceptible to symbiont-incurred damage.

Given that *H. defensa* confers variable resistance against a range of aphid parasitoid species, comparative developmental studies among wasp species could reveal whether prolonged sequestration of the embryo inside a protective chorion is a general strategy to circumvent symbiont-mediated defense. However, such a strategy is clearly not universal. First, in black bean aphids, *H. defensa* protection is not only specific to particular wasp species, but also to particular genotypes within wasp species (Cayetano & Vorburger 2015; Rouchet & Vorburger 2012; Rouchet & Vorburger 2014). Second, in *A. pisum*, mortality to *A. ervi* owing to both aphid-encoded and *H. defensa* strains with APSE2 often occurs after this 72h window, and *P. pequodorum*, whose larva is usually

exposed by this point, is just as successful at attacking pea aphid with these types of resistance to *A. ervi*. And finally, *H. defensa* confers protection against *A. colemani* in black bean aphids, but not in cowpea aphids (Asplen *et al.* 2014; Cayetano & Vorburger 2015), although developmental studies show that *A. colemani* emerges from its egg as late as two days after parasitism (Hofsvang & Hagvar 1978) so it possible that this is important in cowpea aphid, but not black bean aphid due to the timing of symbiont-induced harm. Clearly, more work is needed to understand mechanisms underlying the specificity of parasitoid resistance.

5.5 Conclusions

Here we show that multiple resistance mechanisms that pea aphids use to combat their most abundant parasitoid *A. ervi*, including protective bacterial symbionts and resistant genotypes, are ineffective against a related, but less common parasitoid, *P. pequodorum.* Given that *A. ervi* is a superior external competitor, pea aphid resistance to *A. ervi* may be one mechanism, which allows *P. pequodorum* to persist in North American populations of pea aphids, where all other parasitoids have been eliminated. However, in a more evenly matched competitive interaction, the introduction of resistance could potentially give a strong selective advantage of one natural enemy over another, which could lead to dramatic shifts in natural enemy composition. Nevertheless, our findings suggest that host-symbiont infections and intrinsic resistance are important not only their ecology, but the ecology of their competing natural enemies, potentially influencing the guilds of natural enemies attacking a host species in a given area.

5.6 References

Angalet GW, Fuester R (1977) The *Aphidius* parasites of the pea aphid *Acyrthosiphon pisum* in the eastern half of the United States. *Annals of the Entomological Society of America* **70**, 87-96.

Asplen MK, Bano N, Brady CM, *et al.* (2014) Specialisation of bacterial endosymbionts that protect aphids from parasitoids. *Ecological Entomology* **39**, 736-739.

Beckage NE, Gelman DB (2004) Wasp parasitoid disruption of host development: Implications for New Biologically Based Strategies for Insect Control. *Annual Reviews in Entomology* **49**, 299-330.

Belshaw R, Quicke DL (1997) A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). *Molecular phylogenetics and evolution* **7**, 281-293.

Benassi V, Frey F, Carton Y (1998) A new specific gene for wasp cellular immune resistance in *Drosophila*. *Heredity* **80**, 347-352.

Bensadia F, Boudreault S, Guay J-F, Michaud D, Cloutier C (2006) Aphid clonal resistance to a parasitoid fails under heat stress. *Journal of insect physiology* **52**, 146-157.

Campbell A (1974) Seasonal changes in abundance of the pea aphid and its associated parasites in the southern interior of British Columbia. *Dissertation Abstr lnt* **35**, 1234.

Campbell A, Mackauer M (1973) Some Climatic Effects on the Spread and Abundance of Two Parasites of the Pea Aphid in British Columbia (Hymenoptera: Aphidiidae-Homoptera: Aphididae). *Zeitschrift für angewandte Entomologie* **74**, 47-55.

Carton Y, Frey F, Nappi A (1992) Genetic determinism of the cellular immune reaction in *Drosophila melanogaster*. *Heredity London* **69**, 393-393.

Carver M, Sullivan D, Niemczyk E, Dixon A (1988) Encapsulative defence reactions of aphids (Hemiptera: Aphididae) to insect parasitoids (Hymenoptera: Aphidiidae and Aphelinidae). *Ecology and Effectiveness of Aphidophaga*, 299-303.

Cayetano L, Vorburger C (2015) Symbiont-conferred protection against Hymenopteran parasitoids in aphids: how general is it? *Ecological Entomology* **40**, 85-93.

Chow FJ, Sullivan DJ (1984) Developmental stages of *Praon pequodorum* Viereck (Hymenoptera: Aphidiidae), a pea aphid parasitoid. *Annals of the Entomological Society of America* **77**, 319-322.

Cloutier C, Douglas AE (2003) Impact of a parasitoid on the bacterial symbiosis of its aphid host. *Entomologia Experimentalis et Applicata* **109**, 13-19.

Condon MA, Scheffer SJ, Lewis ML, *et al.* (2014) Lethal interactions between parasites and prey increase niche diversity in a tropical community. *Science* **343**, 1240-1244.

Danyk T, Mackauer M (1996) An Extraserosal Envelope in Eggs of *Praon pequodorum* (Hymenoptera: Aphidiidae), a Parasitoid of Pea Aphid. *Biological Control* **7**, 67-70.

Danyk TP (1992) *Competitive Interactions Between the Pea Aphid Parasitoids, Aphidius ervi and Praon pequodorum (Hymenoptera: Aphidiidae): Influence on Guild Composition in Southern British Columbia*, Simon Fraser University.

Davis JJ (1913) The Cyrus Thomas Collection of Aphididae and a Tabulation of Species Mentioned and Described in His Publications. *Bulletin of the Illinois state laboratory of natural history*.

Davis JJ (1915) *The pea aphis with relation to forage crops* US Department of Agriculture.

Decaestecker E, Vergote A, Ebert D, De Meester L (2003) Evidence for strong host clone-parasite species interactions in the *Daphnia* microparasite system. *Evolution* **57**, 784-792.

Digilio MC, Isidoro N, Tremblay E, Pennacchio F (2000) Host castration by *Aphidius ervi* venom proteins. *Journal of insect physiology* **46**, 1041-1050.

Douglas A, Francois C, Minto L (2006) Facultative 'secondary'bacterial symbionts and the nutrition of the pea aphid, *Acyrthosiphon pisum*. *Physiological Entomology* **31**, 262-269.

Falabella P, Tremblay E, Pennacchio F (2000) Host regulation by the aphid parasitoid *Aphidius ervi*: the role of teratocytes. *Entomologia Experimentalis et Applicata* **97**, 1-9.

Fellowes M, Godfray H (2000) The evolutionary ecology of resistance to parasitoids by *Drosophila*. *Heredity* **84**, 1-8.

Fisher RC (1961) A study in insect multiparasitism II. The mechanism and control of competition for possession of the host. *Journal of Experimental Biology* **38**, 605-629.

Fluke CL (1925) Natural enemies of the pea aphid (*Illinoia pisi* Kalt.); Their abundance and distribution in Wisconsin. *Journal of Economic Entomology* **18**, 612-616.

Godfray HCJ (1994) *Parasitoids: behavioral and evolutionary ecology* Princeton University Press.

Halfhill JE, Featherston PE, Dickie AG (1972) History of the *Praon* and *Aphidius* parasites of the pea aphid in the Pacific Northwest. *Environmental Entomology* **1**, 402-405.

He XZ (2008) *Reproductive behaviour of Aphidius ervi Haliday (Hymenoptera: Aphidiidae)*, Massey University.

He XZ, Wang Q, Teulon DAJ (2005) The effect of parasitism by *Aphidius ervi* on development and reproduction of the pea aphid, *Acyrthosiphon pisum*. *New Zealand Plant Protection* **58**, 202-207.

Henter HJ, Via S (1995) The potential for coevolution in a host-parasitoid system. I. Genetic variation within an aphid population in susceptibility to a parasitic wasp. *Evolution*, 427-438.

Himler AG, Adachi-Hagimori T, Bergen JE, *et al.* (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* **332**, 254-256.

Hofsvang T, Hagvar E (1978) Larval morphology and development of *Aphidius colemani* Viereck and *Ephedrus cerasicola* Stary (Hymenoptera, Aphidiidae). *Norwegian journal of entomology*.

Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ (2010) Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science* **329**, 212-215.

Kraaijeveld A, Van Alphen J, Godfray H (1998) The coevolution of host resistance and parasitoid virulence. *Parasitology* **116**, S29-S45.

Kraaijeveld AR, Godfray HCJ (2009) Evolution of host resistance and parasitoid counter-resistance. *Advances in parasitology* **70**, 257-280.

Lamb R, Pointing P (1972) Sexual morph determination in the aphid, *Acyrthosiphon pisum*. *Journal of insect physiology* **18**, 2029-2042.

Laughton AM, Garcia JR, Altincicek B, Strand MR, Gerardo NM (2011) Characterisation of immune responses in the pea aphid, *Acyrthosiphon pisum*. *Journal of insect physiology* **57**, 830-839.

Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology* **32**, 1295-1309.

Mackauer M, Finlayso T (1967) Hymenopterous parasites (Hymenoptera - Aphidiidae et Aphelinidae) of pea aphid in Eastern North America. *Canadian Entomologist* **99**, 1051-&.

Marsh P (1977) Notes on the taxonomy and nomenclature of *Aphidius* species [Hym.: Aphidiidae] parasitic on the pea aphid in North America. *Entomophaga* **22**, 365-372.

Martinez AJ, Ritter SG, Doremus MR, Russell JA, Oliver KM (2014a) Aphid-encoded variability in susceptibility to a parasitoid. *BMC Evol Biol* **14**, 127.

Martinez AJ, Weldon SR, Oliver KM (2014b) Effects of parasitism on aphid nutritional and protective symbioses. *Molecular ecology* **23**, 1594-1607.

McBrien H, Mackauer M (1991) Decision to superparasitize based on larval survival: competition between aphid parasitoids *Aphidius ervi* and *Aphidius smithi*. *Entomologia Experimentalis et Applicata* **59**, 145-150.

McBrien HL (1992) *Oviposition decisions and larval competition between the aphid parasitoids Aphidius ervi and Aphidius smithi (Hymenoptera: Aphidiidae)*.

McLean AH, Godfray HCJ (2015) Evidence for specificity in symbiont-conferred protection against parasitoids **282**, 20150977.

Meisner M, Harmon JP, Ives AR (2007) Presence of an unsuitable host diminishes the competitive superiority of an insect parasitoid: a distraction effect. *Population ecology* **49**, 347-355.

Morgan A, Craig Maclean R, Buckling A (2009) Effects of antagonistic coevolution on parasite-mediated host coexistence. *Journal of evolutionary biology* **22**, 287-292.

Oliver KM, Campos J, Moran NA, Hunter MS (2008) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B: Biological Sciences* **275**, 293-299.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325**, 992-994.

Oliver KM, Martinez AJ (2014) How resident microbes modulate ecologically-important traits of insects. *Current Opinion in Insect Science* **4**, 1-7.

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences* **102**, 12795-12800.

Oliver KM, Noge K, Huang EM, *et al.* (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC biology* **10**, 11.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1803-1807.

Parker BJ, Garcia JR, Gerardo NM (2014) Genetic variation in resistance and fecundity tolerance in a natural host–pathogen interaction. *Evolution* **68**, 2421-2429.

Pennacchio F, Digilio M (1989) Morphology and development of larval instars of Aphidius ervi Haliday (Hymenoptera, Braconidae, Aphidiinae). *Bollettino del Laboratorio di Entomologia Agraria'Filippo Silvestri'* **46**, 163-174.

Poirie M, Frey F, Hita M, *et al.* (2000) Drosophila resistance genes to parasitoids: chromosomal location and linkage analysis. *Proceedings of the Royal Society of London B: Biological Sciences* **267**, 1417-1421.

Price PW (1972) Parasitiods utilizing the same host: adaptive nature of differences in size and form. *Ecology*, 190-195.

Rouchet R, Vorburger C (2012) Strong specificity in the interaction between parasitoids and symbiont-protected hosts. *Journal of evolutionary biology* **25**, 2369-2375.

Rouchet R, Vorburger C (2014) Experimental evolution of parasitoid infectivity on symbiont-protected hosts leads to the emergence of genotype specificity. *Evolution* **68**, 1607-1616.

Russell JA, Weldon S, Smith AH, *et al.* (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. *Molecular ecology* **22**, 2045-2059.

Sanchis A, Latorre A, González-Candelas F, Michelena JM (2000) An 18S rDNA-based molecular phylogeny of Aphidiinae (Hymenoptera: Braconidae). *Molecular phylogenetics and evolution* **14**, 180-194.

Schellhorn NA, Kuhman TR, Olson AC, Ives AR (2002) Competition between native and introduced parasitoids of aphids: nontarget effects and biological control. *Ecology* **83**, 2745-2757.

Shi M, Chen X (2005) Molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae) based on DNA sequences of 16S rRNA, 18S rDNA and ATPase 6 genes. *European Journal of Entomology* **102**, 133.

Smith AH, Łukasik P, O'Connor MP, *et al.* (2015) Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. *Molecular ecology* **24**, 1135-1149.

Smith HS (1929) Multiple parasitism: its relation to the biological control of insect pests. *Bulletin of entomological research* **20**, 141-149.

Smith PT, Kambhampati S, Völkl W, Mackauer M (1999) A phylogeny of aphid parasitoids (Hymenoptera: Braconidae: Aphidiinae) inferred from mitochondrial NADH 1 dehydrogenase gene sequence. *Molecular phylogenetics and evolution* **11**, 236-245.

Start P (1970) Biology of aphid parasites (Hymenoptera: Aphidiidae) with respect to integrated control. *Series Ent.* **6**, 1-643.

Starý P (1974) Taxonomy, origin, distribution and host range of *Aphidius* species (Hym., Aphidiidae) in relation to biological control of the pea aphid in Europe and North America. *Zeitschrift für angewandte Entomologie* **77**, 141-171.

Starý P (2006) *Aphid parasitoids of the Czech Republic (Hymenoptera: Braconidae, Aphidiinae)* Academia.

Starý P, Gerding I, Norambuena I, Remaudière G (1993) Environmental research on aphid parasitoid biocontrol agents in Chile (Hym., Aphidiidae; Hom., Aphidoidea). *Journal of Applied Entomology* **115**, 292-306.

Strand MR (2008) The insect cellular immune response. *Insect Science* **15**, 1-14.

Strand MR, Pech LL (1995) Immunological basis for compatibility in parasitoid host relationships. *Annual Review of Entomology* **40**, 31-56.

Takada H, Tada E (2000) A comparison between two strains from Japan and Europe of *Aphidius ervi*. *Entomologia Experimentalis et Applicata* **97**, 11-20.

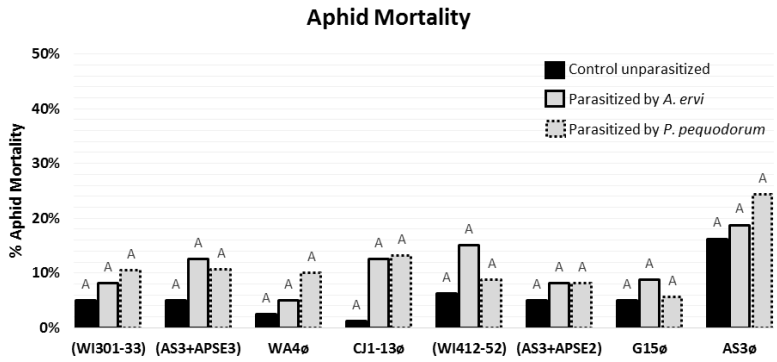
Thiboldeaux R, Hutchison W, Hogg D (1987) Species composition of pea aphid (Homoptera: Aphididae) primary and secondary parasitoids in Wisconsin. *The Canadian Entomologist* **119**, 1055-1057.

Thomas C (1879) Noxious and beneficial insects of the State of Illinois. *Report of the State Entomologist (Illinois)* **8**, 1-212.

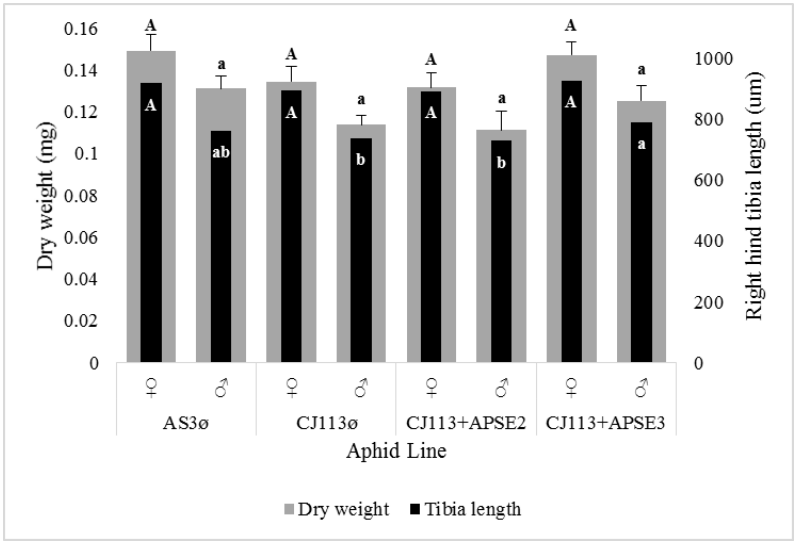
Vorburger C (2014) The evolutionary ecology of symbiont-conferred resistance to parasitoids in aphids. *Insect Science* **21**, 251-264.

Xi ZY, Khoo CCH, Dobson SL (2005) *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* **310**, 326-328.

Xie JL, Winter C, Winter L, Mateos M (2015) Rapid spread of the defensive endosymbiont *Spiroplasma* in *Drosophila hydei* under high parasitoid wasp pressure. *Fems Microbiology Ecology* **91**, 11.



Supplemental figure 5.1. Comparison of mortality (not resulting in mummification) among aphid lines. Overall significance: p = 0.0002 (GzLM df = 23, χ^2 = 55.8); Control significance: p = 0.0330 (GzLM df = 7, χ^2 = 15.2); see main text and Figure 5.2 for analyses involving dual mortality due to *A. ervi* and *P. pequodorum* parasitism. Letters indicate lack of significant differences among treatments, within aphid lines (Tukey's HSD α = 0.05).



Supplemental figure 5.2. Fitness measures of adult *P. pequodorum* emerging from susceptible (AS3) and resistant (CJ113) aphid lines. Dry weight, p = 0.2061♀ / 0.0687♂. Tibial length, p = 0.1431♀ / 0.0015♂ (ANOVA). Letters indicate significant differences (Tukey's HSD α = 0.05). Note: Error bars are for dry weight only.

CHAPTER 6

CONCLUSIONS

Virtually all multicellular eukaryotes, including insects, are attacked by a wide range of natural enemies which place strong selective pressures on the development and maintenance of resistance. In addition to a diverse array of endogenous defense mechanisms, there is a growing appreciation that insects also form protective mutualisms with symbiotic microbes to aid in their defense. The pea aphid, for example, appeared to rely primarily on the bacterium *Hamiltonella defensa* for protection against parasitoids, but my findings show that both host *and* symbiont contribute substantial variation in resistance and that some aphids maintain both endogenous and symbiotic types of defenses against parasitism. These findings suggested that there are balancing selective pressures to maintain both types of resistance in aphid populations and resulted in new questions regarding each's advantages or disadvantages. I found that the maintenance of endogenous protection apparently carried no fitness costs, while infection with *H. defensa* often decreased aphid fecundity and longevity and was only conditionally advantageous to aphids depending on compatibility between symbiont-host genotypes and parasitism pressure. Some endogenously-protected aphids, though, did still benefit from *H. defensa*'s additional protection. In an unexpected twist, however, I found that both *H. defensa*-conferred and endogenously-encoded resistance traits were specialized towards the pea aphid's most common parasitoid, *Aphidius ervi*, and were completely ineffective against the related but less common parasitoid, *Praon pequodorum,* a finding

that likely contributes to the latter's persistence on this host despite being a poorer external competitor. Finally, I found that parasitism by *A. ervi* influences the aphid's nutritional and defensive symbioses, with significant changes in *Buchnera*'s and *H. defensa*-APSE's titers occurring concomitantly with important transitions in wasp development, this may signify wasp manipulation of aphid symbioses and/or the mobilization of symbiotic defensive factors. Together, my results show that pea aphid resistance to parasitic wasps is much more complicated than previously thought and this new appreciation will help further our understanding of insect-microbe-parasitoid interactions. These works also informs how natural enemies influence infection frequencies of protective symbionts and, in turn, how protective symbionts and host resistance influence composition of natural enemies. Clearly there is much need for continued examination of host-microbe-natural enemy interactions, their underlying mechanisms, and how these processes influence biodiversity of all interacting players, extending through trophic levels above and below.