Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I herby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of the thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of the thesis or dissertation.

Signature:

_____ _____

Date

ECOLOGICAL FACTORS DETERMINE IMMUNE COSTS AND SUSCEPTIBILITY OF PEA APHIDS TO A NATURAL FUNGAL PATHOGEN

By
Benjamin James Parker
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Population Biology, Ecology, and Evolution

Nicole Gerardo, Ph.D.
Advisor

Meghan Duffy, Ph.D.
Committee Member

Kerry Oliver, Ph.D.
Committee Member

Jacobus de Roode, Ph.D.
Committee Member

Todd Schlenke, Ph.D.
Committee Member

Approved:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

ECOLOGICAL FACTORS DETERMINE IMMUNE COSTS AND SUSCEPTIBILITY OF PEA APHIDS TO A NATURAL FUNGAL PATHOGEN

By
Benjamin James Parker
A.B. The University of Chicago, 2007

Advisor: Nicole M. Gerardo

An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Science—
Population Biology, Ecology and Evolution

2013

ABSTRACT

Studying the immune system in the context of ecology has been critical for our understanding of host-parasite interactions. Fundamental to this approach is the recognition that immunity is costly to organisms. From this research field we have learned that internal constraints caused by the high costs of immunity have frequently led to measurable variation among individuals in parasite defense. Understanding the factors influencing the evolution of host defenses is of interest not just because of the devastating effects of parasites on host populations, but also for understanding a major class of interspecific interactions.

However, studies frequently fail to measure immune costs. One explanation is that immune responses only impact host fitness under certain ecological conditions. Studies have implicated host nutrition as one such condition, but it is likely that other ecological factors play an important role as well. In this dissertation, I use pea aphids (*Acrythosiphon pisum*) and their natural microbial communities to study how ecological factors influence susceptibility to pathogens and the link between immunity and host fitness. I first measure aphid reproductive fitness in response to several natural microbial pathogens, and demonstrate that exposure to several aphid-specific fungal pathogens is costly. I then show that the expression of these costs is influenced by ecological factors: exposure to environmental stressors that lead to the production of a winged dispersing morph, and the presence of intracellular bacteria that protect their hosts from fungal pathogens. By combining experiments, immune assays, and measures of gene expression, this work aims to increase our understanding of the link between immunity and host fitness, and to enhance our mechanistic understanding of immune costs.

ECOLOGICAL FACTORS DETERMINE IMMUNE COSTS AND SUSCEPTIBILITY OF PEA APHIDS TO A NATURAL FUNGAL PATHOGEN

By

Benjamin James Parker
A.B. The University of Chicago, 2007

Advisor: Nicole M. Gerardo

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Science—
Population Biology, Ecology and Evolution

2013

ACKNOWLEDGEMENTS

This work was supported by Emory University, NSF grant IOS-1025853 to Nicole Gerardo, an NIH Training Grant in Population Biology of Infectious Diseases (T32-AI055404), and an NSF Graduate Student Fellowship to Benjamin Parker.

I could not have asked for a better advisor than Nicole Gerardo. I am looking forward to our future collaborations. Thanks to my thesis committee, Meghan Duffy, Kerry Oliver, Jaap de Roode, and Todd Schlenke, for their insightful feedback over the years. Thanks to the Gerardo lab and the PBEE program, especially Alice, Anand, Eleanore, Erin, Lynn, Nancy, Nelle, Josh, Justine, Pearl, & Stephanie, all of whom have helped make the last five years fly by. Lastly, thanks to my mom the librarian, Melinda Welch, who always ensures that at least one person is looking up my papers on the internet, and my dad, Stephen Parker, PhD, who through words and example taught me how to take on big projects one day at a time.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

COSTS OF IMMUNITY

Organisms face a diverse array of pathogens and parasites, and have evolved a set of defenses to protect themselves from infection. Genetics and molecular biology have uncovered many of these immunological mechanisms, and have focused primarily on how they work. The emerging field of 'ecological immunology,' in contrast, explores the impact of pathogens on host life-history traits and population dynamics with specific reference to the mechanisms of immunity in their environmental context (Schulenburg et al. 2007; Sadd and Schmid-Hempel 2009). Fundamental to this approach is the recognition that immunity is costly to organisms in both an ecological and evolutionary context (Rolff and Siva-Jothy 2003).

The simplest empirical demonstrations of the idea that parasite defenses are costly come from studies that directly measure fitness traits (Ahmed et al. 2002; Armitage et al. 2003). For example, bacterial infection decreases the reproductive success of burying beetles (Cotter et al. 2010), and exposure to a vaccine decreases parental effort in blue tits (Råberg et al. 2000). These studies demonstrate that there are fitness consequences of the activation of immune mechanisms. Similarly, increasing investment in life-history traits can have consequences for immunity. For example, inducing faster developmental rate in juvenile *Daphnia* increases susceptibility to a bacterial pathogen (Allen and Little 2011). In addition, immune mechanisms can be costly even in the absence of

pathogens and parasites. For example, immunodeficient *Drosophila* have longer life-spans compared to immunocompetent flies (Valtonen et al. 2010), suggesting that the maintenance of immune mechanisms can be costly even when an immune response is not elicited.

Another approach to studying immune costs focuses on evolutionary trade-offs between fitness and immunity. Early studies showed tradeoffs in response to artificial selection for parasite defense. For example, *Drosophila* larvae that were selected for wasp resistance showed a reduced ability to compete for resources with unselected lines (Kraaijeveld and Godfray 1997), which was show to be a result of reduced feeding rate (Kraaijeveld et al. 2001). Other studies have shown negative pleiotropy between increased immunity and other traits (Kraaijeveld and Godfray 1997; Kraaijeveld et al. 2001; Sadd and Schmid-Hempel 2006; Cotter et al. 2010; Valtonen et al. 2010), for example with a negative correlation between *Drosophila* genotypes with high fecundity in the absence of infection and those that are resistant to bacterial infection (Mckean et al. 2008). These studies suggest that parasite defense can also be costly in an evolutionary context.

IMMUNE COSTS GENERATE VARIATION IN HOST DEFENSES

The most obvious way that immune costs influence host-pathogen coevolution is by generating variation among hosts in parasite defense. An important distinction needs to be made between two types of host defenses: mechanisms of resistance, which actively reduce parasite burden, and mechanisms of tolerance, which limit the impact of infection on host fitness without reducing parasite

infection or growth. In populations exposed to parasites, hosts that are able to resist parasites have high fitness, and pressure from parasites therefore selects for parasite defenses. This in turn reduces parasite prevalence, and makes costly defense mechanisms less beneficial, leading to negative frequency-dependent selection and variation among individuals in resistance (Lively and Dybdahl 2000; Woolhouse et al. 2002; Gandon et al. 2008). Costs of resistance can therefore contribute to the maintenance of variation in parasite defense among hosts.

Like resistance, mechanisms of tolerance protect host fitness and therefore benefit hosts. But in contrast to mechanisms of resistance that reduce pathogen fitness, mechanisms of tolerance benefit pathogens by increasing transmission and pathogen prevalence (Roy and Kirchner 2000; Miller et al. 2005; Svensson and Råberg 2010). Tolerance mechanisms are therefore predicted to generate positive feedback and the absence of genetic variation. The role of immune costs in the evolution of tolerance is less clear, but some studies have suggested that there can be costs to parasite tolerance (Kraaijeveld and Godfray 2008). More concretely, empirical studies have shown negative correlations between resistance and tolerance (Fineblum and Rausher 1995), and theoretical work has shown that these tradeoffs, coupled with immune costs, can lead to variation in tolerance (Best et al. 2008).

Variation in pathogen defenses has been tied to polymorphisms in genes of the immune system. For example, allelic diversity at a specific locus in *Arabidopsis thaliana* explained patterns of resistance to microbial infection and herbivory (and also explained why more resistant *A. thaliana* strains had slower

leaf production and lower biomass) (Todesco et al. 2010). Similarly, genetic polymorphisms have been linked to variation in *Drosophila* resistance to several pathogens (*e.g.* bacteria (Lazzaro et al. 2004); fungi (Tinsley et al. 2006)), and this variation has been linked to patterns of gene expression (Ye et al. 2009).

INVESTMENT IN IMMUNITY AND CONTEXT-DEPENDENT COSTS:

A LIMITED RESOURCE POOL

The idea that immune responses are costly for hosts is supported by a growing number of empirical studies, yet there are also studies that have failed to measure immune costs (*e.g.* in European starlings (Williams et al. 1999); in *Daphnia* resistance to a bacterial pathogen (Labbé et al. 2010)). In addition there are a number of studies that only measure immune costs under certain conditions, for example, in conditions of starvation or low nutrient availability (Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000; Mckean et al. 2008). In the first of these studies, healthy bumblebees were shown not to suffer from immune activation while starved bees showed a decrease in survival after immune activation (Moret and Schmid-Hempel 2000). The explanation for these context-dependent immune costs is that in resource-rich conditions, the pool of available resources is sufficient to produce an immune response without negatively influencing measured fitness-traits, but in resource-limited conditions immunity trades-off with fitness.

This idea is supported by another area of research showing that individuals raised in optimal environmental conditions can invest more in immune responses

than those raised in poor conditions (Triggs and Knell 2011). Of particular interest is the influence of nutritional status on immunity. Some studies have linked changes in susceptibility to pathogens or parasites to differences in food availability (Vass and Nappi 1998; Klemola et al. 2007); others have linked such changes to food quality (Lee et al. 2006; 2008). Differences in host susceptibility have been explained by changes in specific mechanisms of immunity—for example, dietary protein quality was shown to influence lysozyme-like activity in caterpillar larvae (Myers et al. 2011), and nutritional deprivation was shown to down-regulate phenoloxidase activity in beetles (Siva-Jothy and Thompson 2002).

Together, these studies suggest that immune mechanisms are costly to hosts only under certain ecological conditions. Host nutrition has been identified as one such condition, but it is possible that there are other ecological factors that influence the expression of costs as well. In this thesis, I use pea aphids (*Acrythosiphon pisum*) and their suite of associated microbes with the goal of gaining a better understanding of the role of ecological factors in how the fecundity costs of immunity are expressed. In chapters 3 and 6 of this thesis, I examine the roles of two ecological contexts: a stress-induced wing polyphenism, and associations with symbiotic bacteria, as factors influencing the expression of immune costs. A study of immune costs using aphids that ignored these contexts would not have found immune costs in this system, and this work therefore suggests that our lack of understanding of the context-dependency of the expression of costs represents a significant gap in our understanding of

ecological immunology. Immune costs are an important factor underlying host-pathogen coevolution, and understanding the ecological conditions under which immunity influences host fitness is critical for our general understanding of host-pathogen interactions.

THE INVERTEBRATE IMMUNE SYSTEM

Insects are particularly attractive for studies of immune costs because they have relatively simple immune systems and they are amenable to experimentation. Insects do not have an adaptive, antigen–based response typical of vertebrates. They do, however, have an innate immune response, which is typically divided into cellular and humoral responses. Cellular responses are mediated by blood cells, which include encapsulation, phagocytosis, and melanization (Strand 2008). These responses are induced immediately after recognition of a foreign invader, and it has been suggested that cellular responses clear a large proportion of pathogens quickly after infection (Haine et al. 2008). Humoral responses are characterized by signaling cascades that culminate in the production and activation of immune cells or effector molecules that attack invasive organisms (Govind 2008). This component of innate immunity takes longer to generate, on the order of several hours (*e.g.* Lavine et al. 2005), and can persist for weeks (Sadd and Schmid-Hempel 2006). The specificity of insect humoral pathways is poorly understood (Dionne and Schneider 2008), but some studies have linked signaling pathways to defense against specific classes of parasites. For example, the immunodeficiency (IMD) pathway has been

implicated in defense against Gram-negative bacteria in *Drosophila* (Lemaitre et al. 1997).

Invertebrates also defend themselves from infection without using the conventional mechanisms of the immune system (Figure 1-1). Hosts can reduce the likelihood they are infected by reducing the probability that they are exposed to a parasite. For example, the honey bee (*Apis mellifera*) displays a social grooming behavior that actively removes *Varroa* ectoparasitic mites from colony members, a behavior that appears to be a main mechanism of resistance to the parasite (Sammataro et al. 2000). Hosts can also sequester compounds that reduce infection probability–a form of 'prophylactic self-medication'. By mixing resin (a plant material rich in volatile compounds) with nest material, wood ants (*Formica paralugubris*) can increase their survival rates if challenged with bacterial and fungal parasites (Chapuisat et al. 2007). Hosts can also reduce the growth or burden of parasites once infection has occurred. For example, parasitoid-infected arctiid caterpillars (*Grammia incorrupta*) can increase the uptake of anti-parasitic chemicals from their food, which reduces parasitoid success (Singer et al. 2009). Also, parasite-infected monarch butterflies (*Danaus plexippus*) preferentially lay their eggs on plants that reduce parasite growth in their offspring (Lefèvre et al. 2010). These are two forms of therapeutic medication. The microbial symbiont community of a host can also influence parasite growth after infection. Invertebrates are often protected from parasites by symbiotic bacteria (Haine 2008; Brownlie and Johnson 2009), which can strongly influence population dynamics (e.g. Jaenike et al. 2010). For example,

southern pine beetles harbor a beneficial fungus that provides nourishment for developing larvae. This beetle fungus mutualism can be disrupted by a second, pathogenic fungus. Recent work has shown that an actinomycetous bacterium produces antibiotics that inhibit the pathogenic fungus, showing a complex relationship between host and multiple microbes that influences disease dynamics (Scott et al. 2008). Lastly, even if they cannot reduce the probability of infection or reduce parasite growth, some hosts can compensate for the fitness lost from infection through 'fecundity compensation'. This is where an individual changes the timing of her reproductive efforts in response to parasitism. The water-flea (*Daphnia magna*) produces more offspring early in life when exposed to the microsporidian parasite *Glugoides intestinalis* compared with uninfected controls (Chadwick and Little 2005; Vale and Little 2012).

Our current model of insect innate immunity relies heavily on a few model organisms (*e.g., Drosophila melanogaster* (Lemaitre et al. 1997; Lemaitre and Hoffmann 2007), *Anopheles gambiae* (Christophides et al. 2004)*, Tribolium castaneum* (Zou et al. 2007)*.* The immune systems of some insects, however, differ from these models of innate immunity (Smith et al. 2011a,b). For example, annotation of the immune system of the honey bee revealed that they have approximately one-third as many immune-system genes as *Drosophila* or *Anopheles* mosquitoes, and that expected genes are missing from multiple pathways. Some have suggested that social defenses such as hygienic and grooming behaviors are effective in reducing parasite pressure, and as a result honey bees are not as dependent on the mechanisms of innate immunity. The

exploration of the immune systems of non-model invertebrates has called into question the generality of our current picture of insect immunity, and suggests that complicated interactions between ecological factors and various forms of immune defense shapes animal immune systems.

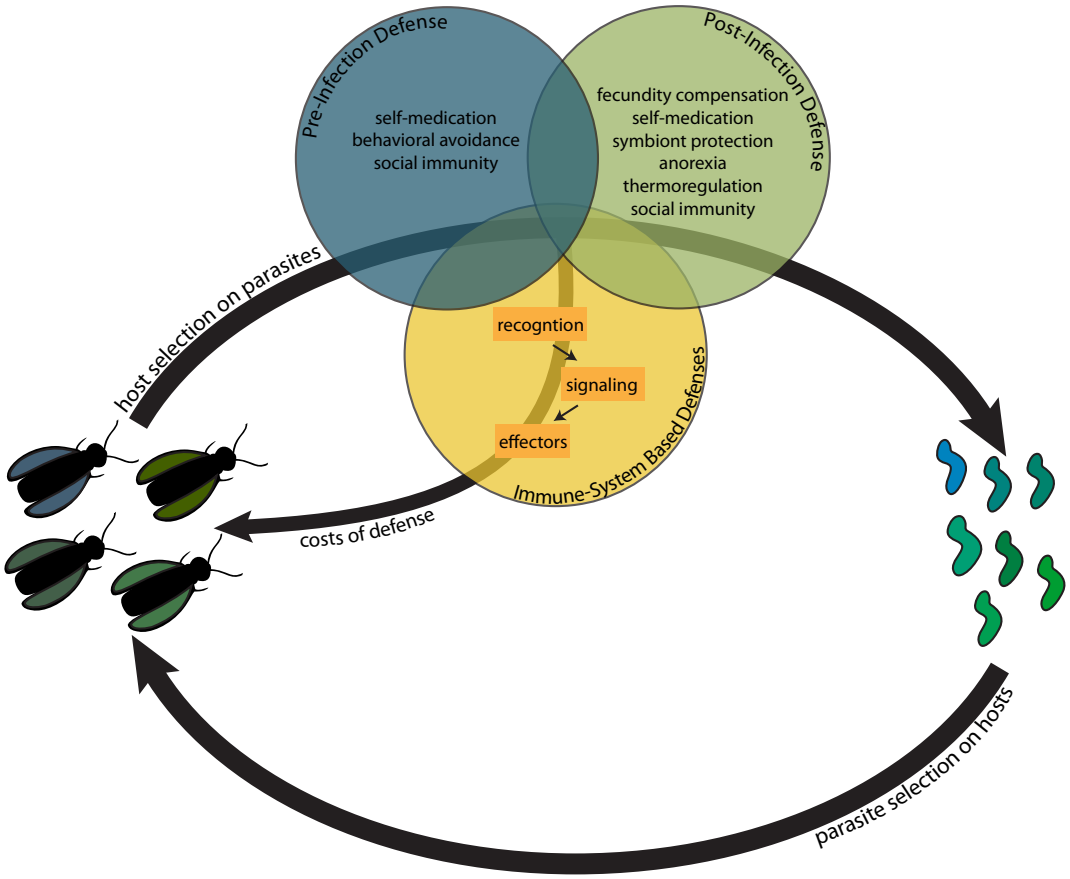


Figure 1-1. An expanded view of host–parasite coevolution. Host–parasite coevolution is driven by parasites selecting for host defense (bottom arrow) and host defense selecting for parasites that can overcome this defense (top arrow). Host selection on parasites is mediated not only through evolution of immune system-based pathways and processes, but also by pre-infection and post-infection mechanisms of non-immunological defense. Non-immunological and immune system-based defenses in turn influence the evolution of each other as well as other host processes due to the cost of defense (middle arrow).

APHIDS AND THEIR MICROBIAL COMMUNITY

Aphids are soft–bodied, hemimetabolous insects that feed on plant phloem and cause hundreds of millions of dollars in lost agricultural production each year (Brisson and Stern 2006). They face infection by a diverse array of pathogens and parasites, including parasitoid wasps, viruses, bacteria, and fungal pathogens, some of which have been shown to cause significant decline of natural aphid populations (VandenHeuvel et al. 1997; Hufbauer 2002). Aphids are also dependent on microbes for their survival. All aphids harbor intracellular Gram–negative bacteria called *Buchnera aphidicola*, which have the ability to synthesize the required amino acids that are not available from a diet of plant phloem. In addition, some aphids also harbor one or more additional Gram–negative bacterial symbionts, the best characterized of which are *Hamitonella defensa, Serratia symbiotica*, and *Regiella insecticola* (Moran and Telang 1998). Pea aphids and their associated microbial partners have emerged as the primary insect system to study host-symbiont interactions due to extensive genomic resources available for both aphid and bacterial associates (International Aphid Genomics Consortium 2010; Hansen et al. 2012), the relative ease with which their diverse symbionts can be systematically manipulated (*i.e.* introduced or eliminated) within or among clonal aphid lines (Oliver et al. 2010), and their utility in experimental studies investigating natural host-enemy interactions (Henter and Via 1995; Ferrari and Godfray 2003).

Experimental studies have documented a wide range of ecologically important traits conferred to aphids by harboring facultative symbionts (Oliver et

al. 2010). The focus of this thesis is on one common gamma-proteobacterial secondary symbiont, *Regiella insecticola*. *Regiella* has been shown to confer protection to aphids against several entomopathogenic fungi (Scarborough et al. 2005; Lukasik et al. 2012; Parker et al. 2013) including *Pandora neoaphidis*, a pea aphid fungal pathogen. In chapter 5, I expand our understanding of this protection by showing that *Regiella* protects aphids from several species of aphid specialist fungal pathogens, but not against a generalist insect fungal pathogen. It is not known how this protection works, though hypotheses include the production of toxins that directly impact fungal growth or that *Regiella* provides resources that boost pea aphid immunity (Brownlie and Johnson 2009). Because *Pandora* must kill its host in order to complete its life cycle, strong selective forces exist that shape the characteristics and maintenance of this defensive symbiosis.

Though they are beneficial under certain ecological conditions, recent work has also shown that infection with the facultative symbionts has a negative effect on aphid fitness (Vorburger and Gouskov 2011). Symbionts are expected to have their own requirements for energy, which they must obtain from their hosts (Haine 2008). Although such energetic costs have not been measured directly, they have been measured indirectly. Oliver et al. (2008) conducted cage experiments that monitored the population dynamics of aphids infected with *Hamiltonella defensa* (a facultative symbiont that has been found to offer protection against parasitoid wasps) (Oliver et al. 2003). The frequency of aphids harboring *H. defensa* increased in the presence of parasitic wasps. However, the

frequency of aphids harboring the symbiont decreased in the absence of parasites, suggesting that harboring symbionts is costly.

It is perhaps no surprise that symbiont-mediated protection against *Pandora* arose in aphids, as this fungus is one of its host's primary natural pathogens (Pell et al. 2001). Aphid-*Pandora* interactions have been extensively studied because *Pandora* is used as a means of biocontrol for this important agricultural pest (Hajek and Delalibera 2010; Jackson et al. 2010). For many years aphids were used as an example of the tremendous clonal variation found among wild populations in susceptibility to fungal pathogens (Henter and Via 1995; Ferrari et al. 2001). Initially it was assumed that this variation was due to genetic differences among aphid clones, but protection from *Regiella* brings this assumption into doubt. However, in chapter 4 I study variation among aphid lineages, and find that genotypes vary in their ability to resist *Pandora* infection in the absence of protective symbionts. This finding suggests that variation among clones is likely due to a combination of genetic variation in host immunity, variation in symbiont presence, and variation in symbiont genotype.

Symbionts may, however, have had an impact on the evolution of the aphid immune system. As part of the recent manual annotation of the newly available pea aphid genome sequence, we identified genes thought to be involved in the aphid immune system based on homologs of other insects sequenced to date (e.g. *Drosophila melanogaster, Anophales gambiae, Tribolium castenatum*) (Gerardo et al. 2010). These experiments provided us with a preliminary picture

of what the aphid response to a variety of pathogens might look like, and in particular, indicated that genes thought to be part of responses to fungal pathogens in other insects might be present in the aphid genome (Figure 1-2). However, our annotation efforts also revealed that aphids are missing some genes thought to be critical for immunity. Annotation of the pea aphid genome showed that pea aphids are missing much of the immune deficiency (IMD) pathway, are lacking peptidoglycan recognition proteins (PGRPs) (Gerardo et al. 2010), and produce no homologs of known antibacterial peptides (Altincicek et al. 2008). Functional studies found a surprisingly reduced upregulation of transcripts and proteins upon infection in aphids compared with other insects. This suggests that aphids have a much different immune system than other insects studied to date (Gerardo et al. 2010). While alternative immune-system based defenses might still be uncovered through additional functional assays, one explanation for this reduction is that the benefits afforded to the aphid by obligate and facultative symbionts have selected for reduced use of the immune system in the host to facilitate these symbiotic relationships. Alternatively, symbiont-conferred resistance might cause immune mechanisms to become redundant (Altnicicek et al. 2008).

Overall, our understanding of the aphid immune system from the aphid genome project, the variety of pathogens and parasites developed for lab assays, and a depth of research on the ecology of aphids and their microbial communities make aphids an ideal system to explore the role of ecological factors in aphid immunity. In chapter two I first test for costs of immunity to a

broad array of pathogens, and measure fecundity costs of resistance to fungal pathogens. In chapter three I show that these costs are limited to a winged polyphenic morph, and identify mechanisms of immunity that may explain this pattern. Immune costs can contribute to the maintenance of variation in susceptibility among individuals, and in chapter four I measure variation in susceptibility among aphid genotypes in the absence of protective symbionts. In chapter five I test for symbiont-conferred protection against several fungal pathogens, and suggest that pathogen specialization may be necessary for symbiont-conferred protection to evolve. Lastly, in chapter 6 I show that protective symbionts are also an important ecological factor underlying the expression of immune costs. Overall, this work demonstrates a role for several previously unrecognized factors in determining host susceptibility to pathogens and in determining the link between immunity and fitness, and in general highlights the importance of ecological context for studies of host-pathogen interactions.

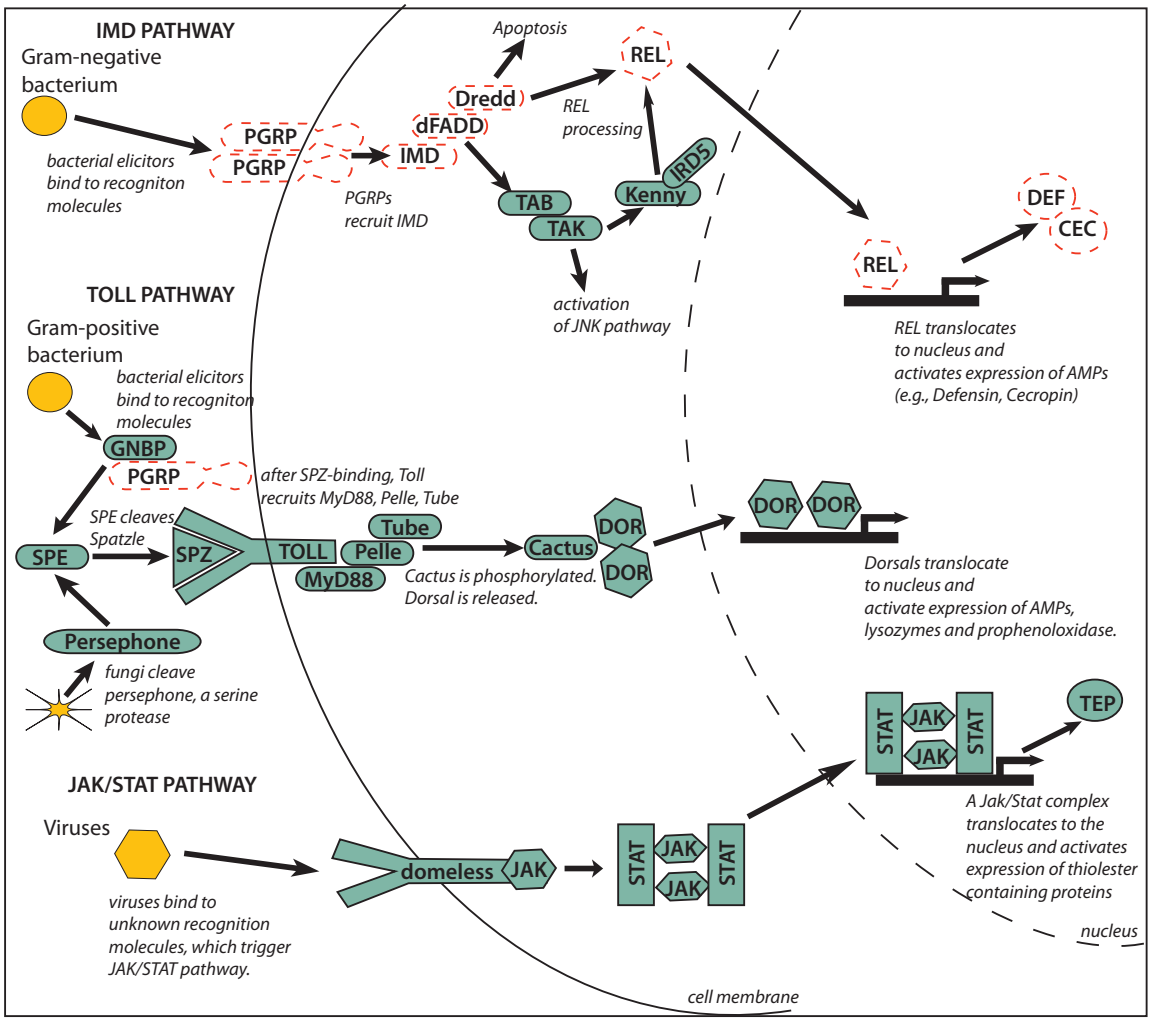


Figure 1-2: Pea aphid humoral immunity: Studies of several invertebrate immune systems have indicated that innate immune signaling pathways, shown here, are conserved across insects. Many of the genes involved in these pathways were found in pea aphids (shown in green), but a number of genes thought to be critical to these pathways were found to be missing in pea aphids (shown in red dotted lines).

CHAPTER 2: EXPOSURE TO NATURAL PATHOGENS REVEALS COSTLY APHID RESPONSE TO FUNGI BUT NOT BACTERIA

ABSTRACT

Immune responses are costly, causing trade-offs between defense and other host life history traits. Aphids present a special system to explore the costs associated with immune activation since they are missing several humoral and cellular mechanisms thought to be important for microbial resistance, and it is unknown whether they have alternative, novel immune responses to deal with microbial threats. Here we expose pea aphids to an array of heat-killed natural pathogens, which should stimulate immune responses without pathogen virulence, and measure changes in life-history traits. We find significant reduction in lifetime fecundity upon exposure to fungal pathogens, but not to bacterial pathogens. This finding complements recent genomic and immunological studies indicating that pea aphids are missing mechanisms important for bacterial resistance, which may have important implications for how aphids interact with their beneficial bacterial symbionts. In general, recent exploration of the immune systems of non-model invertebrates has called into question the generality of our current picture of insect immunity. Our data highlights that taking an ecological approach and measuring life-history traits to a broad array of pathogens provides valuable information that can complement traditional approaches.

INTRODUCTION

Invertebrates rely on innate immune mechanisms for protection against diverse parasitic organisms. Our current model of insect innate immunity relies heavily on knowledge from relatively few model organisms (*e.g., Drosophila melanogaster* (Lemaitre and Hoffmann 2007), *Anopheles gambiae* (Christophides et al. 2004)*, Tribolium castaneum* (Zou et al. 2007))*.* The immune systems of some insects, however, differ from these models of innate immunity (Evans et al. 2006; Smith et al. 2011a,b), questioning the generality of our current picture of insect immunity. For example, genomic and experimental studies using traditional immunological approaches revealed that pea aphids (*Acyrthosiphon pisum*) seem to lack many critical immune genes (*e.g.,* bacterial recognition molecules, common antimicrobial peptides, are missing much of the IMD (immunodeficiency) pathway) (Gerardo et al. 2010), have relatively few hemocytes (Laughton et al. 2011a), have weakly functioning lysozymes (Altincicek et al. 2008), and have no detectable antimicrobial peptides via standard functional (Laughton et al. 2011a) and proteomic assays (Gerardo et al. 2010). We expect that exploration of immune mechanisms across more diverse hosts, which is being facilitated by the declining costs of genome sequencing and thus of comparative genomics, will lead to a growing number of examples of organisms that do not fit the conventional models of immunity. In these situations, it is difficult to determine whether organisms are unable to respond to particular parasites or whether they are responding to parasite challenge using unknown mechanisms. Addressing these two possibilities will facilitate

investigation of the evolution of host-microbe associations in many non-model systems of host-parasite coevolution and symbiosis.

Evolutionary theory suggests an alternative way to capture an immune response. Immune responses come at a high energetic cost, causing a trade-off between pathogen defense and other life history traits (Sheldon and Verhulst 1996; Rolff and Siva-Jothy 2003). Here we illustrate that by measuring life-history traits of organisms after pathogen exposure we can reveal immune responses that are recalcitrant to traditional approaches (Boughton et al. 2011). We expose aphids to several heat-killed natural aphid pathogens—two species of Gram-negative bacteria, a Gram-positive bacterium, and two species of aphid-specific entomopathogenic fungi—and measure fitness traits after exposure.

METHODS

Fungal pathogens. *Zoophthora occidentalis* and *Pandora neoaphidis* are both aphid specific fungal entomopathogens. We cultured *Zoophthora occidentalis* in 100 mL potato dextrose broth shaking at room temperature for two days. We then passed the total culture through a vacuum filter and scraped the filtered fungal culture into 250 μL Ringers solution. We cultured *Pandora neoaphidis* on plates of SDAEY (Fungi: Entomophthorales 1997) for 14 days, and scraped 1 cm^2 of fungal growth into 250 μL of Ringer's solution. To expose insects to fungal signals but not to pathogen virulence, we heat-killed both fungal pathogens by autoclaving the exposure solutions at 121°C for 20 minutes.

Bacterial Pathogens. We isolated bacteria from laboratory stock aphids by crushing individual sick aphids in 500 μL Carlson's solution and plating a portion of this solution onto Luria Broth (LB) plates, which were then cultured at 28°C overnight. We sequenced a portion of the 16s RNA gene (primers 27F: 5-AGAGTTTGATYMTGGCTCAG, 1492R: 5'- TACCTTGTTAYGACTT) and identified the bacteria using the Ribosomal Database Project (Cole et al. 2009), NCBI BlastN and phylogenetic analyses (data not shown). Strain Ng5b is *Enterobacter* c.f. *cloacae*, strain n1324b is *Bacillus* c.f. *pumilus*, and strain s8d is *Serratia* c.f. *fonticola.*

To assess pathogenicity of the bacteria strains, we plated bacteria onto LB from glycerol stocks and grew them overnight at 30°C. We then picked multiple colonies and grew then to OD_{600} = 0.5. We stabbed six-day old aphids (line 5A0) with a minutin pin dipped into either sterile LB (control) or the live bacterial solution. In two experiments (first experiment: control sterile stab, Ng5b, s8d; second experiment: control sterile stab, Ng5b, n1324b), we stabbed 12 aphids per treatment sub-cuticularly into the ventral side of the abdomen and to one side of the midline to avoid rupturing the gut. Thirty minutes after stabbing, aphids were transferred from sterile Petri dishes to fava bean plants and monitored for survival.

As with the fungal elicitors, to measure the costs associated with mounting an immune response rather than the damage caused by bacterial pathogen virulence, we exposed the insects to heat-killed pathogens. We cultured bacteria Ng5b and n1234b in LB overnight at 37°C, standardized a final volume of 250 μL

to OD_{600} = 0.5, spun each suspension at 2000 x g for two minutes and resuspended the pellets in 250 μL Ringers solution. To make a more concentrated solution of s8d, we followed the same procedure but resuspended a pellet from 2000 μL bacterial solution (OD_{600}= 0.5) in 250 μL Ringers solution. Finally, we heat-killed the bacteria by autoclaving the solutions at 121°C for 20 minutes.

Cost of Pathogen Signal Exposure

We maintained aphids asexually on fava bean (*Vicia faba*) plants in 16 hr light: 8 hr dark conditions at 20°C. We used aphid clones 5A0 (Oliver et al. 2003) and LSR1-01 (International Aphid Genomics Consortium 2010), which are free of secondary, facultative symbionts but harbor the obligate bacterial symbiont, *Buchnera aphidicola*. Pea aphids produce two distinct phenotypic morphs, a dispersing winged morph and a more sedentary unwinged morph. Immune costs are often context dependent, only appearing under energetically limiting conditions (*e.g.* Moret and Schmid-Hempel 2000). Therefore, we targeted costs of pathogen exposure in winged aphids since they have the additional energetic burden of producing wings and the associated musculature (Artacho et al. 2011). To induce the production of winged offspring, we exposed developing aphids to the alarm pheromone (E)-*β*-farnesene (EBF) (5 μL of 1000 ng/μL EBF every 48 hrs for 10 days). We then grew offspring of these EBF-exposed aphids for six days, and exposed them to a suspension of heat killed pathogen by stabbing them ventrally (Altincicek et al. 2011) in the thorax with a minutin pin

contaminated with heat killed pathogen solution. All aphids were born within 24 hours of one another to reduce differences among individuals. We allowed aphids to heal in a clean dish before we put them individually onto plants. We monitored survival and counted their offspring every 2-4 days. We removed offspring from plants after counting to prevent overcrowding, and trimmed the plants as necessary.

We conducted two experiments. In Experiment 1, we used heat-killed solutions of the bacterial pathogens *Enterobacter* Ng5b (Gram –) and *Bacillus* n1324b (Gram +) and the aphid-specific fungal pathogen *Z. occidentalis.* We also included two control conditions by stabbing aphids with sterile Ringers solution and by handling unstabbed aphids. We blocked Experiment 1 into two replicates, and used aphid genotype 5A0 (66 aphids per treatment). In Experiment 2, to extend our experiment to additional pathogen species and an additional aphid genotype, we stabbed aphids with either sterile Ringers solution, a solution of heat-killed bacterial pathogen *Serratia* s8d (Gram –), or a solution of heat killed *P. neoaphidis*. We used two genotypes (LSR1-01 and 5A0) and included 56 individuals per treatment per genotype.

STATISTICAL METHODS

We used survival analysis to confirm bacterial virulence, fitting a non-parametric (cox proportional hazard) model to analyze survival after confirming that the assumption of proportional hazards was met. We conducted a post-hoc multiple

comparisons test using the 'multcomp' package in R to determine which levels were significantly different within bacterial treatment.

For the first assay of costs to pathogen signal exposure (Experiment 1) we analyzed total reproduction, last day of reproduction, and day of death using analyses of variance (ANOVA), followed by Tukey's HSD *post hoc* tests, after Yeo-Johnson power transformations (lambda = 3.164, 1.964, 1.621 respectively, using the 'car' package in R) to correct for deviation from assumptions of normality and homogeneity of variance. We analyzed total reproduction from Experiment 2 in the same fashion (lambda = 2.306). In both experiments we excluded individuals that had fewer than 10 offspring or that died within the first six days, as these were likely damaged from the experimental exposure. We used R (2.10.0, R Development Core Team, 2010) for all analyses.

RESULTS

Confirming Pathogen Virulence

Both fungal pathogens, *Z. occidentalis* and *P. neopahidis* significantly reduce aphid survival upon infection (Ferrari et al. 2001; Parker et al. 2013). Exposure to each of the three bacterial strains used here also significantly reduced aphid survival (Supplemental Figure S2-1). In both infection assays, bacterial treatment significantly reduced survival (Infection 1 – ng5b and s8d, $\chi^2 = 40.51$, 2 Df, $P < 0.0001$; Infection 2 – ng5b and n1324b, $\chi^2 = 13.67$, 2 Df, $P = 0.001$). The Gram-negative *Enterobacter* bacterium ng5b was the most virulent, killing aphids

significantly faster than the Gram-negative bacterium *Serratia* s8d (z = 3.25, P = 0.003) and the Gram-positive bacterium *Bacillus* n1324b (z = 2.34, P = 0.048).

Costs of Pathogen Signal Exposure

Experiment 1. Exposure to heat-killed pathogens significantly influenced lifetime reproduction (Figure 2-1; $F_{4, 282} = 5.91$, $P < 0.001$) with aphids exposed to the entomopathogenic fungus *Z. occidentalis* having lower reproduction than any other exposure group, although it was statistically indistinguishable from aphids exposed to the Gram-positive bacteria n1324b. Exposure did not significantly influence the length of reproductive period or longevity (Figure S2-2 & S2-3, $F_{4, 282} = 2.10$, $P = 0.08$; $F_{4, 282} = 1.85$, $P = 0.12$, respectively). Block had a significant effect on total reproduction ($F_{1,282} = 11.69$, P = 0.0008), but there was no significant interaction between replicate block and exposure indicating that the treatment effects were consistent across blocks.

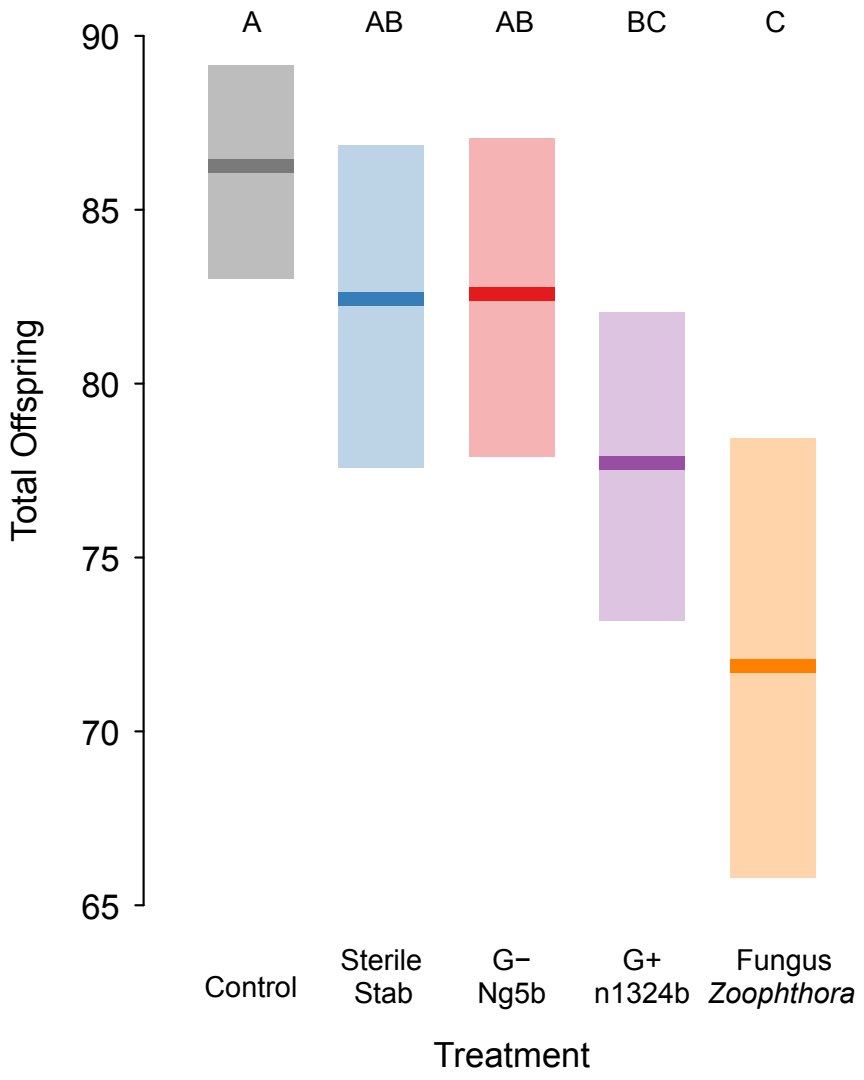


Figure 2-1. Mean lifetime reproduction ± bootstrapped 95% confidence intervals for naïve control aphids (clone 5A0) or aphids exposed to a sterile, Gram-negative, Gram-positive or fungal challenge (Experiment 1). Letters denote Tukey's HSD groups.

Experiment 2. Aphids of two genotypes given the second suite of heat-killed pathogens again had significantly reduced lifetime reproduction (Figure 2-2, $F_{2,}$

$_{302}$ = 25.26, $P < 0.0001$) with exposure to the fungal pathogen *P. neoaphidis* reducing fecundity. Exposure to the Gram-negative bacteria s8d did not significantly alter reproduction. The genotypes differed in overall fecundity, but there was no significant interaction between aphid genotype and treatment.

Figure 2. Mean lifetime reproduction ± bootstrapped 95% confidence intervals for aphids from two clones (5A0, LSR1-01) exposed to a sterile, Gram-negative or fungal challenge (Experiment 2). Letters denote Tukey's HSD groups.

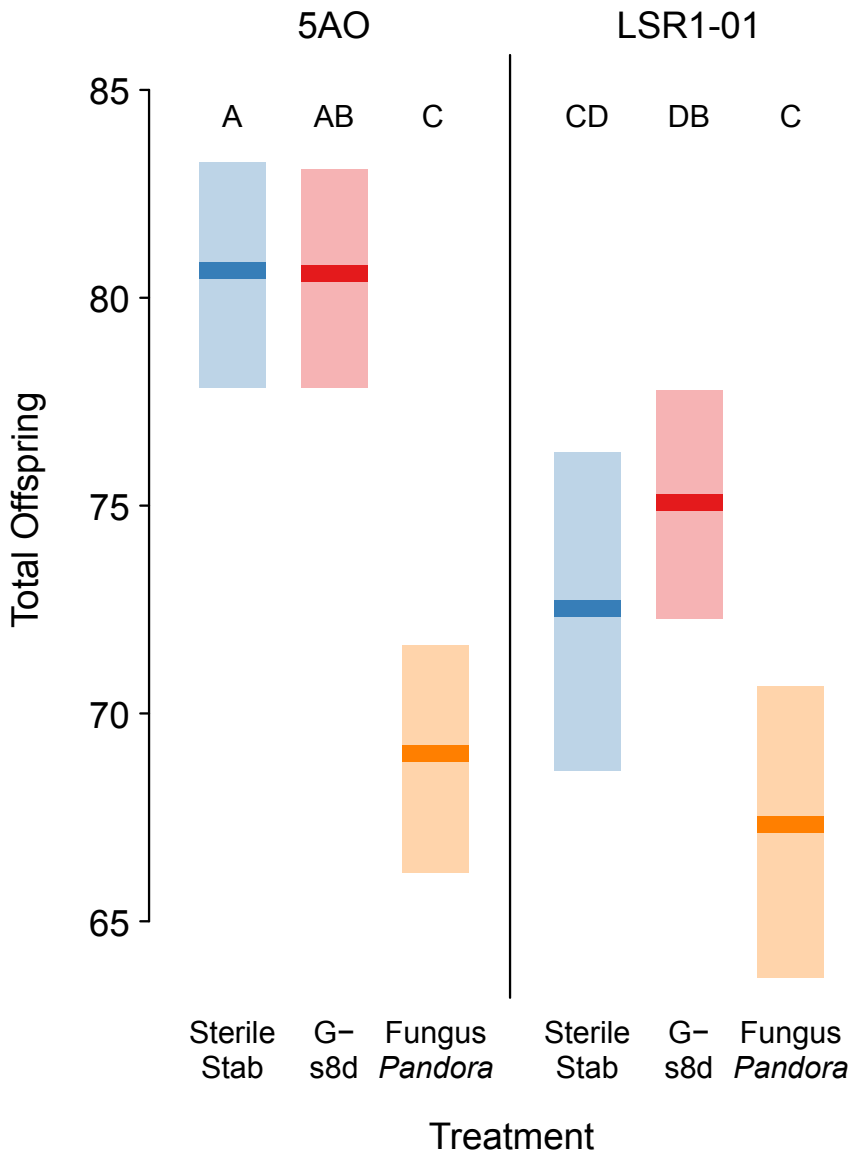


Figure 2-2. Mean lifetime reproduction ± bootstrapped 95% confidence intervals for aphids from two clones (5A0, LSR1-01) exposed to a sterile, Gram-negative or fungal challenge (Experiment 2). Letters denote Tukey's HSD groups.

DISCUSSION

Aphids suffered fitness costs after exposure to signals of two ecologically relevant fungal pathogens. There was no difference between sterile-stab and

unstabbed control aphids, indicating that the fitness loss was the result of pathogen exposure not of wounding. Exposure to heat-killed Gram-negative bacteria failed to significantly reduce any fitness measures, although exposure to the Gram-positive antigen did slightly reduce reproduction. To increase the likelihood of seeing a response to Gram-negative bacteria in Experiment 2 we used a higher concentration of bacterial elicitors, and yet we still did not detect a cost of immunity. Across the two experiments, we also used two species of Gram-negative bacteria, one highly and one moderately virulent (Figure S2-1) to limit the possibility of a lack of response being due to immune evasiveness of a selected pathogen. These results are consistent with previous studies that did not detect substantial immune responses to bacterial challenge using transcriptomic (Altincicek et al. 2008; Gerardo et al. 2010), proteomic (Gerardo et al. 2010) and immunological assays (Altincicek et al. 2008; 2011; Laughton et al. 2011a).

Thus, the fitness costs of an immune response can be detected in pea aphids, and these costs are only apparent when aphids are given fungal cues. Pea aphids lack most of the IMD pathway and many of the effector molecules presumed necessary to deal with infection (Gerardo et al. 2010). In this way we use the pea aphid immune system as a natural knockout, as it lacks one arm of the insect immune response. The specificity of insect humoral pathways is poorly understood (Dionne and Schneider 2008), but the IMD pathway is critical for fighting many Gram-negative bacteria in *Drosophila* (Lemaitre et al. 1997), and

studies have found changes in susceptibility to some fungal pathogens in IMD knock-out *Drosophila* (Dionne and Schneider 2008). Pea aphids do, however, retain other important pathways (*i.e.,* Toll, JNK and JAK/STAT pathways) and can phagocytose invading microbes (Laughton et al. 2011a; The cellular immune response of the pea aphid to foreign intrusion and symbiotic challenge. 2012). Our finding that aphids respond to fungus through a costly response opens the door for investigations into the molecular mechanisms behind aphid-fungal resistance and will facilitate study of the evolution of aphid-fungal pathogen interactions and how these interactions are shaped by the coupling of host immune responses and aphid bacterial symbionts known to confer protection against fungal pathogens (Scarborough et al. 2005; Parker et al. 2013). In general, when studying the immune system of a non-model host, it is not clear what parasite challenge one should study, and how to measure a fitness response to parasite challenge (Boughton et al. 2011). This work suggests that measuring life-history traits after pathogen exposure provides valuable information about natural host-pathogen interactions in non-model systems.

Acknowledgements
Thanks to Stephanie Chiang, Dan Sok, and Nancy Lowe for laboratory assistance, and Meghan Duffy for comments.

SUPPLEMENTARY FIGURES

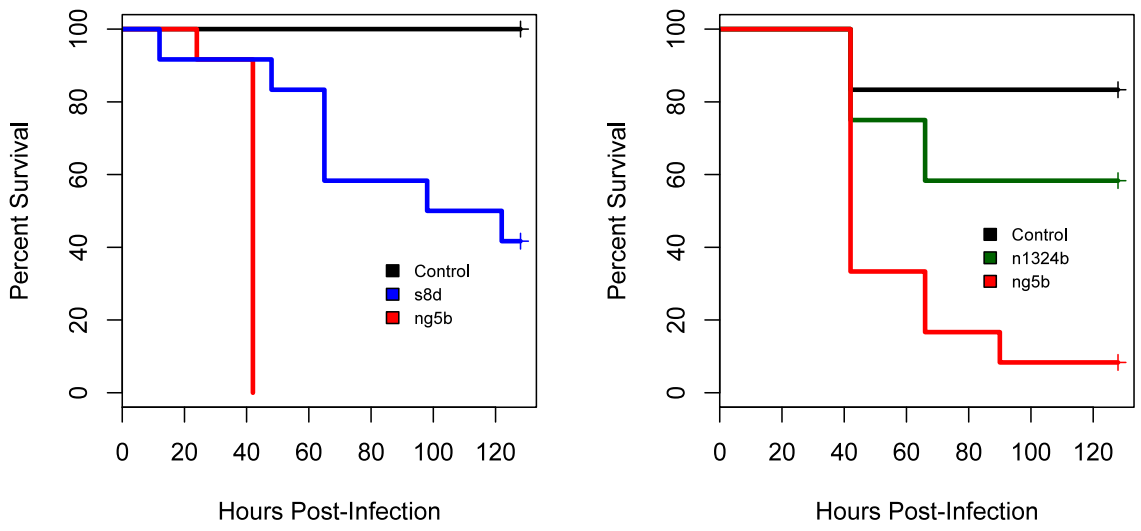


Figure S2-1. Survival curves from live bacterial virulence trials. The survival of aphids stabbed with a minutin pin dipped in sterile LB (Control) or live bacterial solution was monitored for five days. Two trials were conducted, **(Left):** comparing the two Gram negative bacteria (s8d and ng5b) and **(Right):** comparing the Gram positive bacteria (n1324b) with ng5b.

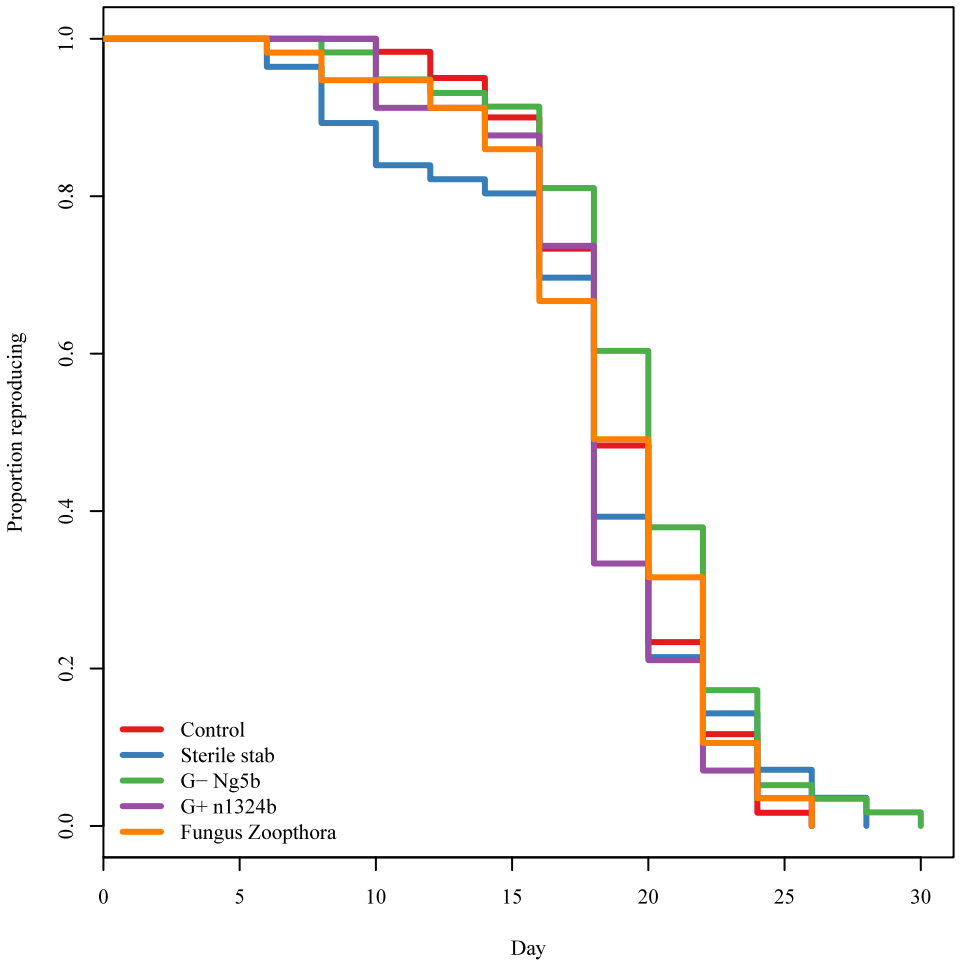


Figure S2-2. Proportion of aphids reproducing over time after challenge with heat killed pathogens or sterile stab.

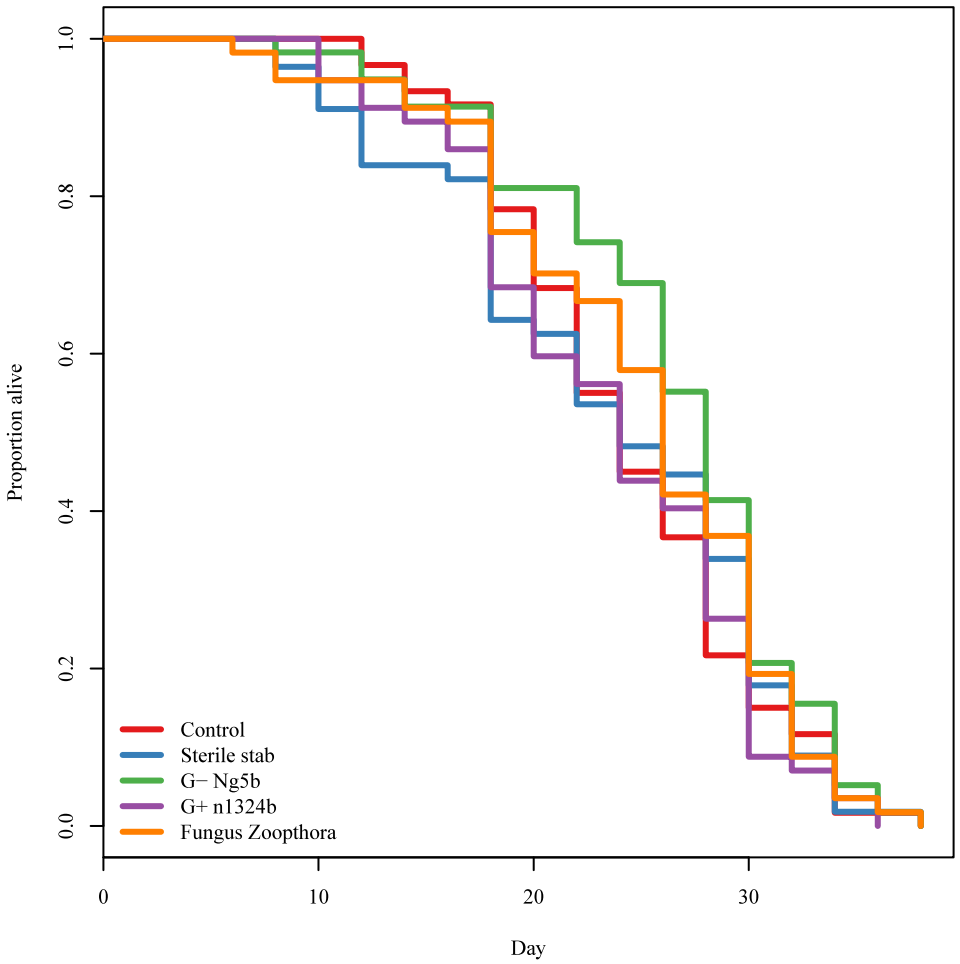


Figure S2-3. Proportion of aphids alive after challenge with heat killed bacteria or sterile stabs.

CHAPTER 3: INCREASED PATHOGEN SUSCEPTIBILITY AND IMMUNE COSTS IN A DISPERSAL POLYPHENISM

ABSTRACT

Animal dispersal is an important factor influencing host-pathogen interactions. Here we test the idea that dispersal can influence dynamics by increasing host susceptibility. We study a classic example of dispersal polylphenism, pea aphids (*Acyrthosiphon pisum*), which produce a winged morph in response to crowding, predation, or pathogen exposure. We first measure the fecundity costs of an immune response in both winged and unwinged morphs, and find that immune costs are limited to winged aphids. We then show that winged aphids are more susceptible to infection with a natural aphid-specific fungal pathogen (*Pandora neoaphidis*), and we tie this to differences between morphs in measures of cellular immunity and gene expression. Our findings are consistent with the idea that energetic limitations from costly wing production lead to decreased investment in immunity. This suggests that the costs associated with dispersal can influence host—pathogen ecology and evolution by increasing host susceptibility. Our findings also suggest that the pool of available resources in winged aphids is insufficient to produce an immune response without influence host fitness, showing that polyphenism can influence the expression of immune costs.

INTRODUCTION

Animal dispersal can influence the between-host transmission of pathogens and parasites, and therefore has important consequences for the ecological and evolutionary dynamics of host-pathogen interactions. Most studies of this link focus on the role of host dispersal in the spatial spread of pathogens (Boots and Mealor 2007; Best et al. 2011), exposure risk (Dwyer 1991; Parker et al. 2010), and in contacting new pathogen strains (Waldenström et al. 2002). An additional possibility is that dispersal might influence host-pathogen interactions because the associated energetic demands increase host susceptibility. Tests of this idea are rare, but a few studies have linked the effects of long-distance flight to changes in immune measures in migrating birds, suggesting that the energetic demands of migration can influence susceptibility through host immunity (Owen and Moore 2008a,b; Altizer et al. 2011).

The costs of dispersal are often discussed in the context of life-history. Studies focus on clear dispersal polyphenisms (e.g. winged vs. wingless or short-winged morphs (Simpson et al. 2011)), varying life-history strategies (Krug et al. 2012), or on variation in dispersal behavior (Hanski et al. 2006; Guershon and Ayali 2012), and have generally shown tradeoffs between dispersal and fitness (Karlsson and Johansson 2008; Bonte et al. 2012). Similarly, the field of ecological immunology has demonstrated life history tradeoffs between immunity to pathogens and host fitness, indicating that immunity is costly for hosts in terms of evolving (Kraaijeveld and Godfray 1997; Kraaijeveld et al. 2001; Valtonen et al. 2010) and using (Jacot et al. 2004) immune mechanisms. Fundamental to this

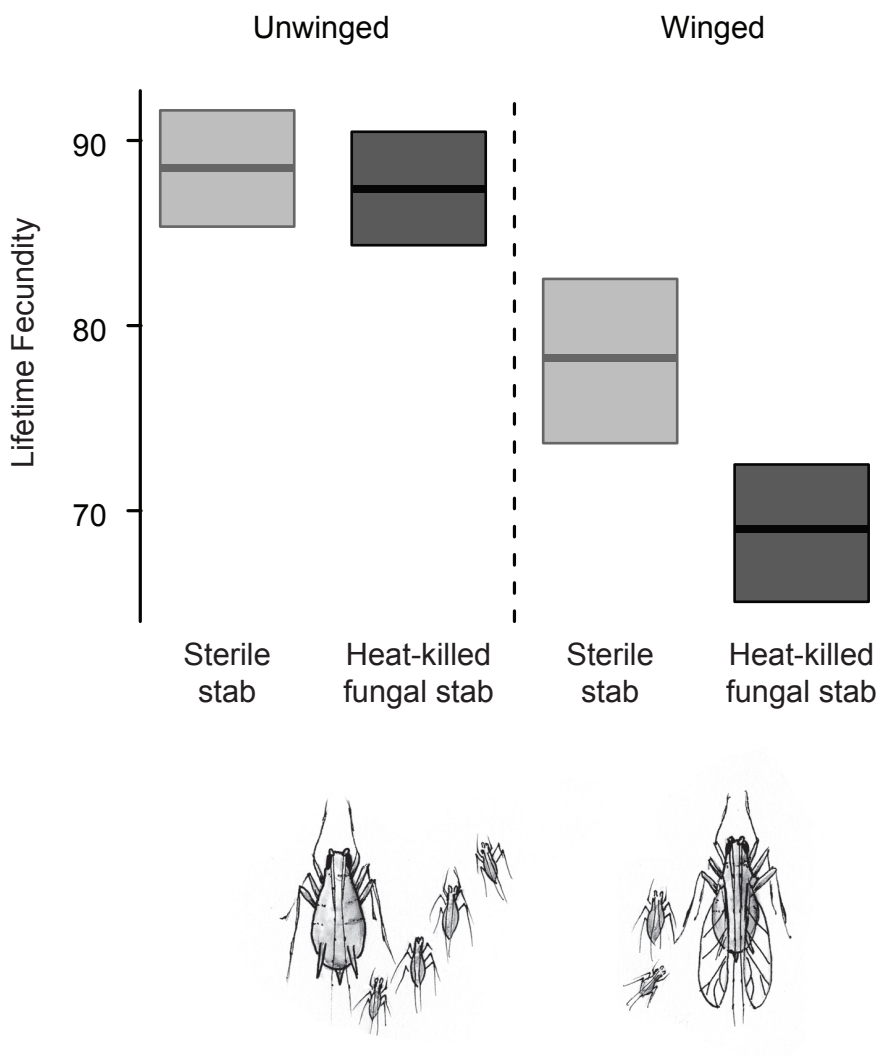
approach is the idea that a limited pool of resources fuels the immune system and other fitness-related traits (Hamilton and Zuk 1982; Simms and Rausher 1987). This idea is further supported by work linking differences in susceptibility to parasites and immune function to host nutritional condition (Vass and Nappi 1998; Lee et al. 2006; Klemola et al. 2007; Lee et al. 2008; Myers et al. 2011). For example, starvation increases the susceptibility of *Rhodinus prolixus* bugs to bacterial infection (Feder et al. 1997), and was shown to down-regulate immune activity in beetles (Siva-Jothy and Thompson 2002).

Here we investigate the possibility that the energetic demands associated with the production of a dispersing morph lower the resource pool available for mounting an immune response and increase host susceptibility. We use a classic example of wing polyphenism, the pea aphid *Acrythosiphon pisum* (Brisson and Stern 2006), where the production of a winged, dispersing morph is triggered by exposure to signals of danger, including high host density (Brisson and Stern 2006), predation (Kunert et al. 2005; Balog et al. 2013), or exposure to fungal pathogens (Hatano et al. 2012). Dispersing aphids invest in wings and associated musculature, and as a result winged aphids have been shown to have different energetic requirements (Hatano et al. 2010) and produce fewer offspring than genetically identical unwinged aphids (Groeters and Dingle 1989). We first show that the costs of mounting an immune response to a heat-killed fungal pathogen signal are limited only to the dispersing morph. We then show, using live infections, that winged aphids are more susceptible to fungal infection than unwinged aphids, and we uncover differences in immune mechanisms between

morphs. These results suggest that increasing pathogen susceptibility is an additional and under-recognized mechanism by which animal dispersal can influence host-pathogen interactions.

RESULTS & DISCUSSION

Our goal was first to measure the fitness consequences of immune responses in aphids. We measured reproductive fitness in winged and unwinged aphids by counting offspring produced over the course of an aphid's lifespan. To assess the impacts of immune reaction without the consequences of pathogen virulence, we measured the fitness of control aphids vs. those exposed to a heat-killed pathogen (*Pandora neoaphidis*, a natural aphid-specific fungal entomopathogen). We found that winged aphids had lower lifetime fecundity than unwinged aphids, and that aphids exposed to heat-killed *Pandora* solution had lower fecundity (Table 3-1, Figure 3-1), confirming the results presented in Chapter 2. Most importantly, there was also a significant interaction between exposure and morph (Table 3-1, Figure 3-1), and post-hoc tests found no effect of *Pandora* exposure on unwinged aphids, suggesting that the effect of *Pandora* was limited to winged aphids. These findings were consistent across three experimental replicates, each using a different pea aphid genotype (Table 3-1), although there were differences among replicates in overall fecundity (Table 3-1, Supplementary Information 3A). We found no effects of heat-killed *Pandora* on aphid survival (Supplementary Information 3B).



3-1) Fecundity cost of exposure to heat-killed *Pandora*: We measured lifetime reproductive fitness of winged (left panel) and unwinged (right panel) aphids that were either stabbed with a needle dipped in sterile PBS (light grey) or with a needle dipped in a solution of PBS and heat-killed *Pandora* mycelium (dark grey). Boxes show the 95% confidence estimates of lifetime fecundity.

Table 3-1

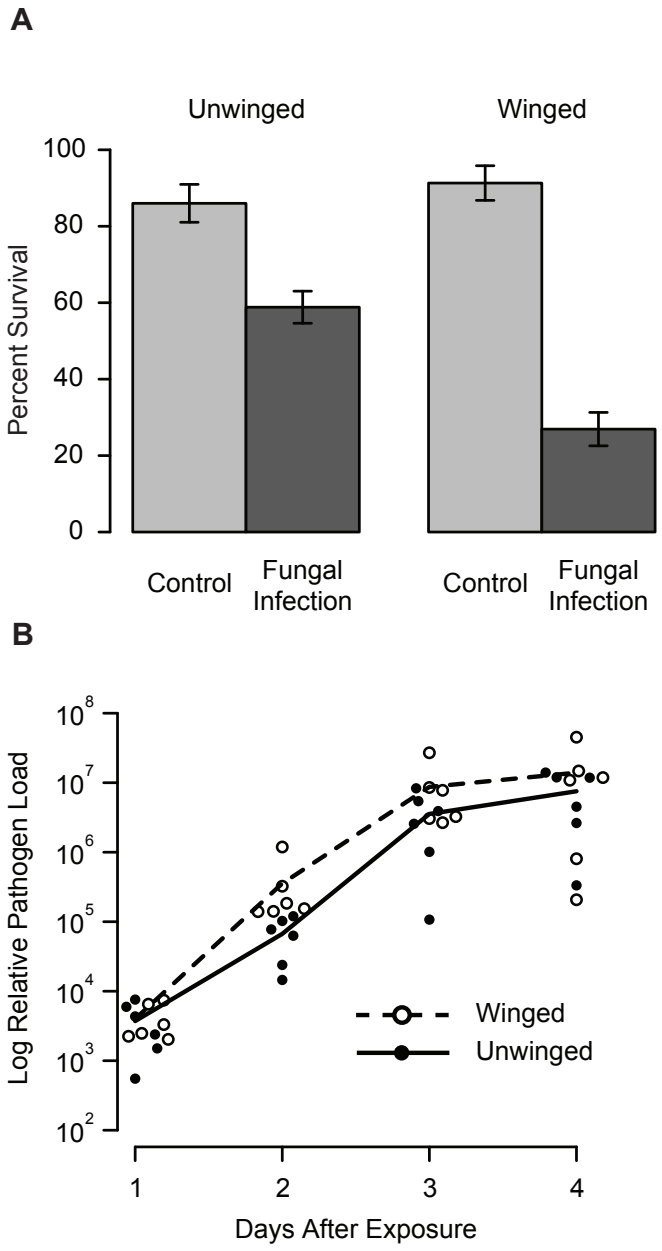
	Test Statistic	d.f.	*p*
Cost of Exposure			
Morph	F = 113	1	p < 0.0001
Exposure	F = 5.33	1	p = 0.02
Block/Genotype	F = 69.6	2	p < 0.0001
Morph * Exposure	F = 6.87	1	p = 0.009
Morph * Block/Genotype	F = 11.2	2	p < 0.0001
Exposure * Block/Genotype	F = 1.25	2	p = 0.28
Morph * Exposure * Block/Genotype	F = 0.076	2	p = 0.92
Cost of Exposure – Post-hoc comparisons			
Unwinged:Control vs. Unwinged:Fungus		N.S.	
Unwinged:Control vs. Winged:Control		p < 0.001	
Winged:Control vs. Winged:Fungus		p < 0.05	
Survival to 8 days after live infection			
Morph	χ^2 = 13.3	1	p < 0.0001
Treatment	χ^2 = 64.7	1	p < 0.0001
Morph * Treatment	χ^2 = 7.08	1	p = 0.0080
Genotype	χ^2 = 17.0	2	p = 0.0002
Morph * Genotype	χ^2 = 3.59	2	p = 0.16
Treatment * Genotype	χ^2 = 4.06	2	p = 0.13
Morph * Treatment * Genotype	χ^2 = 1.04	2	p = 0.59
Pathogen Load			
Morph	F = 6.76	1	p = 0.01
Day	F = 63.4	1	p < 0.0001
Genotype	F = 6.30	2	p = 0.0045
Morph * Day	F = 0.16	1	p = 0.688
Morph * Genotype	F = 2.80	2	p = 0.742
Day * Genotype	F = 0.09	2	p = 0.916
Morph * Day * Genotype	F = 0.23	2	p = 0.797
Cell concentration after heat-killed challenge			
Morph	F = 11.4	1	p = 0.0015
Treatment	F = 11.5	2	p < 0.0001
Morph * Treatment	F = 7.96	2	p = 0.0011
Cell concentrations after live infection			
Morph	F = 4.35	1	p = 0.04
Treatment	F = 6.53	1	p = 0.01
Time Point	F = 2.61	1	p = 0.11
Morph * Treatment	F = 1.43	1	p = 0.24
Morph * Time Point	F = 0.14	1	p = 0.70
Treatment * Time Point	F = 4.40	1	p = 0.04
Morph * Treatment * Time Point	F = 14.6	1	p = 0.0003

Effect of Pre-treatment			
Morph	$\chi^2 = 8.46$	1	$p = 0.0036$
Pre-Treatment	$\chi^2 = 11.1$	1	$p = 0.00085$
Morph * Pre-Treatment	$\chi^2 = 19.6$	1	$p < 0.0001$

Two hypotheses could explain the finding that immune costs are limited to winged aphids. The first possibility is that winged aphids mount a stronger immune response than unwinged aphids. Winged aphids are produced in response to ecological conditions, such as crowding, that could increase the probability of pathogen exposure. Winged aphids may be investing more heavily in immunity in response to this risk, and therefore experience greater costs than unwinged aphids. The second possibility is that the immune costs we observed in this system are condition dependent. The production of wings and the associated musculature requires host resources, and leads to lower lifetime fecundity (Figure 3-1, Groeters and Dingle 1989)—as a result winged aphids may be energetically limited such that the pool of resources in winged aphids is insufficient to produce an immune response without negatively influencing fecundity. Such condition-dependent immune costs have been observed in other systems (Kraaijeveld and Godfray 1997; Mckean et al. 2008; Cotter et al. 2010). For example, studies have reported costs only in conditions of starvation or low nutrient availability (Moret and Schmid-Hempel 2000).

To test these hypotheses, we characterized the susceptibility of both morphs to a live *Pandora* infection. We reasoned that if winged aphids were investing more in an immune response they should be less susceptible to *Pandora* infection than unwinged aphids. If, on the other hand, winged aphids are

energetically limited leading to context-dependent immune costs, we expected that winged aphids would be more or equally susceptible to live *Pandora* infection than unwinged aphids. We infected aphids from both morphs with *Pandora* spores and recorded whether each aphid died after pathogen exposure. We found that winged aphids were significantly more susceptible to fungal infection than unwinged aphids (Table 3-1, Figure 3-2A), and that this trend was consistent across multiple genotypes (Table 3-1, Supplementary Information 3C). In addition to measuring survival, we also recorded whether each aphid produced *Pandora* spores after death. *Pandora* is transmitted after it produces a sporulating cadaver, and the success or failure of spore production is therefore relevant to pathogen transmission. We found that winged aphids were significantly more likely to produce a sporulating cadaver than unwinged aphids (Supplementary Information 3D). We performed a second experiment where we stabbed aphids with infectious spores instead of infecting aphids through the cuticle, and again found that winged aphids were more susceptible than unwinged aphids to *Pandora* (Supplementary Information 3E). This suggests that differences in *Pandora* susceptibility that we measured with a natural infection route were not driven by differences in the ability of spores to penetrate the host cuticle.



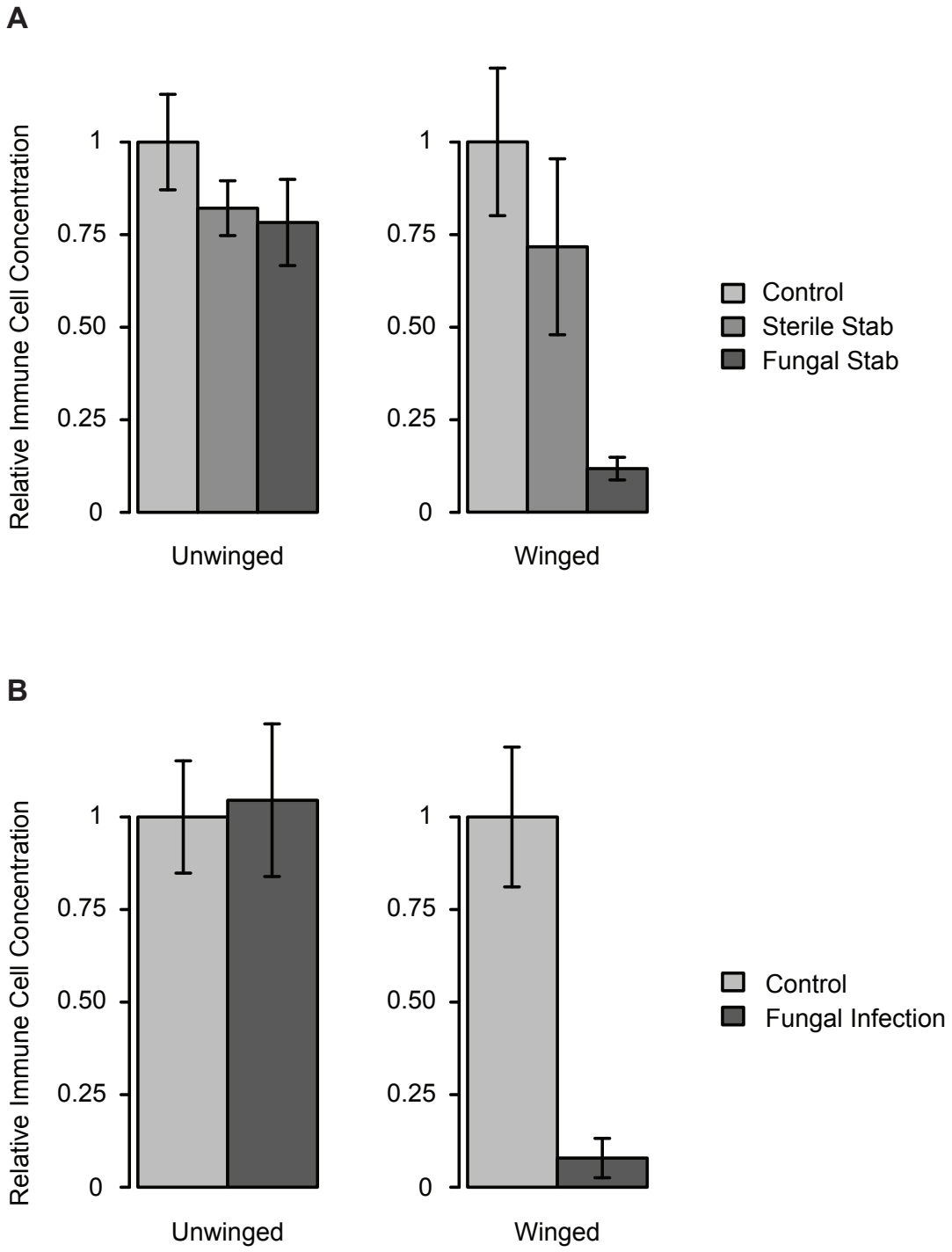
3-2) Susceptibility of morphs to live *Pandora* infection: A. Percent survival: Shows the percent survival 8 days after *Pandora* infection of winged (left) and unwinged (right) aphids. Control aphids (unexposed) are shown in light grey, and exposed aphids are dark grey. Error bars show standard error of the mean. **B. Pathogen load:** Shows the number of copies of *Pandora* 18S measured with quantitative PCR for winged (dotted line, open circles) and unwinged (solid line, solid circles) aphids at 1, 2, 3, and 4 days after exposure. The y-axis is a log scale.

We next used quantitative PCR to measure the pathogen load of unwinged and unwinged aphids over the course of a *Pandora* infection. Recent work has suggested that studies in ecological immunology should measure pathogen load in addition to other fitness traits because of complex relationships between host fitness and within-host pathogen load (Graham et al. 2011). *Pandora* grew logarithmically during the first four days of infection (Table 1, Figure 2B). Winged aphids had higher pathogen loads than unwinged aphids across the time course of infection (Table 3-1, Figure 3-2B), and this effect was consistent across genotypes though genotypes differed significantly in pathogen load (Table 3-1). Together these results show that winged aphids are more susceptible than unwinged aphids to *Pandora* infection.

We next aimed to link these results to measures of immunity. As a first step, we investigated the cellular immune response of winged and unwinged aphids. Previous work on aphid immunity has shown that aphids possess several distinct cell (hemocyte) types (Laughton et al. 2011a; Schmitz et al. 2012). Of these cell types, granulocytes were shown to have a role in the aphid immune response (Laughton et al. 2011a; Schmitz et al. 2012), and so we quantified the number of granulocytes in fixed volumes of hemolymph from winged and unwinged aphids that were either exposed to heat-killed *Pandora*, control stabbed, or unstabbed. In a pilot study using only control aphids, we found that winged aphids had about half as much hemolymph as unwinged aphids, but had a higher concentration of granulocytes. We therefore do not make direct comparisons between morphs

using cell concentrations, and instead compare changes in cell titer as a result of *Pandora* exposure between morphs. We found that winged aphids showed a significant depletion of immune cells when exposed to heat-killed *Pandora* compared to sterile stab and control aphids, but found no significant changes in cell concentration in unwinged aphids (Table 3-1, Figure 3-3A).

We next quantified the number of granulocytes in winged and unwinged aphids after live *Pandora* infection. We sampled aphids at two time points (48hrs and 96hrs after infection), and found that at 96 hours we again saw the depletion phenotype observed above in winged aphids, and again found no effect in unwinged aphids (Table 3-1, Figure 3-3B). We found no changes in cell titers at 48 hours post-exposure in either morph.



3-3) Cell counts: The y-axes of these plots show the relative concentrations of immune cells (granulocytes) among treatments—for each bar, the number of cells in 0.25μL hemolymph was divided by the number of cells of control aphids for that morph. Unwinged aphids are shown on the left, winged aphids are shown on the right. **A. Cellular immunity after heat-killed pathogen exposure:** Here

we measured cell concentration of control (not stabbed) aphids (light grey), aphids stabbed with a needle dipped in sterile PBS (medium grey), or with a needle dipped in a solution of PBS and heat-killed *Pandora* mycelium (dark grey). Error bars show standard error. These data were collected 24 hrs after stabs. **B. Cellular immunity after live infection:** Here we measured cell concentrations of control (light grey) and *Pandora* exposed (dark grey) aphids 96 hours after exposure. Error bars show standard error. We conducted these live infections on adult aphids.

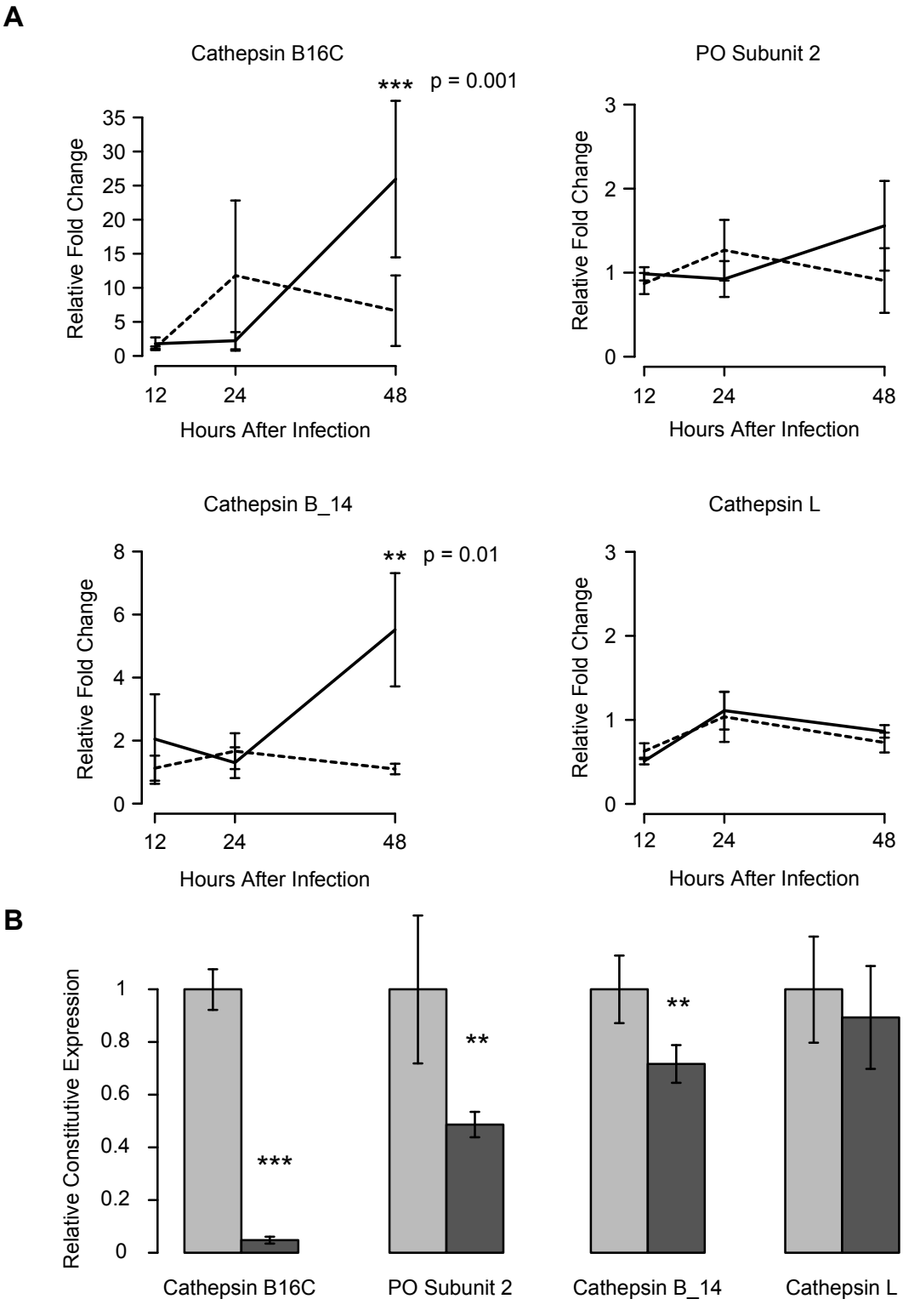
A number of studies have interpreted changes in hemocyte counts as evidence for differences in immune activity. For example, higher number of hemocytes in solitary vs. gregarious lepidopterans was taken as greater investment in immunity (Wilson et al. 2003), and increases in hemocyte numbers have been reported in response to parasitic wasp infection in *Drosophila* (Sorrentino et al. 2002; Márkus et al. 2009). In our study, neither winged or unwinged aphids showed increases in hemocyte counts after fungal infection, and we only observed a decrease in hemocyte numbers in winged aphids, which are less resistant to *Pandora*. There are fewer studies in the literature to use as precedent for interpreting decreases in cell concentrations, but work in bumblebees has shown that when worker bees transition from nursing to foraging activities, they show a decrease in hemocyte number. This result is interpreted as a reaction to selection to optimal resource allocation (Amdam et al. 2005)—most bees perish shortly after transitioning to foraging activities, and so selection does not favor investing in immunity. The results we report in aphids could reflect a shift in resource allocation from immunity to other energetic needs (e.g. repair or fecundity). Alternatively, the cell depletion we observe could reflect

winged aphids' inability to sustain granulocyte titers after mounting a cellular immune response to *Pandora* infection.

We next turned to patterns of gene expression in response to *Pandora* infection. Information on genes involved in the invertebrate immune response to fungi is limited, but previous work has implicated several innate immune signaling pathways as important to fungal defense in *Drosophila* (Lemaitre et al. 1997; Lemaitre and Hoffmann 2007). We previously measured the expression of a number of pea aphid homologs to these genes in response to fungal infection and found no significant patterns of differential expression (Gerardo et al. 2010). To overcome this problem here, we used RNAseq to sequence mRNA from winged and unwinged aphids to determine which genes were differentially expressed in response to *Pandora* infection. A small number of genes were significantly differentially expressed in response to fungal exposure (winged: 625; unwinged: 419). This gene list contained a number of potential immune genes, including phenoloxidase, which is a key component of immune function in invertebrates (Laughton et al. 2011b), and a group of cathepsins, which are proteases that have been shown to be expressed in hemocytes and to have lysozymal activity against bacterial and viruses in other invertebrates (Tryselius and Hultmark 1997; Serbielle et al. 2009; Nishikori et al. 2009; Hamilton et al. 2011).

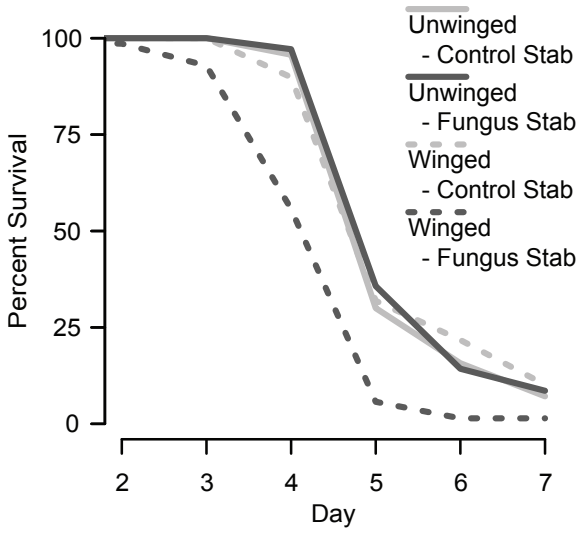
We then used quantitative real-time PCR to assess patterns of gene expression in winged and unwinged aphids in response to *Pandora* infection across multiple time-points during infection. We included three of the putative

immune genes identified above, and also included Cathepsin L (which was not found to be differentially expressed in our RNAseq analysis). We first compared the expression of these genes between uninfected winged and unwinged aphids as a comparison of constitutive expression. Unwinged aphids consistently expressed these genes more strongly than winged aphids (Figure 3-4). This pattern of higher constitutive expression of putative immune genes in unwinged aphids matched patterns seen in the transcriptome (Figure 3-SF). When comparing expression between control and infected aphids, we found no evidence of up- or down-regulation of putative immune genes early during the infection (12 and 24 hours), but found that several of these genes were upregulated 48 hours after infection (Figure 3-4). We further found that these genes were upregulated more strongly in unwinged than winged aphids (Figure 3-4). Together these results suggest that unwinged aphids invest more strongly in immunity and in an immune response than winged aphids. This lends support to the second hypothesis described above; that winged aphids are more susceptible to *Pandora* infection and invest less in an immune response because they are energetically limited as a result of wing production.


A
Cathepsin B16C
*** p = 0.001
Relative Fold Change
Hours After Infection
PO Subunit 2
Cathepsin B_14
** p = 0.01
Cathepsin L
B
Relative Constitutive Expression
Cathepsin B16C
PO Subunit 2
Cathepsin B_14
Cathepsin L

3-4) Putative immune gene expression data: We used quantitative PCR to measure the expression of our putative aphid fungal immunity genes. **A. Differential expression:** The y-axis shows the relative fold change of *Pandora* infected vs. control aphids. Differential expression was measured for winged (dotted lines) and unwinged (solid) lines at three time points after *Pandora* exposure (12 hrs, 24 hrs, and 48 hrs). The error bars show standard error across three biological replicates for each point. Asterisks indicate statistically significant differences, as determined by the interaction effect between morph and treatment at each time-point. **B. Constitutive expression:** We averaged the expression of control aphids (unexposed) across the three timepoints, and compared the expression of these unexposed aphids between unwinged (light grey) and winged (dark grey) aphids as a measure of constitutive gene expression. Statistical significance is indicated by asterisks, error bars show standard error.

We last explored the effects of morph on an aphid's ability to mount a subsequent immune response after an initial heat-killed immune challenge. In many systems, active upregulation of immune mechanisms because of preliminary exposure leads to later immune protection in a mechanism termed 'immunological priming' (Moret and Siva-Jothy 2003; Konrad et al. 2012). We instead found that winged aphids were more susceptible to *Pandora* infection after a heat-killed fungal pre-treatment, but found no effect in unwinged aphids (Table 3-1, Figure 3-5). This result indicates that after pre-treatment, winged aphids were less successful at mounting a subsequent response to live infection, but that unwinged aphids were not affected by pre-treatment. Some have speculated on the interaction between immune costs and immunological priming (Little and Kraaijeveld 2004), and this finding suggests that increased susceptibility to a subsequent pathogen challenge after priming is an additional way in which immunity can be costly for hosts.



3-5) Survival curves of *Pandora* aphids after pre-treatment: We measured survival between 2 and 7 days after exposure to *Pandora* of winged (dotted lines) and unwinged (solid lines) aphids that were exposed to one of two pre-treatments: a stab with a needle dipped in sterile PBS (light grey) or with a needle dipped in a solution of PBS and heat-killed *Pandora* mycelium (dark grey).

CONCLUSIONS

We found that measurable costs of immunity to *Pandora* are limited to winged aphids both in terms of lifetime fecundity and in terms of the ability of aphids to respond to a subsequent pathogen infection after initial immune challenge. We further found that winged aphids are more susceptible to *Pandora* infection than unwinged aphids, and that they do not invest as heavily in immune mechanisms. These findings are consistent with the idea that because winged aphids are energy limited from the production of wings and associated musculature, the pool of available resources is not sufficient to produce an immune response without negatively influencing lifetime fecundity.

Recent work has emphasized the importance of winged aphids for the spread of fungal pathogens (Feng et al. 2004; Chen and Feng 2004; 2005; 2006), and has suggested that dispersing winged aphids may be the primary source of transmission of aphid fungal pathogens (Feng et al. 2007). The increased susceptibility of dispersing aphids is likely an important driver of disease dynamics in this system, and future efforts to characterize fungal pathogen epidemics in aphid populations (Plantegenest et al. 2001) and in the use of fungal pathogens for aphid biocontrol (Hajek and Delalibera 2010) will benefit from considering this difference. More generally, many species exhibit physiological differences associated with dispersal. A well-studied example comes from locusts, where high population densities lead to a switch from a solitary non-dispersing morph to a swarming migratory morph (Simpson et al. 2011; Guershon and Ayali 2012). Other examples include monarch butterflies, where individuals from migrating populations are larger than those from non-migratory populations (Altizer and Davis 2010), and termites that can produce winged sexual and unwinged worker castes (Korb and Katrantzis 2004). There are also energetic demands from dispersing itself that influence resource availability for immunity (Roff 1977; Owen and Moore 2008b). We therefore expect that dispersal can influence disease dynamics through host susceptibility in a wide variety of organisms, but clearly more research is needed.

Lastly, these findings have important implications for evolution. The rates of dispersal of hosts and pathogens affect the evolution of local adaptation (Gandon et al. 1996; Lively 1999). An increase in host susceptibility because of the

physiological demands of dispersal could increase parasite transmission, which would therefore influence host-pathogen co-evolution. In addition, we found that costs of immunity were condition dependent, with effects only in the dispersing morph. Condition-dependent immune costs have been found in several previous studies (Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000; Mckean et al. 2008; Cotter et al. 2010), focusing mostly on the role of host nutritional state. Our results suggest that polyphenism is an additional and previously unrecognized condition under which immune costs can be expressed, which may be important in a number of well-characterized host-pathogen systems where hosts exhibit polyphenism (Simpson et al. 2011). The fitness costs associated with immunity have been used in the explanations of a number of observations in host-parasite interactions, from why we see variation among individuals in parasite susceptibility (Schmid-Hempel 2003; Sadd and Schmid-Hempel 2009) to how parasite virulence evolves (Gandon and Michalakis 2000; Mackinnon and Read 2004; de Roode et al. 2011). Determining the conditions under which immunity has an impact on host fitness is therefore critical for our understanding of host-pathogen interactions.

METHODS

Study organism: We maintained pea aphids asexually on fava bean (*Vicia faba*) plants in 16 hr light: 8 hr dark conditions at 20°C. All aphids used in a given experiment were born within 24 hours of one another to reduce ontogenetic differences among individuals. We exposed developing aphids to the alarm

pheromone (E)-*β*-farnesene (EBF) at a dose that causes them as adults to give birth to approximately 50% winged and 50% unwinged offspring (dose based on preliminary experiments, 5 μL of 1000 ng/μL EBF every other day for 10 days). For the experimental work described below (cost of exposure experiments and assays of resistance) we used three genotypes: LSR1-01, 5AO, and G6 (Table 3-S1). For the immunological assays we used genotype LSR1-01.

Cost of Exposure Assays: We obtained an isolate of *Pandora* (genotype ARSEF 2588) from the USDA ARS Collection of Entomopathogenic Fungal Cultures and grew it for 2 weeks on SDAEY plates at 20°C (Papierok and Hajek 1997). We added approximately 1 cm^2 of mycelium to 250μl Ringers solution, and autoclaved this solution at 121°C for 20 minutes and then homogenized the solution. We exposed 6 day old aphids to this heat-killed *Pandora* by stabbing them ventrally in the thorax with a 0.10mm minutin pin contaminated with the heat-killed pathogen, or with a sterile minutin pin dipped in Ringers solution as a control (Altincicek et al. 2008; Barribeau et al. 2010). We then allowed the aphids 30 minutes to heal in a clean Petri dish before we put them individually onto fava bean plants in cup cages. We performed three replicates of the experiment, each in a different host genotype (LSR1-01, 5AO, G6). Every 48 hours we counted the number of offspring produced by each aphid. During each check, offspring were removed from the plants after counting to prevent overcrowding, and plants were trimmed as necessary. We replaced the plants every 14 days, and continued the experiment until all subjects stopped reproducing. We analyzed these data using

generalized linear models, with a quasipoisson distribution and log link function, implemented in R version 2.11. We included morph, treatment, and block factors. Note that for each of the three blocks we used a different genotype to ensure that results were consistent across both genotype and experimental replicate. Minimal models were derived by removing terms followed by model comparisons using ANOVA. Terms were retained if their removal significantly reduced the explanatory power of the model. An interaction effect between morph and treatment indicated that immune costs differed between morphs. We then performed multiple comparison tests using the multcomp package in R (Hothorn et al. 2008) to determine whether significant effects of treatment were limited to the winged morph. Although we used a sterile stab as a control, our previous work has shown that there is no effect of stabbing on aphid survival or fecundity (Barribeau et al., in review).

Live infection: We infected aphids with *Pandora* by exposing them to a 'spore shower' (based on (Scarborough et al. 2005; Baverstock et al. 2006; 2008; Hatano et al. 2012; Parker et al. 2013)). *Pandora* was grown on SDAEY plates as above, and small pieces of fungal mycelium were cut and placed onto 1.5% tap water agar. After approximately 15 hours, the fungus begins to sporulate, at which time it was inverted over hollow tubes with aphids at the bottom of the chamber. Fungus plates were rotated among treatment groups to ensure that each treatment received an equal inoculation dose, and control aphids were handled similarly but were not exposed to spores. We exposed winged and

unwinged aphids (same three genotypes used above) to fungus. At 8 days after infection we recorded the survival of each aphid, and analyzed these data using a binomial GLM (with logit link function). We included morph, treatment, and genotype as factors in the model, and performed model comparisons as above.

Quantifying pathogen load: We used quantitative PCR to measure spore load of infected aphids. A live infection was performed as described above, but using a high spore dose designed to produce 100% lethality. Aphids were flash frozen in liquid nitrogen at 24, 48, 72, and 96 hours after infection, and then stored at -80°C, after which DNA was extracted using Bender buffer (with Proteinase K) and ethanol precipitation (based on (Bender et al. 1983)). At each time point, we collected two biological replicates of five aphids. Both winged and unwinged aphids were collected from three. Primers for *Pandora* 18S ribosomal RNA gene (Accession: EU267189.1) were designed using Primer Express 3.0 (supplementary information), and primer and template DNA efficiencies were optimized to 100 +/- 5% efficiency. We used the Invitrogen TOPO TA cloning kit with pCR 2.1 vector to clone our target fragment into One Shot TOP10 competent *e. coli* cells, and we extracted amplified plasmids using GE Healthcare illustra plastmidPrep Mini Spin Kit under recommended conditions. The cloned fragment was sequenced with M13F primer to confirm its identity. We used the standard curve method on an Applied Biosystems Step One Plus platform, measuring target amplification in experimental samples and in a standard dilution series (using 6 dilutions of 1:5 starting with 3.2×10^6 copies),

with three technical replicates. The comparative threshold cycle (Ct) was averaged across technical replicates, and *Pandora* 18S copy number was determined using Applied Biosystems Step One Plus software. We then used a quasipoisson GLM as above with morph, genotype, and day as factors.

Cellular Immunity Assays: Our protocol for characterizing the cellular immune response is based on (Laughton et al. 2011a), and involves collecting and staining hemolymph and performing cytological identification under a light microscope. Hemolymph was collected from leg wounds from several aphids until 0.25μl was obtained, and samples were then smeared onto a slide. Hemolymph was then fixed and stained using a Diff-Quick stain kit, and the number of granulocytes was counted under a light-microscope. We performed two experiments using this basic protocol. In the first experiment, we stabbed aphids with a heat-killed *Pandora* solution as above, with a sterile stab control and a no stab control, and collected hemolymph 24 hours after infection. In the second experiment we performed a live infection as described above, and collected hemolymph from infected and uninfected aphids at 48 and 96 hours after exposure.

Identifying candidate immune genes: To identify genes that play a role in aphid fungal immunity, we used RNAseq to measure the transcriptional response of winged and unwinged aphids to *Pandora* infection. Aphids were infected as above, but here we used spores from three fungal strains (ARSEF 2588, 2755,

and 5403). RNA was extracted from aphids 48 and 72 hours after pathogen exposure using Trizol and isopropanol precipitation. For each combination of treatment (control, *Pandora* infected) and morph (winged, unwinged), RNA from 10 aphids (from two host plants) from each timepoint was pooled, and libraries were constructed using Illumina kit B. CDNA libraries were then sequenced on an Illumina hi-seq machine. Reads were mapped to the pea aphid genome assembly version 2 using tophat (v.1). Differential expression of mapped transcripts was determined using cufflinks v. 2.1 (Trapnell et al. 2012), and genes were assigned function based on published annotation from the pea aphid genome project (International Aphid Genomics Consortium 2010).

qPCR assays of candidate immune gene expression: We infected winged and unwinged aphids with *Pandora* as above (with *Pandora* strain 2588), and flash-froze aphids in liquid nitrogen at 12, 24, and 48 hours after exposure. Aphids were stored at -80°C until RNA extraction using the trizol protocol described above. Genomic DNA contamination was reduced using the Invitrogen Turbo DNA-free kit and recommended protocols, and RNA was converted to cDNA using Invitrogen SuperScript III First-Strand Synthesis under recommended protocols. Primers were designed based on the RNAseq data generated above and sequence from the aphid genome sequencing project using Primer Express 3.0. Primer and template cDNA concentrations were optimized to 100 +/- 5% efficiency (Supplementary Information). *A. pisum* Ef1α was used as an endogenous control (Wilson et al. 2006). We subtracted the critical threshold

value (Ct) for the endogenous control from the target gene for each sample (ΔCt). We analyzed the differential expression of each gene at each time-point by using ANOVA to compare the ΔCt values for control and infected aphids from both morphs. A significant interaction effect between morph and treatment indicated that the magnitude of difference in the change in expression of the target gene differed between morphs.

Resistance after pre-treatment: We tested the effect of a heat-killed fungal 'pre-treatment' on resistance of winged and unwinged aphids. Stabs were carried out as described above, this time on 9-day old LSR1-01 aphids with heat-killed *Pandora* and a sterile stab control. Aphids were placed in a Petri dish for 3 hours and were then exposed to spores as described above. Aphid survival was recorded every 24 hours for 7 days. Data were analyzed using a non-parametric survival model with a Coxph distribution using the Survival package in R version 2.11. A test of proportional hazards was conducted to ensure that the data fit model assumptions. Significance was determined using model comparisons as described above.

ACKNOWLEDGEMENTS

Members of the Gerardo lab provided valuable feedback on a draft of this manuscript.

SUPPLEMENTARY INFORMATION

Supplementary Information 3A: Costs of immunity across genotypes

We performed three replicates of the cost of exposure experiment, each using a different aphid genotype. We found a significant effect of block/genotype on overall fecundity (Table 1), suggesting that genotypes differed in their overall reproductive fitness under experimental conditions. We did not find a significant interaction between morph, exposure, and block/genotype, suggesting that the trend of stronger immune costs in winged vs. unwinged aphids was consistent across replicate/block. However, when we analyze the data from each genotype separately, we find no significant interaction between morph and treatment in genotype G6 (stats). This could be the result of a smaller sample size in this genotype (n =). Alternatively, this could suggest that immune costs are not found in all genotypes, and future work will need to determine the role of host genotype in immune costs in this system.

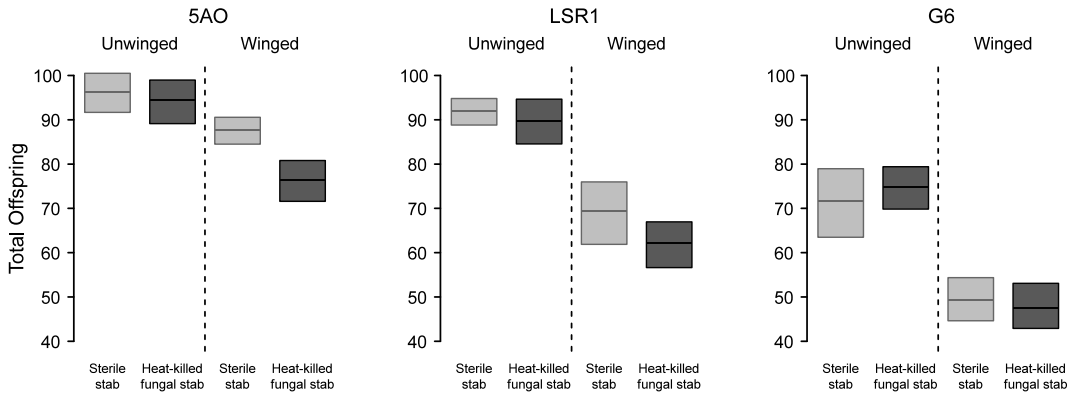


Figure SA: Cost of exposure data by replicate/genotype. Total lifetime fecundity is shown for the aphids used in the cost of exposure experiment, broken down into the three replicates of the experiment, each of which used a different host genotype. Boxes show the 95% confidence intervals; lighter colored boxes show control aphids, darker boxes show aphids exposed to heat-killed fungus. Data is broken down into unwinged (left panels) and winged aphids (right panels).

Supplementary Information 3B: Survival analysis of aphids exposed to heat-killed *Pandora* solution:

We found no effect of heat-killed *Pandora* exposure on survival of winged aphids. Data were analyzed using non-parametric survival models with a Coxph distribution using the Survival package in R version 2.11. A test of proportional hazards was conducted to ensure that the data fit model assumptions. Minimal models were derived by removing terms followed by model comparisons using ANOVA. Terms were retained if their removal significantly reduced the explanatory power of the model. Winged aphids had significantly higher survival across the course of the experiment (χ^2 = 10.3, 1DF, p = 0.001), but there was no effect of treatment or block so these terms were removed from the model (Figure SB).

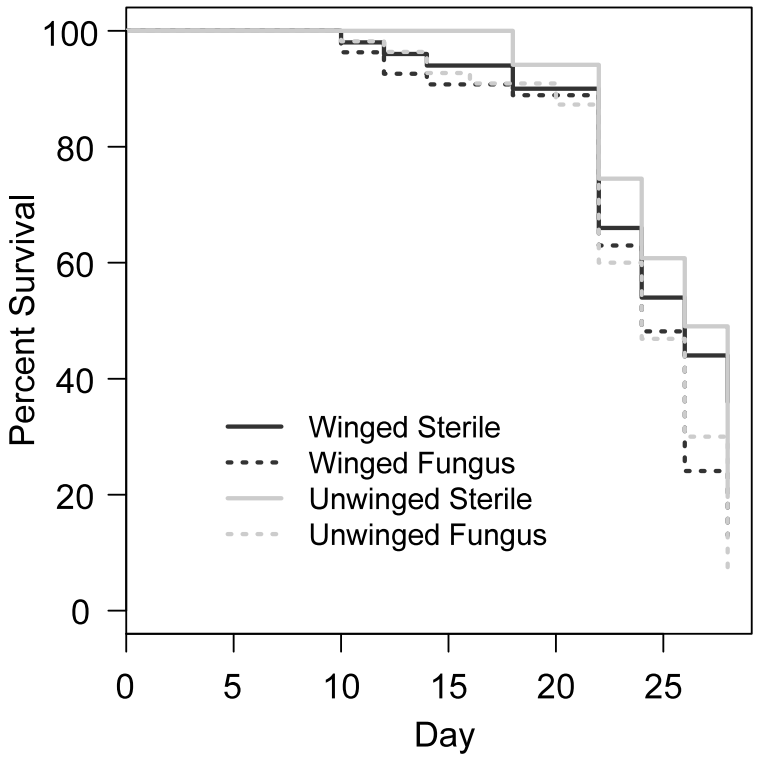


Figure SB: Survival of aphids exposed to heat-killed pathogen solution. Dark grey lines show survival of winged aphids, light grey shows survival of unwinged aphids. The dotted line indicates that aphids were exposed to heat-killed *Pandora*, the solid lines represent control stabbed aphids.

Supplementary Information 3C: Live *Pandora* fungal infections across genotypes

We used a binomial GLM to analyze infection status 8 days after *Pandora* exposure. There were significant differences among genotypes in overall survival (Table 1), but neither the interaction between genotype and treatment, or the three-way interaction between genotype, morph, and treatment, were significant. This suggests that the trend of higher susceptibility in winged aphids was consistent across genotypes.

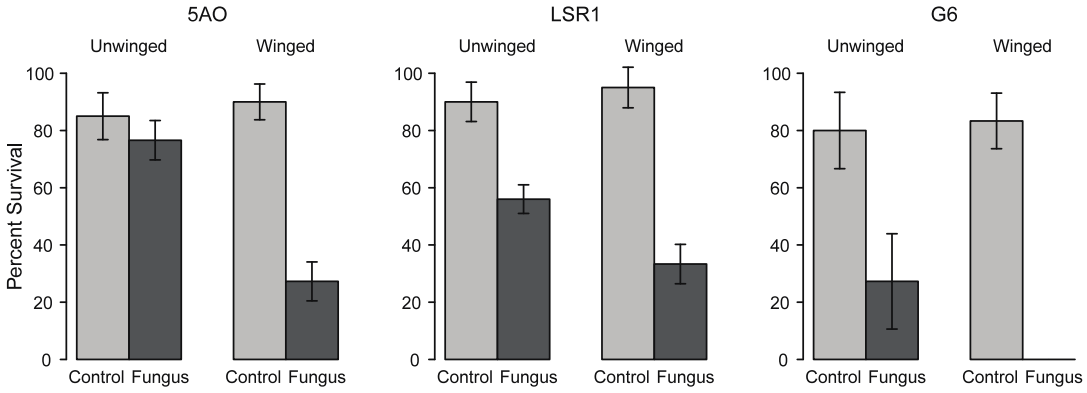


Figure SC: Survival of aphids 8 days after *Pandora* exposure. The light bars show unexposed aphids, and the dark bars show aphids exposed to *Pandora*. Overall winged aphids were more susceptible to *Pandora* infection. The error bars show standard error.

Supplementary Information 3D: Sporulation after live *Pandora* fungal exposure

Of aphids exposed to fungus, morph significantly impacted the likelihood of producing a sporulating cadaver ($\chi^2_{1,219} = 32.9$, $p < 0.0001$).

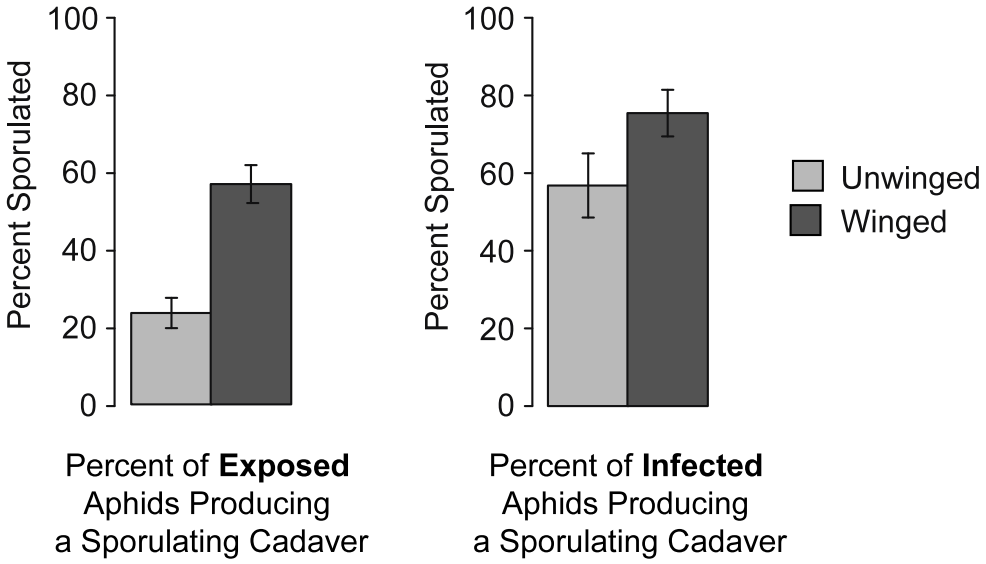


Figure SD: The percent of aphids that produce a sporulating cadaver. No control aphids produced a sporulating cadaver—they are not included in the plot. Aphids either survived until the end of the experiment, died but did not produce a cadaver, or produced a sporulating cadaver. In the wild, producing a sporulating cadaver is necessary for *Pandora* transmission. The left panel shows the frequency of aphids that were exposed to fungus that produced a sporulating cadaver. The right panel shows the frequency of the aphids that were both exposed, and died before the end of the experiment, that produced a sporulating cadaver. The lighter grey shows unwinged aphids, the darker grey shows winged aphids. For both measures, winged aphids produced a sporulating cadaver more frequently than unwinged aphids.

Supplementary Information 3E: Survival of aphids stabbed with infectious *Pandora* spores

We made a solution of live spores by crushing up three sporulating aphid cadavers in 100μl PBS. Winged and unwinged aphids were reared as described in the methods, and at 9 days old were stabbed in the thorax with a 0.10mm minutin pin contaminated with the spore solution. We measured the daily survival of stabbed aphids for 5 days after exposure. Data were analyzed using a coxph survival model after testing for the proportional hazards assumption. Significance was determined using a likelihood ratio test comparing survival models with and without morph as a factor. Winged aphids had significantly lower survival than unwinged aphids (χ^2 = 16.241, 1 DoF, $p < 0.0001$). This suggests that like the other measures of aphid fitness we report, winged aphids are more susceptible to fungal pathogens even when spores are injected directly into the hemolymph. This suggests that the differences in susceptibility we measure through infection by spore showere were not solely due to differences in winged and unwinged aphid cuticle thickness (Although winged aphids have ticker cuticles than unwinged aphids (Brisson and Stern 2006), suggesting that if cuticular thickness were an issue, we would see the reverse trend of winged aphids being more resistant than unwinged aphids).

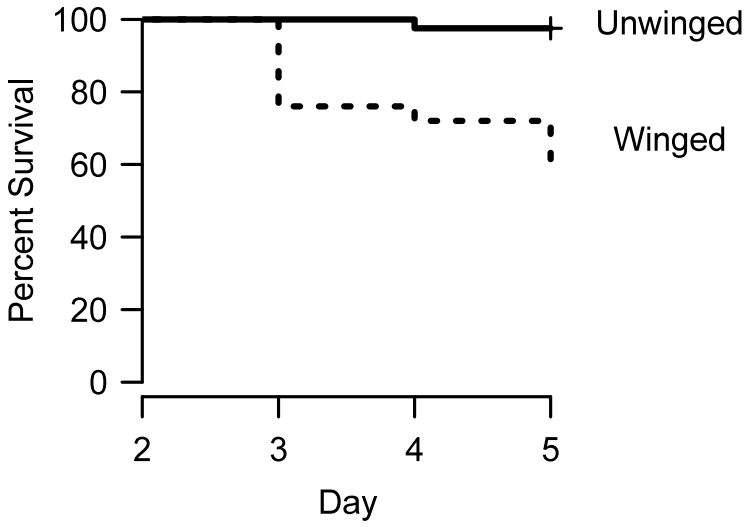


Figure SE: Survival of Winged and Unwinged aphids after *Pandora* stabs. The dotted line shows survival of winged aphids, and the solid line shows unwinged aphids.

Supplementary Information 3F: Constitutive gene expression from RNAseq data

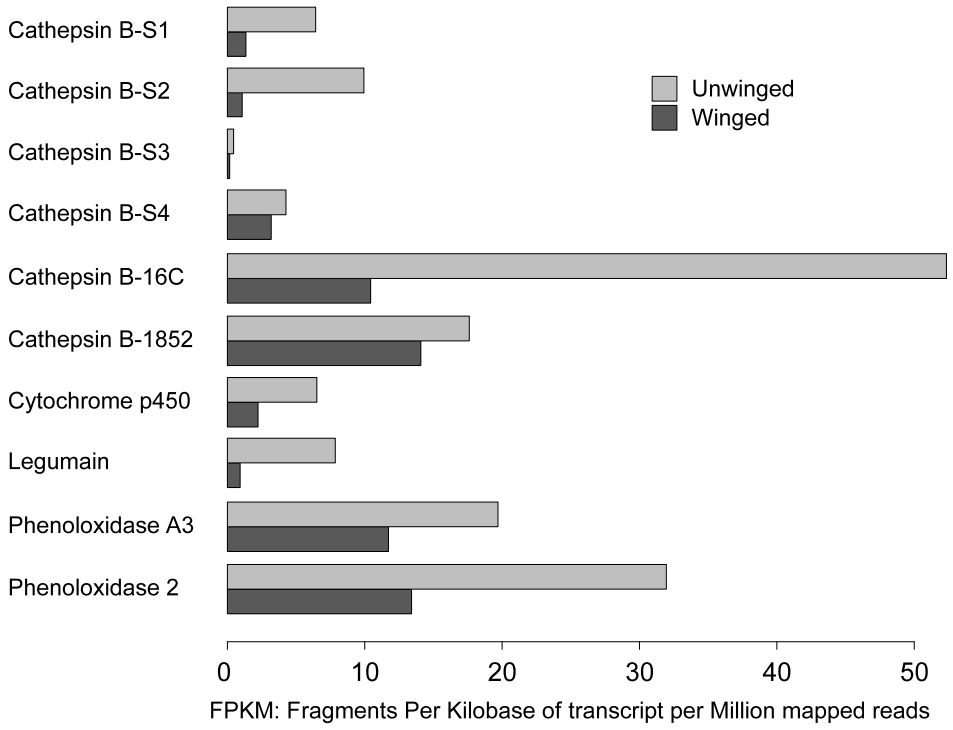


Figure SF: Constitutive expression of putative immune genes. FPKM (Fragments Per Kilobase per million Mapped reads) is compared between uninfected unwinged (light grey) and winged (dark grey) aphids.

Table 3-S1: Aphid Genotypes:

LSR1-01	Ithaca, NY	Alfalfa	1998	(International Aphid Genomics Consortium 2010)
G6	Atlanta, GA	Mixed weeds	2008	(Barribeau et al. 2010)
5AO	Madison, WI	Alfalfa	1999	(Russell & Moran 2006)

Pathogen Load Primers:

Quantitative PCR	
Pandora_18S_F	TCTTTGGGCTTAGTTGGTACTTTACTG
Pandora_18S_R	GCCCGCTTTGAACACTCTAATT

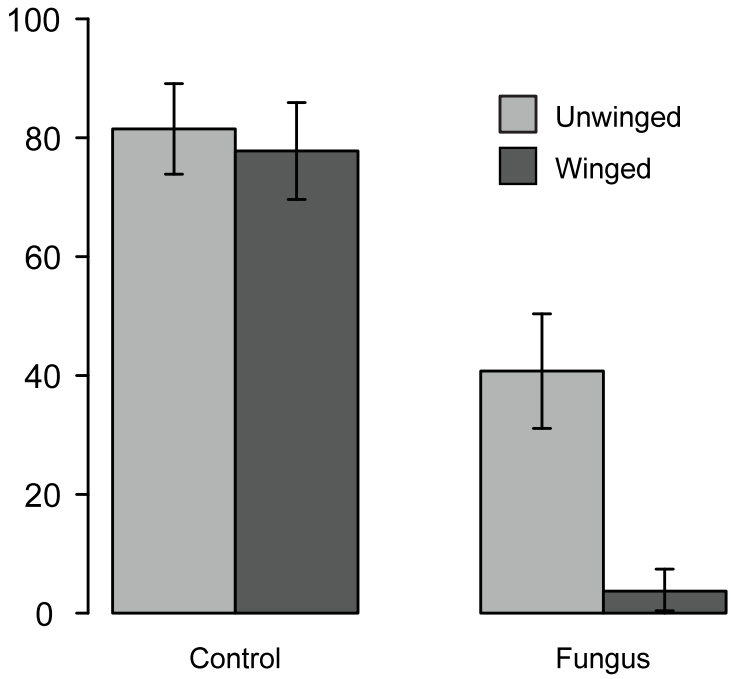
qPCR primer efficiencies:

Gene (Primer Concentration)	DNA Concentration (ng/μl)	Mean C^T	Slope	Efficiency
PO sub2 (100nM)	50	21.7	-3.32	100.2%
	10	23.9		
	2	26.4		
PO sub2 (200nM)	50	20.9	-3.22	104.2%
	10	23.1		
	2	25.5		
PO sub2 (300nM)	50	20.9	-3.38	97.6%
	10	23.1		
	2	25.6		
Cathepsin B16C (100nM)	50	19.6	-3.20	105.4%
	5	22.3		
	0.5	26.0		
Cathepsin B16C (200nM)	50	19.9	-2.96	117.5%
	5	22.8		
	0.5	25.9		
Cathepsin B16C (300nM)	50	19.8	-2.96	117.9%
	5	22.6		
	0.5	25.7		
Cathepsin BS00014 (100nM)	50	21.4	-3.21	105.1%
	10	23.7		
	2	25.9		
Cathepsin BS00014	50	21.3	-3.26	102.5%

(200nM)	10	23.5		
	2	25.9		
Cathepsin BS00014 (300nM)	50	21.0	-3.38	97.5%
	10	23.3		
	2	25.7		
Cathepsin L (100nM)	50	21.8	-3.34	99.4%
	10	24.0		
	2	26.4		
Cathepsin L (200nM)	50	21.3	-3.32	100.0%
	10	23.7		
	2	26.0		
Cathepsin L (300nM)	50	21.4	-3.23	103.8%
	10	23.6		
	2	25.9		

Supplementary Information 3G: Survival of aphids used in transcriptional analysis

We infected aphids in addition to those used to produce RNA for sequencing to verify that the *Pandora* infection worked and that the pattern of susceptibility in winged and unwinged aphids was consistent throughout all of our infections. We recorded survival at Day 8 of winged and unwinged aphids that were exposed to *Pandora* or were unexposed as a control (n = 27 per treatment per morph). We analyzed these data using a binomial GLM, and performed model comparisons using ANOVA. Morph (χ^2 = 4.45, 1DF, p = 0.034) and treatment (χ^2 = 40.3, 1DF, p < 0.0001) both significantly influenced the proportion of aphids alive at Day 8, as did the interaction between Morph and Treatment (χ^2 = 5.36, 1DF, p = 0.021).



This indicates that as before, winged aphids were more susceptible to *Pandora* infection than unwinged aphids.

Figure SG: Survival at Day 8 for the transcriptome experiment. We found that winged aphids were more susceptible to *Pandora* infection than unwinged aphids, consistent with previous experiments (Figure 3).

CHAPTER 4: GENETIC VARIATION IN RESISTANCE AND FECUNDITY TOLERANCE IN A NATURAL HOST—PATHOGEN INTERACTION

ABSTRACT

Not all host defenses against pathogens are subject to the same selective pressures. One important distinction is between mechanisms of resistance, which actively fight off infection, and tolerance, which limit the impact of infection on host fitness. Theory predicts variation among genotypes in resistance, but not in all mechanisms of tolerance. In addition, environmental factors can complicate host—pathogen coevolution. For example, pea aphids are protected from fungal pathogens by several species of symbiotic bacteria. Here we look for variation among aphid genotypes in measures of resistance and tolerance in the absence of protective bacteria. We find variation in resistance among symbiont-free genotypes, suggesting that selection can maintain variation in host resistance even when host-pathogen evolutionary dynamics are influenced by protective symbionts. We also find evidence of variation among genotypes in tolerance of the effects of pathogen infection on host fecundity, but no variation in measures of tolerance of pathogen-induced mortality, validating theoretical predictions. These results highlight the complexity of the evolutionary pressures generating variation in host defenses.

INTRODUCTION

Pathogens are ubiquitous in nature and impose strong selective pressures on their hosts. In turn, hosts have evolved ways to defend against pathogens. A fundamental insight into this interaction is that there can be measurable variation in these defenses among hosts (Rolff and Siva-Jothy 2003; Sadd and Schmid-Hempel 2009). This variation has consequences for host-pathogen coevolution, and determining the evolutionary forces generating variation in defenses has been critical for addressing a number of biological questions (*e.g.* variation in disease prevalence across ecological gradients (Altizer et al. 2011; Møller et al. 2011); the evolution of pathogen virulence (Gandon and Michalakis 2000; Mackinnon and Read 2004; de Roode et al. 2011)).

This task is difficult, however, because there are a variety of ways that hosts defend against pathogens, and not all of these defenses have the same effects on host-pathogen coevolution. One important distinction is often made between mechanisms of resistance, which actively reduce pathogen burden, and mechanisms of tolerance, which limit the impact of infection on host fitness without reducing pathogen infection or growth. Because they have different effects on pathogen fitness, theory predicts differences in the patterns of variation found in resistance and tolerance in wild populations. Resistance mechanisms reduce pathogen fitness by directly combating infection, and pressure from pathogens selects for resistant hosts. This in turn reduces pathogen prevalence, makes expensive resistance mechanisms less beneficial (see Chapters 2 & 3), and leads to negative frequency-dependent selection,

generating variation in resistance mechanisms among hosts (Lively and Dybdahl 2000; Woolhouse et al. 2002; Gandon et al. 2008). Tolerance mechanisms also alleviate the reduction of host fitness due to pathogen infection, but in contrast to mechanisms of resistance, tolerance also benefits the pathogen by increasing transmission and pathogen prevalence (Roy and Kirchner 2000; Miller et al. 2005; Svensson and Råberg 2010). Tolerance mechanisms are therefore predicted to generate positive feedback and the absence of genetic variation. Recent theory has made an additional distinction between tolerance of pathogen-induced mortality ('mortality tolerance') and tolerance of the effects of pathogen infection on host fecundity ('fecundity tolerance'). While mortality tolerance has a positive effect on pathogen fitness, fecundity tolerance is predicted to be neutral for horizontally transmitted pathogens (Best et al. 2008). We might therefore expect to find variation among genotypes in fecundity tolerance due to genetic drift, but empirical validation of these predictions is needed.

In addition, researchers have recently started to recognize the importance of microbial symbionts in host defense (reviewed in Haine 2008; Brownlie and Johnson 2009). The human gut, for example, is host to symbiotic bacteria that defend against colonization by opportunistic pathogens (Round and Mazmanian 2009; Maynard et al. 2012). Other examples of symbiont-mediated protection include fungi that protect plants from pathogen attack (Arnold et al. 2003) and *Spiroplasma* bacteria that protect *Drosophila* flies from parasitic nematodes (Jaenike et al. 2010). The number of examples in the literature of protective associations is rapidly increasing in a taxonomically diverse set of hosts.

However, how symbiont-mediated protection influences the evolution of hosts' immune system-based defenses is unclear (Boughton et al. 2011; Parker et al. 2011). One possibility is that symbiont-mediated protection leads to relaxed selection on immune mechanisms (Altincicek et al. 2008).

Understanding the complexity of host defenses within an ecological and evolutionary context is critical for understanding a major class of interspecific interactions. Here we use pea aphids (*Acyrthosiphon pisum*) and a natural aphid fungal entomopathogen, *Pandora neoaphidis,* to look for empirical validation of existing theory about the evolutionary sources of variation in host defenses. Initial characterizations of natural aphid populations revealed a large amount of variation in susceptibility to *Pandora* (Henter and Via 1995; Ferrari et al. 2001), and it was presumed that this variation was due to genetic differences among aphid clones. It was eventually discovered, however, that pea aphids are protected from *Pandora* and other fungal pathogens by several Gram-negative bacterial symbionts (Scarborough et al. 2005; Lukasik et al. 2012; Parker et al. 2013). In this system where symbiont-mediated protection is known to play a substantial role in host-pathogen coevolution, we measure fitness (survival, percent sporulation, and fecundity) of aphid genotypes that do not harbor protective symbionts to determine whether we still find genetic variation in resistance and tolerance in the absence of protective symbionts.

METHODS

Pea aphids reproduce asexually by parthenogenesis during the summer, producing genetically identical offspring that are born live—a single female can produce ~15 offspring per day during her peak reproductive period. Before infection, aphids were reared in identical conditions of 16L:8D at 20ºC on *Vicia faba* plants. The pea aphid lineages used were collected in North America (see Table S1 in the Supplementary Information). PCR was used to determine that aphids did not harbor secondary symbionts (primer sequences and PCR protocols can be found in Table S2). The absence of secondary symbionts was also confirmed by cytological staining (Laughton et al. 2011a).

We amplified microsatellite loci to look for genetic differences among lineages. DNA was extracted from 20 aphids of each lineage using Bender buffer (with Proteinase K) and ethanol precipitation (Bender et al. 1983). We used the Qiagen Type-it Microsatellite PCR Kit under recommended conditions with primers Ap02, Ap03, and Ap05 (Kurokawa et al. 2004) and s17b (Wilson et al. 2004). Amplified products were run on a capillary sequencing instrument, and data was analyzed using GeneMarker version 2.4.0 (see the Supplementary Information for details).

Pandora is transmitted among individuals as infectious spores, which, upon contact with a suitable host, penetrate the host's cuticle. Recent work has emphasized the importance of horizontal transmission through movement behavior (Baverstock et al. 2005a), overwintering spores (Fournier et al. 2010), and the production and dispersal of winged aphid morphs (Feng et al. 2004;

2007) in aphid—*Pandora* dynamics. Vertical transmission likely also occurs between sporulating aphids and closely located offspring, but the relative importance of these two modes of transmission is unknown. After exposure, mycelia fill the host until new spores are produced and released into the environment. A pea aphid infected with *Pandora* will die and begin to sporulate between 4 and 10 days after exposure. To mimic a natural mode of transmission under the controlled environment of the laboratory, we infected aphids with *Pandora* by placing them under a spore shower (based on (Scarborough et al. 2005; Baverstock et al. 2005b; 2006; 2008; Hatano et al. 2012). An isolate of *Pandora* (genotype ARSEF 2588) was obtained from the USDA ARS Collection of Entomopathogenic Fungal Cultures and grown for 2 weeks on SDAEY plates at 20°C (Papierok and Hajek 1997). Twelve hours before infection, small pieces of mycelium (3mm^2) were cut with a sterile instrument and placed on 1.5% tap water agar plates, causing the fungus to sporulate. To infect aphids, the agar plates were inverted over a hollow tube with aphids at the bottom of the chamber. The plates were rotated among aphid lineages during infection to ensure that aphids of each lineage were exposed to equal doses of fungal spores. We included a glass slide in this rotation so that spores could be counted under a light microscope to determine the spore density for each treatment. Infection spore dose was recorded as the number of spores per mm^2 counted on these glass slides, and we used four spore doses in our infections (0 spores / mm^2, 8 spores / mm^2, 16 spores / mm^2, and 24 spores / mm^2). After infection, aphids were assigned a random number so that data collection would be blind to

treatment and lineage, and each aphid was then housed individually on a fava plant. For the first four days after infection, plants were enclosed in a solid plastic cup that kept the aphids in high humidity (> 90%), as *Pandora* requires high humidity to infect aphids (Papierok and Hajek 1997). Every 24 hours following this initial four-day infection period we recorded survival, the number of offspring produced by each aphid, and whether each aphid showed visible signs of sporulation. We continued recording data until 18 days after infection (when all aphids had stopped reproducing). Offspring were removed from each plant each day after counting to prevent overcrowding.

We took a subset of infected aphids and used quantitative PCR to measure pathogen burden. We froze eight aphids per lineage and treatment in liquid nitrogen at four days after infection, and extracted genomic DNA as above. Primers for *P. neoaphidis* 18S ribosomal RNA gene (GenBank Accession: EU267189.1) were designed using Primer Express 3.0 (Table S2), and primer and template DNA concentrations were optimized to 100 +/- 10% efficiency. We used the standard curve method on Applied Biosystems Step One Plus. To generate qPCR standards, the target PCR fragment was cloned using Invitrogen TOPO TA cloning kit with pCR2.1 vector into One Shot TOP10 competent cells, and plasmids were extracted using GE Healthcare illustra plasmidPrep Mini Spin Kit under recommended conditions. The cloned insert was sequenced with the M13F primer to confirm its identity. Target amplification was measured in experimental samples and in a standard dilution series (using 6 dilutions of 1:5 starting with 3.2×10^6 copies), with three technical replicates each. The

comparative threshold cycle (Ct) was averaged across technical replicates, and the absolute quantity of *Pandora* 18S copies was determined using Applied Biosystems Step One Plus software.

STATISTICAL METHODS

All statistical analyses were carried out in R, version 2.11.1. The number of copies of *Pandora* 18S was $\log_{10}$ transformed, and normality was confirmed using a Shapiro-Wilk normality test. A 2-way Analysis of Variance (ANOVA) was conducted using genotype and infection dose as factors.

Survival data were analyzed using non-parametric survival models with a Coxph distribution using the Survival package in R version 2.11. A test of proportional hazards was conducted to ensure that the data fit model assumptions. Other measures of host fitness (whether an aphid produced *Pandora* spores and lifetime fecundity) were analyzed using logistic regression by generalized linear models (GLM). Individuals that died due to handling, and aphids that failed to reproduce (with a lifetime fecundity of zero), were removed from the analysis. We fit a binomial distribution (with logit link function) to analyze the percent of aphids that produced spores. We fit a quasipoisson distribution (with log link function) to analyze the total lifetime fecundity of each aphid. We included uninfected aphids in our analyses for each of these measures to account for the fitness of each aphid genotype in the absence of pathogen infection, allowing us to distinguish between tolerance and general vigor (Råberg et al. 2007; Svensson and Råberg 2010; Lefèvre et al. 2011; Graham et al. 2011;

Baucom and de Roode 2011). For each of these models of pathogen defense we treated genotype and infection dose as fixed effects, although we repeated the analysis using linear mixed-effects models with genotype as a random effect using the nlme package and a poisson distribution (Pinheiro and Bates 2000), which had no effect on the significance of the results. For each model we also fit a quadratic term for infection dose to test for a non-linear relationship between infection dose and host fitness. The quadratic term was significant for sporulation frequency, so we also analyzed sporulation frequency without including uninfected aphids, for which (infection dose)^2 was not significant (Table 1). Minimal models were derived by removing terms followed by model comparisons using ANOVA. Terms were retained if their removal significantly reduced the explanatory power of the model. Here we are measuring tolerance as the slope of host fitness against pathogen load (Råberg et al. 2007; Svensson and Råberg 2010; Baucom and de Roode 2011; Graham et al. 2011)—for each analysis a significant interaction term between infection dose and host genotype indicates that genotypes varied in their tolerance of the pathogen.

RESULTS

Our analysis of microsatellite diversity among the aphid lineages tested found six distinct sets of microsatellite genotypes, suggesting that there are genetic differences among our aphid lineages (Table S3). However, two lineages, G15 and BP15 (which were collected in Atlanta, GA in 2008 and 2010, respectively), were identical at the four microsatellite loci tested. There were no statistically

significant differences between these two lineages in copies of *Pandora* 18S at 4 days after infection ($F = 0.507$, 1 df, $p = 0.561$), survival ($\chi^2 = 0.962$, 1 df, $p = 0.327$), sporulation frequency ($\chi^2 = 2.31$, 1 df, $p = 0.129$), or lifetime fecundity ($F = 11.6$, 1 df, $p = 0.315$). To err on the side of caution we excluded one of these lineages (G15) from the analyses and present the results of analyses with the six remaining genotypes—however, including G15 in the analyses had no effect on the significance of the analyses described below.

The reaction-norm method we employ for measuring tolerance assays the fitness of aphid genotypes at different levels of pathogen burden. An ideal system for this experimental design would allow the experimenter to set a fixed pathogen burden that would remain constant throughout measurements of fitness (although there has been some discussion in the tolerance literature about the relative benefits of using naturally vs. artificially imposed pathogen burden (Tiffin and Inouye 2000; Inouye et al. 2003; Lehtilä 2003; Baucom and de Roode 2011)). In this system, however, we do not have an external indicator of pathogen burden, and any measurement of pathogen burden constantly changes as *Pandora* replicates inside of its aphid host. A solution from other studies of tolerance is to use variable infection doses as a means of experimentally manipulating pathogen burden (Lefèvre et al. 2011). To determine whether this was a suitable approach in our system, we tested whether individuals exposed to higher infection doses had higher pathogen burdens after four days of pathogen growth. We found that pathogen burden was higher with increasing infection doses (ANOVA, $F(1,6) = 97.7$, $p < 0.0001$, Figure S1A). Genotypes varied in day

4 pathogen burden ($F(5,6) = 47.3$, $p < 0.0001$, Figure S1B), but there was no significant interaction between infection dose and genotype ($F(5,6) = 1.70$, $p = 0.268$). This suggests that, as in other studies, we can use variable infection doses as a means of experimentally manipulating pathogen burden.

Higher infection doses led to lower survival (Table 4-1, Figure 4-1A) and higher frequency of sporulation (Table 4-1, Figure 4-1C). We also found significant variation among genotypes in survival (Table 4-1, Figure 4-1B) and sporulation frequency (Table 4-1, Figure 4-1D), indicating that genotypes varied in pathogen resistance. We did not find evidence of an interaction effect between genotype and infection dose for survival or sporulation frequency (Table 4-1), indicating that aphid genotypes did not vary in these measures of mortality tolerance. We found that exposure to fungus significantly reduced lifetime fecundity and that genotypes varied in the number of offspring they produced (Table 4-1, Figure 4-2). Furthermore, we found that aphid genotypes varied in the extent to which fungal exposure reduced their lifetime fecundity, suggesting that aphid genotypes vary in fecundity tolerance.

Table 4-1: Statistical Analysis

Model Term	Test Statistic	d.f.	p-value
Survival			
Infection dose	$\chi^2 = 81.9$	1	$p < 0.0001$ *
$(\text{Infection dose})^2$	$\chi^2 = 1.07$	1	$p = 0.302$
Genotype	$\chi^2 = 38.6$	5	$p < 0.0001$ *
Genotype x Infection dose	$\chi^2 = 2.46$	5	$p = 0.783$
Genotype x $(\text{Infection dose})^2$	$\chi^2 = 4.35$	5	$p = 0.500$
Sporulation Frequency			
Infection dose	$\chi^2 = 102$	1	$p < 0.0001$ *
$(\text{Infection dose})^2$	$\chi^2 = 10.8$	1	$p = 0.0001$ *
Genotype	$\chi^2 = 45.5$	5	$p < 0.0001$ *
Genotype x Infection dose	$\chi^2 = 8.83$	5	$p = 0.116$
Genotype x $(\text{Infection dose})^2$	$\chi^2 = 1.68$	5	$p = 0.892$
Sporulation Frequency (not including control aphids)			
Infection dose	$\chi^2 = 25.0$	1	$p < 0.0001$ *
$(\text{Infection dose})^2$	$\chi^2 = 0.01$	1	$p = 0.935$
Genotype	$\chi^2 = 52.6$	5	$p < 0.0001$ *
Genotype x Infection dose	$\chi^2 = 8.72$	5	$p = 0.121$
Genotype x $(\text{Infection dose})^2$	$\chi^2 = 3.40$	5	$p = 0.639$
Lifetime Fecundity			
Infection dose	$F = 86.8$	1	$p < 0.0001$ *
$(\text{Infection dose})^2$	$F = 0.08$	1	$p = 0.780$
Genotype	$F = 20.3$	5	$p < 0.0001$ *
Genotype x Infection dose	$F = 2.67$	5	$p = 0.0221$ *
Genotype x $(\text{Infection dose})^2$	$F = 1.70$	5	$p = 0.134$

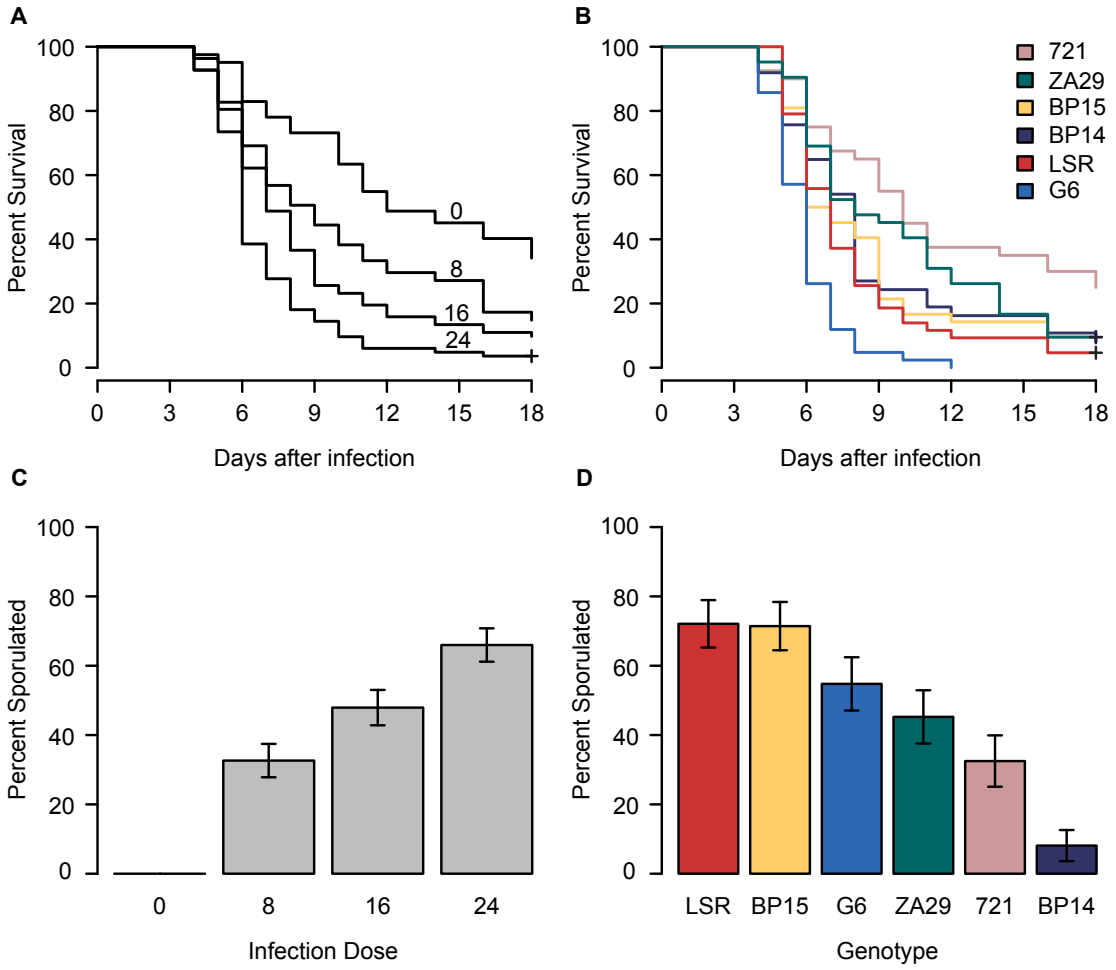


Figure 4-1: Measures of Resistance. Aphids were more susceptible to higher doses of *Pandora* (left column), and there was significant variation among genotypes in resistance (right column. We found no evidence of interaction between genotype and infection dose for either of these measures. **A:** Survival curves averaged across genotypes. Each line represents a different infection dose as indicated. No data was collected during an initial infection period (days 0-3). **B:** Survival curves for each genotype, averaged across infection dose, not including control (infection dose = 0) aphids. **C:** Percent of aphids that showed visible signs of sporulation at each infection dose, averaged across genotypes. **D:** Percent of aphids of each genotype that showed visible signs of sporulation, not including control aphids (infection dose = 0). All error bars show +/- standard error.

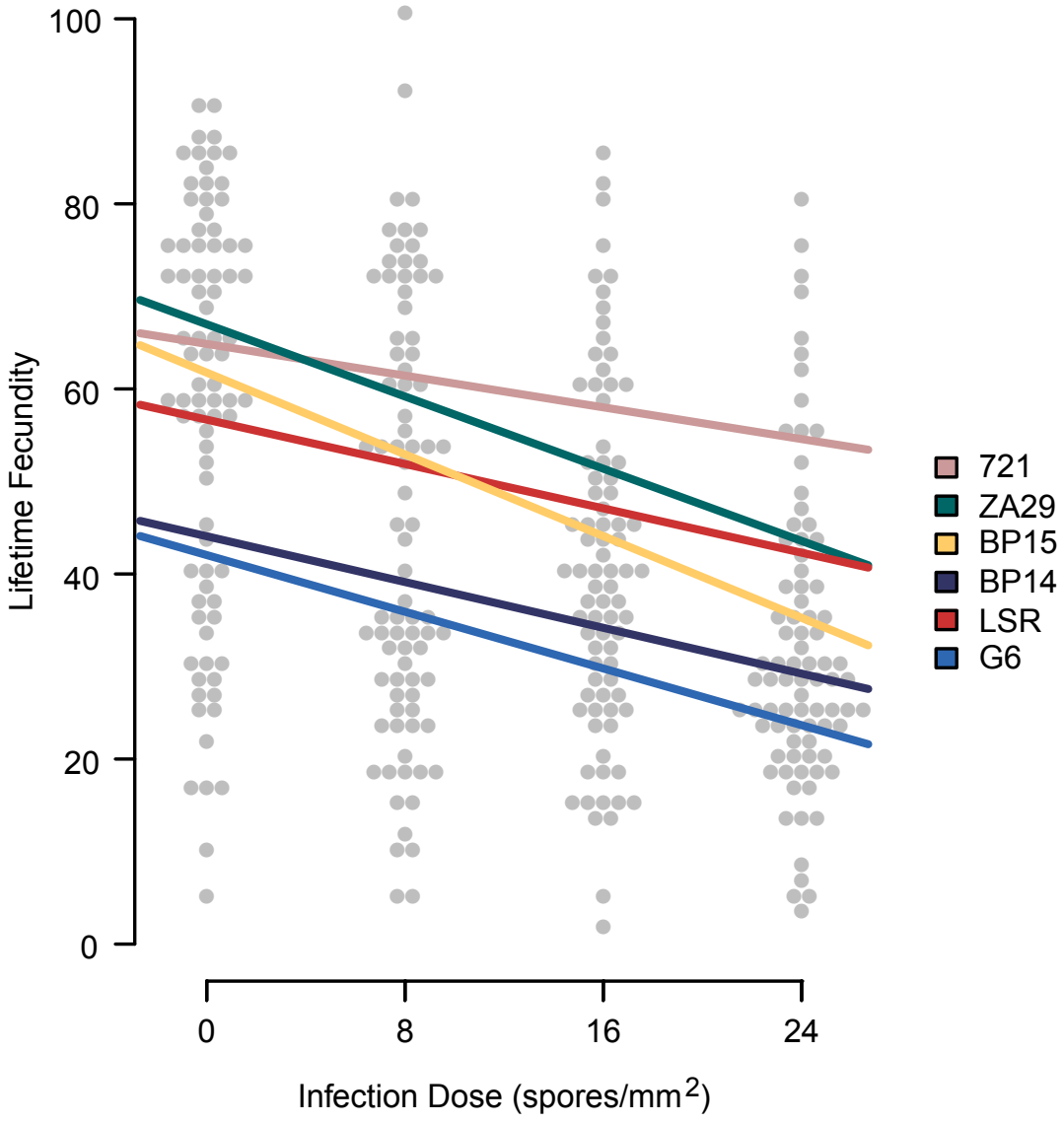


Figure 4-2: Fecundity as a function of infection dose. The grey points indicate the lifetime fecundity of each aphid, which are jittered so that they do not overlap. The lines represent a linear regression of infection dose against lifetime fecundity, with each genotype shown by a different color (using the same colors as in Figure 1). The differences in slope among these lines indicate variation in fecundity tolerance.

DISCUSSION

We found substantial variation among hosts in resistance to *Pandora*, as indicated by significant variation among aphid genotypes in survival (Figure 1B) and the percent of aphids that produced *Pandora* spores (Figure 1D) after fungal infection. We found no differences among genotypes in mortality tolerance when looking at survival or percent sporulation. We did find a significant interaction between infection dose and genotype on lifetime fecundity, a measure of fecundity tolerance, indicating that some aphid genotypes experienced a sharper decline in lifetime fecundity as infection dose increased than other genotypes (Figure 2).

Facultative symbionts play a role in aphid resistance against *Pandora* (Scarborough et al. 2005; Lukasik et al. 2012; Parker et al. 2013). It therefore has been unclear whether aphid genotype contributes to variation in resistance to *Pandora,* and more generally, whether we still find variation among hosts that rely heavily on symbionts for pathogen defense. One possibility is that reliance on symbiont-mediated immunity could lead to relaxed selection on the pea aphid's immune response (Altincicek et al. 2008), and result in lack of variation among host resistance in the absence of symbionts (Oliver et al. 2005). Indeed, investigations of the pea aphid immune system have shown a reduced complement of immune defenses relative to other invertebrates studied to date (Gerardo et al. 2010; Altincicek et al. 2011; Grell et al. 2011; Laughton et al. 2011a). Our findings show that there is substantial variation among hosts in

resistance to *Pandora* in the absence of symbionts. In this system, resistance variation is therefore likely due to a combination of genetic variation in host immunity, variation in symbiont presence, and variation in symbiont genotype (and potentially host genotype by symbiont genotype interactions). More broadly, this work suggests that natural selection can still maintain variation in host defense mechanisms even when host resistance is strongly influenced by other forms of defense. Future work will need to determine the relative influence of host mechanisms and symbionts on resistance (*e.g.,* (Vorburger et al. 2009), and how natural selection acts on resistance in the presence of protective symbionts.

We found no evidence of variation among genotypes in tolerance when measuring survival or percent sporulation, but we did find evidence of variation in tolerance of the effects of pathogen infection on host fecundity. Theoretical work predicts that tolerance can enhance both host and pathogen fitness resulting in fixation and an absence of variation (Roy and Kirchner 2000; Miller et al. 2005; Svensson and Råberg 2010). However, the findings of empirical studies of variation in tolerance have been mixed, and several studies have found evidence of genetic variation in tolerance mechanisms (*e.g.* (Simms and Triplett 1994; Koskela et al. 2002; Kover and Schaal 2002; Råberg et al. 2007; Corby-Harris et al. 2007; Ayres and Schneider 2008). A possible explanation for this discrepancy between theory and empirical studies is that many studies have focused on non-natural host—pathogen interactions (Corby-Harris et al. 2007; Råberg et al. 2007; Ayres and Schneider 2008) or wild-caught individuals that could be subject to environmental influences (Blanchet et al. 2010). Indeed, recent work has

shown how host ecology can influence pathogen tolerance (Sternberg et al. 2012). Another explanation comes from looking at how different mechanisms of tolerance recover host fitness. For example, mechanisms of tolerance that recover host fecundity during infection are predicted to be neutral or costly to pathogen fitness depending on trade-offs in the host, resulting in the maintenance of genetic variation in fecundity tolerance (Best et al. 2008). Our finding of an absence of variation in mortality tolerance and variation among genotypes in fecundity tolerance therefore supports existing theory. One possibility is that tolerant genotypes may be shifting their reproductive efforts earlier in their reproductive period in response to infection (coming at a cost to any future reproduction). This process, termed 'fecundity compensation,' has been shown to occur in aphids in response to bacterial signals (Altincicek et al. 2008; Barribeau et al. 2010), and a recent study in *Daphnia* showed that genotypes can vary in the magnitude of this shift (Vale and Little 2012). Future work will need to determine the mechanisms underlying aphid—*Pandora* interactions, and to tie these mechanisms to immune costs and to patterns of variation in resistance and tolerance.

Acknowledgements
We would like to thank Richard Humber, Karen Hansen, and the USDA ARS Collection of Entomopathogenic Fungal Cultures who provided cultures of *Pandora*. Alex Wilson and Rebecca Duncan assisted with the microsatellite protocol. Kerry Oliver and Nancy Moran provided several aphid genotypes. Jaap de Roode, Eleanore Sternberg, Amanda Pierce, Stephanie Chiang, and Alice Laughton provided valuable assistance with the experiments, analysis, and manuscript.

SUPPORTING INFORMATION

Table S1: Aphid Genotypes

Genotype	Geographic Location	Host-Plant	Date	Reference
721	Cayuga Co., NY	Alfalfa	2001	N.A. Moran, unpublished
ZA29	Montgomery Co., PA	Alfalfa / Clover	2010	K.M. Oliver, unpublished
BP15	Atlanta, GA	Pea	2010	This paper
BP14	Atlanta, GA	Crimson Clover	2010	This paper
G15	Atlanta, GA	Mixed weeds	2008	This paper
LSR	Ithaca, NY	Alfalfa	1998	(International Aphid Genomics Consortium 2010)
G6	Atlanta, GA	Mixed weeds	2008	(Barribeau et al. 2010)

Table S2: Primer Sequences and PCR protocols

Primer name	Primer Sequence	Ref.
Symbiont Screening		
35R	CCTTCATCGCCTCTGACTGC	(Russell et al. 2003)
U1279F	CGAACGTAAGCGAACCTCAT	(Russell et al. 2003)
R1279F	CGAGAGCAAGCGGACCTCAC	(Russell et al. 2003)
T1279F	CGAGGGAAAGCGGAACTCAG	(Russell et al. 2003)
PCR Reaction: Primers - .25μM each, Taq – 1 unit, dNTPs - .2μM each nucleotide, $MgCl_2$ – 2.5mM Cycles: 2 min @ 94°C, 40 cycles x (15 sec @ 94°C, 30 sec @ 48°C, 45 sec @ 70°C), 7 min @ 70°C		
16S_23SF	GCACTGCAGGATCCAGAGTTTGATCATGGCTCAGATTG	This paper
16S_23SR	GCAGGTACCGCGGCCGCGCTCGCGTACCACTTTAAATGGCG	This paper
PCR Reaction: 1X Buffer w/ $MgCl_2$, Primers – .2μM each, Taq – 1 Unit, dNTPs – 300μM each nucleotide Cycles: 2 min @ 94°C, 40 cycles x (30 sec @ 94°C, 30 sec @ 59°C, 2 min @ 70°C), 7 min @ 70°C		

Microsatellite Analysis		
AP02F	CGTCGCGACCTACCTGTAAT	(Kurokawa et al. 2004)
AP02R	GGGTCCGGTGTAAAAATTGA	(Kurokawa et al. 2004)
AP03F	GCAGCAAACAGCAGGTGTAAA	(Kurokawa et al. 2004)
AP03R	ACAATTGCTCGATGGTCCTC	(Kurokawa et al. 2004; Vorburger et al. 2009; Hansen et al. 2012)
AP05F	TCACCAAGGCATCCCTCTAC	(Kurokawa et al. 2004)
AP05R	GTAGCGAATTTTTCCGGTTG	(Kurokawa et al. 2004)
S17bF	TTCTGGCTTCATTCCGGTCG	(Wilson et al. 2004)
S17bR	CGTCGCGTTAGTGAACCGTG	(Wilson et al. 2004)
PCR Reaction: 1X Buffer w/ $MgCl_2$, Primers - .25μM each, Taq – 1 Unit, dNTPs - 375μM each nucleotide Cycles: 2 min @ 94°C, 8 cycles x (30 sec @ 94°C, 1 min @ 62°C (decrease by 1°C per cycle), 45 sec @ 72°C), 22 cycles x (30 sec @ 94°C, 1 min @ 55°C, 45 sec @ 72°C), 6 min @ 72°C		
Quantitative PCR		
Pandora_18S_F	TCTTTGGGCTTAGTTGGTACTTTACTG	This paper
Pandora_18S_R	GCCCGCTTTGAACACTCTAATT	This paper

Microsatellite Analysis:
Microsatellites were analyzed using the GS500_1_old size standard as this standard excludes the 250 bp standard fragment, which can migrate anomalously and result in inaccurate size estimation (Akbari et al. 2008). Size calls were made using the Local Southern algorithm. Each peak was visually inspected to confirm it was a true allele. Two allele sizes are listed under one locus name if the aphid was heterozygous at that locus. The allele sizes shown in Table S2 are rounded to the nearest whole number. Every aphid line had a distinct microsatellite genotype except BP15 and G15, which were identical at these four loci. The 263 and 267 alleles at the Ap05 locus were out of the range of sizes previously reported (Kurokawa et al. 2004).

Table S3: Allele sizes for each microsatellite locus

	Locus							
Genotype	s17b		Ap02		Ap03		Ap05	
721	208	210	228		241		253	262
BP14	211		225		240	242	263	
BP15	208	210	225		235	240	263	267
G15	208	210	225		235	240	263	267
G6	208	210	225	228	241		260	263
LSR	211		220	225	241	254	260	
ZA29	208	210	228		241		253	260

Pathogen Load:

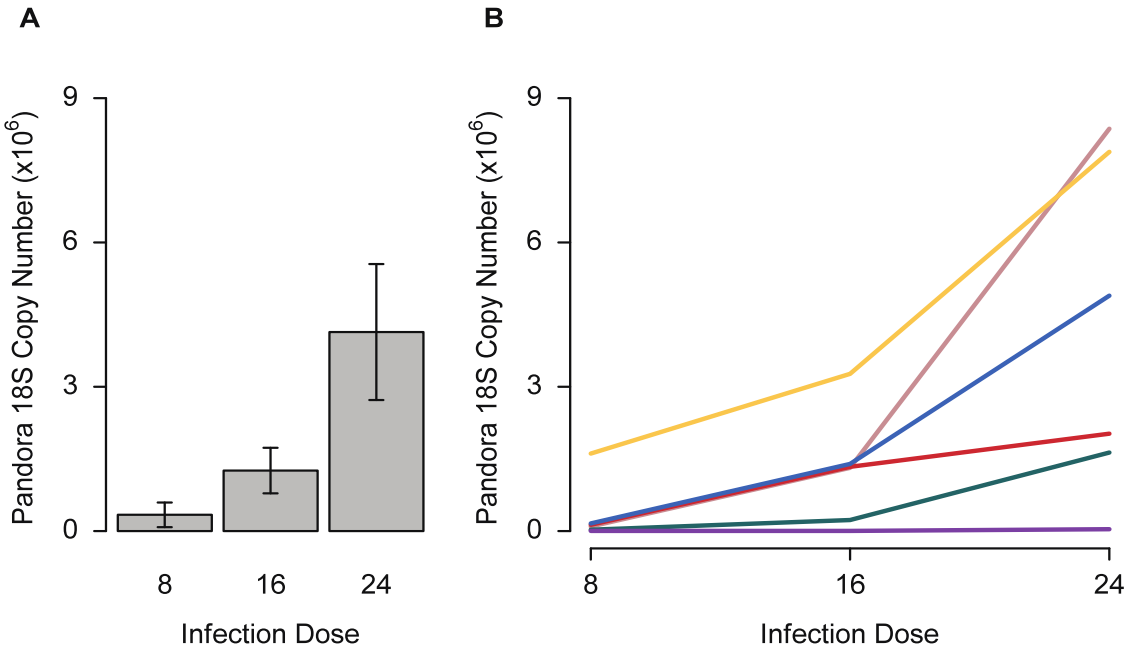


Figure S1: Pathogen burden at 4 days after infection, determined by the number of copies of *Pandora* 18S as measured by qPCR. The y-axes show *Pandora* 18S copy number (x 10^6). **A:** The pathogen load at day 4, averaged across genotypes, is greater at increasing infection doses. Bars show standard error. **B:** Day 4 pathogen load is shown for each genotype at the three infection doses. We found variation among genotypes in pathogen load, but no interaction between infection dose and genotype.

CHAPTER 5: SYMBIONT-MEDIATED PROTECTION AGAINST FUNGAL PATHOGENS IN PEA APHIDS: A ROLE FOR PATHOGEN SPECIFICITY?

Modified from Parker, B.J., Spragg, C.J., Altincicek, B., & Gerardo, N.M. Symbiont-Mediated Protection against Fungal Pathogens in Pea Aphids: a Role for Pathogen Specificity? *Appl. Environ. Microbiol.* 2013, 79(7):2455.

ABSTRACT

Here we show that a bacterial endosymbiont, *Regiella insecticola*, protects pea aphids (*Acrythosiphon pisum*) from the aphid-specific fungal entomopathogen *Zoophthora occidentalis*, but not from the generalist insect fungal pathogen *Beauveria bassiana*. This finding highlights the complex influence of fungi on the dynamics of this economically important agricultural pest.

Symbiotic relationships between invertebrates and vertically transmitted microbes are widespread. One feature of this mutualistic relationship is that symbionts depend on host resources for their own survival and reproduction (Haine et al. 2008), and theory therefore predicts that in the absence of manipulation of host reproduction, beneficial symbionts must provide a fitness advantage to spread through a host population (Brownlie and Johnson 2009). Studying ecologically relevant traits conferred to hosts by symbionts is critical for

understanding host-microbe dynamics, and researchers have therefore searched for fitness advantages of harboring symbionts in a number of systems. Several recent studies have shown that one advantage conferred by some symbionts is protection against pathogens and parasites (Oliver et al. 2005; Scott et al. 2008; Teixeira et al. 2008; Jaenike et al. 2010; Vorburger et al. 2010). For example, pea aphids (*Acyrthosiphon pisum*), which are a model system for the study of host-symbiont dynamics, are protected against the fungal entomopathogen *Pandora neoaphidis* (Zygomycota: Entomophthorales) by several facultative, vertically transmitted bacteria, including the gammaproteobacteria *Regiella insecticola* (Scarborough et al. 2005; Lukasik et al. 2012). Fungi are important natural pathogens of aphids and are used in biocontrol (Pell et al. 2010; Hajek and Delalibera 2010), and symbiont-mediated protection to fungi is likely an important factor influencing the population dynamics of aphids and their symbionts. However, aphids encounter several diverse species of fungal pathogens in the wild (Nielsen and Hajek 2005). It is not known if *Regiella*-conferred protection is specific to *Pandora* or if it extends to other species of fungus as well, which would suggest that multiple fungal species are influencing aphid-*Regiella* dynamics. We therefore exposed pea aphids, with and without symbionts, to two additional species of fungal pathogens: *Zoophthora occidentalis* (Zygomycota: Entomophthorales), a highly aphid-specific entomopathogen, and *Beauveria bassiana* (Ascomycota:: Hypocreales), a generalist that has been found in a variety of hosts, including species of Coleoptera, Hemiptera, and Diptera (Nielsen and Hajek 2005; Vega et al. 2009;

Hatano et al. 2012). These fungal species are highly divergent (with some estimates as high as 1000 mya (Hibbett et al. 2007)), but both species reproduce by passively releasing spores (conidia) that penetrate the cuticle of a suitable host. Mycelia then colonize the host's body tissue until the death of the host, when new spores are produced and released into the environment.

We used two aphid genotypes, both with and without *Regiella* present (5A, collected in the wild in 1999 near Madison, Wisconsin and subsequently injected with *Regiella* symbionts from an aphid collected in Tompkins Co., NY, in 2000; and LSR1, collected on alfalfa near Ithaca, NY in 1998 with a natural *Regiella* infection and then artificially cleared of symbionts.) (International Aphid Genomics Consortium 2010). The use of two aphid genotypes each with and without *Regiella* allowed us to control for effects of aphid genotype on pathogen susceptibility. Before fungal infection, we maintained pea aphids asexually on fava bean (*Vicia faba*) plants at 16hrs light: 8hrs dark at 20°C. We exposed adults to fungus after their final molt (at nine days of age), as we found that molting shortly after exposure strongly reduces infection probability. After exposure, aphids were kept individually for 4 days on fava bean plants under near 100% humidity, after which the humidity was reduced to 70%. This allowed enough time for fungal penetration of the aphid cuticle, which requires high humidity.

Zoophthora (specialist) infection**:**

We infected aphids with *Zoophthora* by placing them under a ‘spore shower' (based on(Scarborough et al. 2005; Baverstock et al. 2005a; 2006; 2008; Hatano et al. 2012). An isolate of *Zoophthora* was obtained from the USDA ARS Collection of Entomopathogenic Fungal Cultures, and was grown for 2 weeks on SDAEY plates at 20°C (Papierok and Hajek 1997). Approximately 15 hours before infection, several small pieces of fungal mycelium ($3mm^2$) were cut with a sterile instrument and placed onto 1.5% tap water agar, which causes the fungus to sporulate. At the time of infection, the agar plates were inverted over a hollow tube for 60 minutes with aphids at the bottom of the chamber. The agar plates were rotated among treatment groups during infection to ensure that each treatment group was exposed to an equal dose of fungal spores (approximately 16.5 spores/mm^2). We included a glass slide in this rotation so that spores could be counted under a light microscope to estimate spore density. Control aphids were handled similarly but were not exposed to fungus.

The *Zoophthora* infection was divided into three blocks conducted several days apart from one another. In each block, we exposed two-thirds of the individuals from each genotype to fungus, and kept one-third under identical conditions as a control. Half of the aphids of each genotype harbored *Regiella*. Nine days after exposure, we recorded the survival of each aphid. We fit a logistic regression model (a Generalized Linear Model (GLM) with quasibinomial error structure and logit link function) to aphid survival with symbiont, fungal exposure, and block as fixed effects. We used R version 2.11 for our statistical

analyses. As expected, aphids exposed to fungus had significantly lower survival than control aphids (odds ratio (OR) = 0.33, 95% confidence interval (CI) = [0.15, 0.72], $p = 5.8*10^{-3}$). There was also a significant interaction between exposure and symbiont-infection on aphid survival (OR = 3.7, CI = [1.2, 11], p = 0.021), but no effect of *Regiella* independently (OR = 0.70, CI = [0.28, 1.73], p = 0.44). This means that in the absence of fungal infection *Regiella* did not have a significant impact on aphid survival, but it increased survival of aphids exposed to fungus (figure 1). The trends were consistent across three blocks, but aphid survival differed across blocks (block 2: OR = 2.6, CI = [1.4, 4.7], $p = 2.5*10^{-3}$ and block 3: OR = 3.4, CI = [1.8, 6.7], $p = 3.2*10^{-4}$). The trend was consistent across both aphid genotypes, and there was no significant effect of aphid genotype on survival; it was therefore removed from the model. More aphid genotypes will need to be tested to determine the effect, if any, of aphid genotype on fungal infection outcome. Using this same protocol and these same genotypes, we also confirmed the results of Scarborough et al. (2005)—that *Regiella* protects pea aphids from *Pandora,* which, like *Zoophthora*, is an aphid-specific fungal pathogen (Supplementary Figure 5A-1). We also conducted a repeat of the *Zoophthora* infection, using a second pathogen genotype, to ensure that our results were consistent across multiple experiments (Supplementary Figure 5A-2).

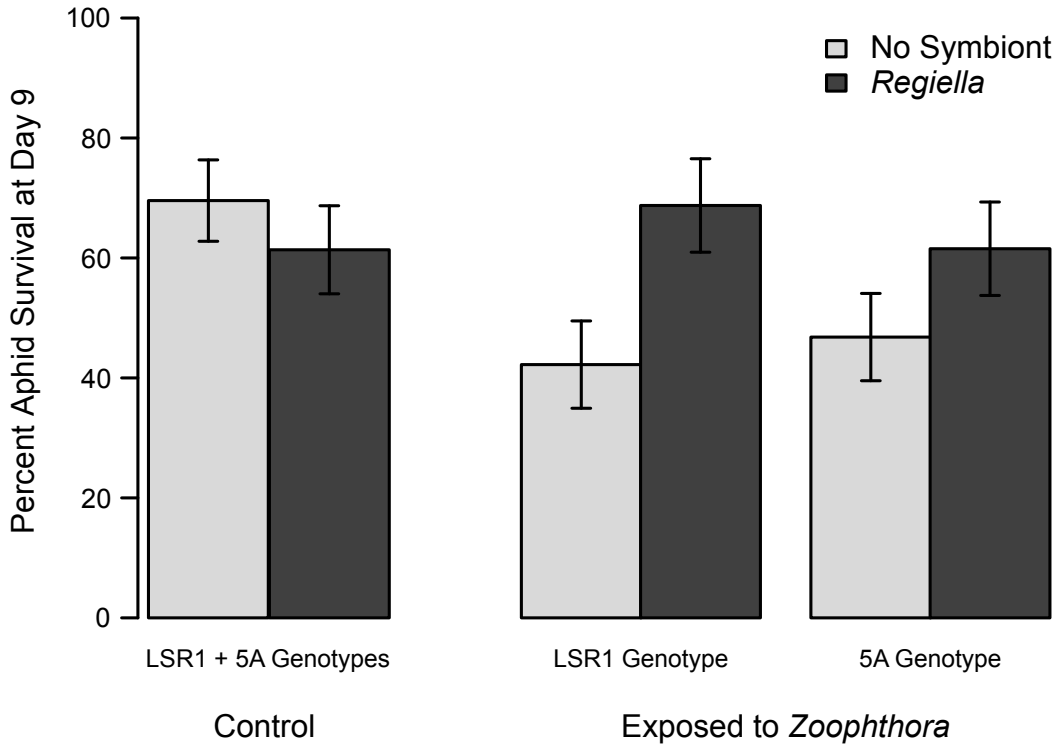


Figure 5-1: Results of infection with Zoophthora. Survival of each aphid was recorded 9 days after infection and is reported as the percent survival of each group. White bars represent aphids with no secondary symbionts (n = 138), and gray bars represent aphids that harbored Regiella (n = 131). Error bars are standard errors.

BEAUVERIA (GENERALIST) INFECTION:

Cultures of *Beauveria* did not sporulate upon transfer to tap water agar, so we instead made up solutions of *Beauveria* spores (Strain GHA, Botanigard es) in distilled water. Spores were washed and separated via centrifuge from inert ingredients, and 0.7μL of the solution was pipetted onto the dorsal side of the abdomen of each aphid. Half of the aphids of each genotype harbored *Regiella*. We exposed aphids to four different spore doses (0, 25, 250, and 2500 spores, estimated using a HYCOR KOVA Glasstic hemocytometer). Aphids became

infected with *Beauveria* faster than with *Zoophthora*, so for the *Beauveria* infections we recorded the survival status of each aphid at 24hr intervals after exposure, and we analyze this data using a survival analysis (Figure 5-2). For the *Beauveria* infection we took the additional precaution of transferring the symbiont from the 5A line into the LSR1 aphid genotype. Symbiont free first instar LSR1 aphids were exposed to the hemolymph of 5A adult aphids that harbored *Regiella* via intrahemocoellic microinjection. Aphids were then kept for at least 10 generations to allow the symbiosis to stabilize and to allow the host lines to adapt to the presence of the bacteria (Koga et al. 2003). This allowed us to compare survival of genetically identical hosts with two different genotypes of symbionts. Survival data were analyzed using age-specific parametric survival models with a Weibull distribution using the Survival package in R version 2.11. The dose of fungal pathogen exposure had a significant impact on aphid survival (minimal model containing spore dose only: $\chi^2 = 935.96$ on 3 d.f., $p < 0.0001$). However, symbiont status had no significant effect on survival, had no interaction with dose, and was removed from the minimal model. This suggests that *Regiella* did not protect aphids from infection with *Beauveria* (Figure 5-2). Aphid genotype had no significant effect on survival and was also removed from the minimal model. We repeated this experiment with aphids that harbored two other species of aphid secondary symbionts, *H. defensa* and *S. symbiotica*, and again found no effect of symbionts on aphid susceptibility to *Beauveria* (Supplementary Figure 5B-1). We also conducted a repeat of the *Beauveria* infection using different spore doses and found no effect of *Regiella* (Supplementary Figure 5B-2). Lastly,

to ensure that this negative result was not due to a sampling effect of *Regiella* genotypes, we conducted an additional experiment where we collected aphids with and without *Regiella* from several geographical locations, and assayed their resistance to *Beauveria*. With this experimental design, we are not controlling for host genotype, as each strain used in the experiment will differ both in terms of symbiont and host genotypic background. However, we are able to determine if multiple wild–collected lines with *Regiella* are, on average, more resistant to *Beauveria* than wild–collected lines without symbionts. We found significant variation in resistance to *Beauveria* among aphid genotypes, but no effect of harboring *Regiella* symbionts (see Supplementary information 5C), suggesting that our results are not due to a lack of diversity in symbiont genotypes.

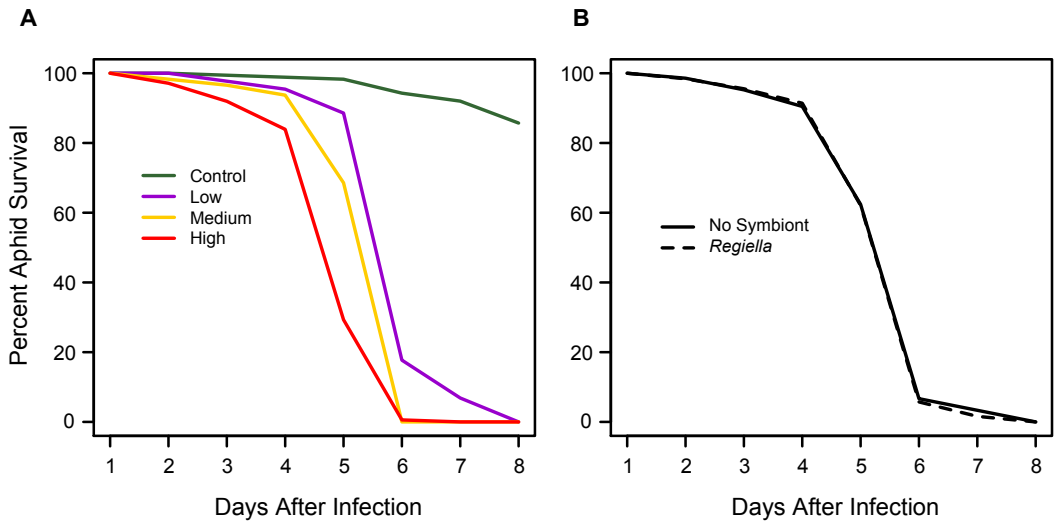


Figure 5-2: Results of infection with *Beauveria*. Survival of each aphid was recorded every 24 h between 0 and 8 days after infection and is reported as survival curves measured in percent survival of each group. **(A) Spore dose**. Each line represents a different spore dose (control, 0 spores/aphid; low, 25; medium, 250; high, 2,500), n = 175 per dose. **(B) Symbiont status**. The solid

line shows infected aphids without *Regiella* (n = 210). The dotted line shows infected aphids that harbored *Regiella* (n = 315).

Together these data suggest that *Regiella* symbionts confer protection against several specialist fungal pathogens, but not against a generalist pathogen. *Regiella* frequencies vary among aphid populations (Tsuchida et al. 2002; Moran et al. 2005a), and researchers have tried to determine the factors that influence symbiont frequencies as a way of better understanding this model host-microbe interaction. Variation in *Regiella* frequency is explained in part as a balance between the benefits of protection from fungal pathogens and the costs of harboring bacteria. Our results suggest that several species of fungal pathogens may be driving this interaction, but that *Regiella* is not beneficial against all species of fungi. In addition, these fungal pathogens are used in aphid biocontrol (Hajek and Delalibera 2010; Jackson et al. 2010), and our results suggest that symbiont-mediated protection against pathogens may be an important consideration when selecting and developing biocontrol agents.

In general, researchers are working to develop an understanding of how evolution acts on alternative defenses (like that conferred from symbionts) organisms have to protect themselves from pathogens and parasites (Parker et al. 2011). One possible explanation for the pattern of symbiont-mediated protection observed here is that *Regiella* protection has evolved in response to pressure from individual species of fungal pathogens. A second possibility is that protection in this system has evolved in response to fungal pathogens that specialize on aphids, but not to generalist insect pathogens, perhaps due to

broad differences found in the infection strategies of generalists vs. specialists (Schlenke et al. 2007). Pathogen specificity has been shown to be an important factor influencing host-enemy interactions (Antonovics et al. 2012; Barrett and Heil 2012), and we therefore highlight the relative specificity of these pathogens as a potential explanation for the pattern of protection observed in this system, but clearly more data are needed. It is possible that close co-evolution between aphid specialists and *Regiella* is needed to develop or maintain protection conferred by symbionts.

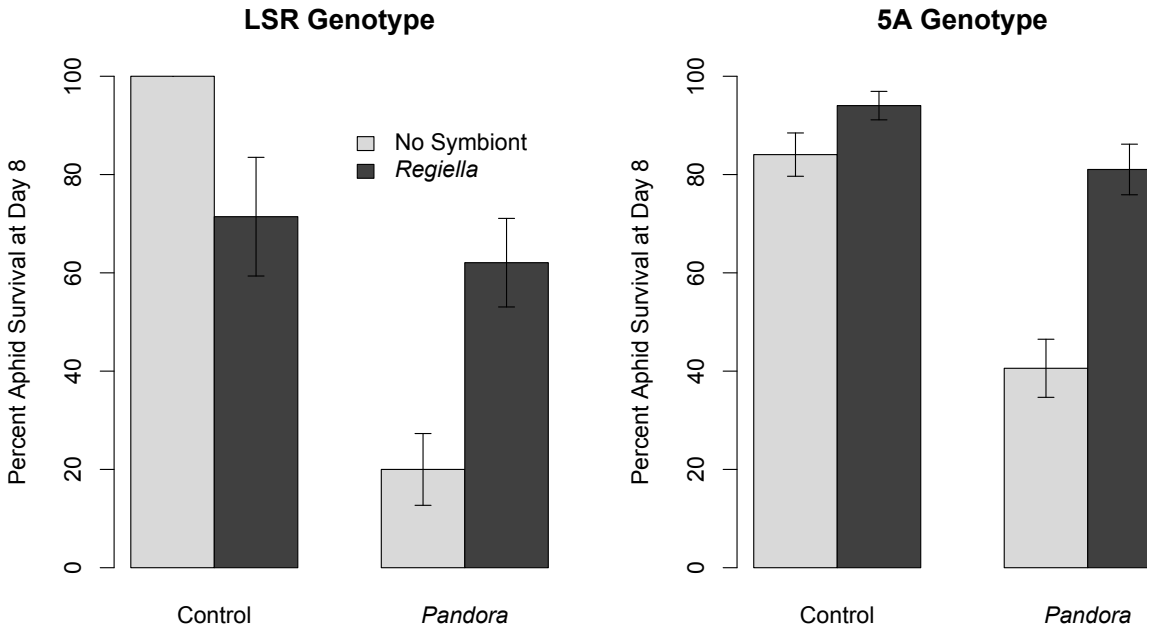
Thierry Lefèvre, Alice Laughton, Eleanore Sternberg, Anand Bhardwaj, and four anonymous reviewers provided valuable assistance with the manuscript. We would like to especially thank Richard Humber, Karen Hansen, and the USDA ARS Collection of Entomopathogenic Fungal Cultures who provided cultures of *Zoophthora*. This research was supported by NSF IOS-1025853 to NMG. BA was supported by a DFG grant (AL902/4-1), and BJP is supported by an NSF Graduate Research Fellowship.

SUPPORTING INFORMATION

Supplementary Information 5A

Scarborough et al. (2005) found that harboring the intracellular symbiotic bacteria *Regiella insecticola* protects pea aphids from the aphid-specific fungal entomopathogen *Pandora neoaphidis*. We confirmed this result by exposing pea aphids from two genotypes, with and without *Regiella,* to *Pandora* spores. We used the same infection protocol as described for *Zoophthora* in the main text. Approximately half of the aphids used harbored symbionts. Eight days after exposure we recorded the survival of each aphid. We fit a logistic regression

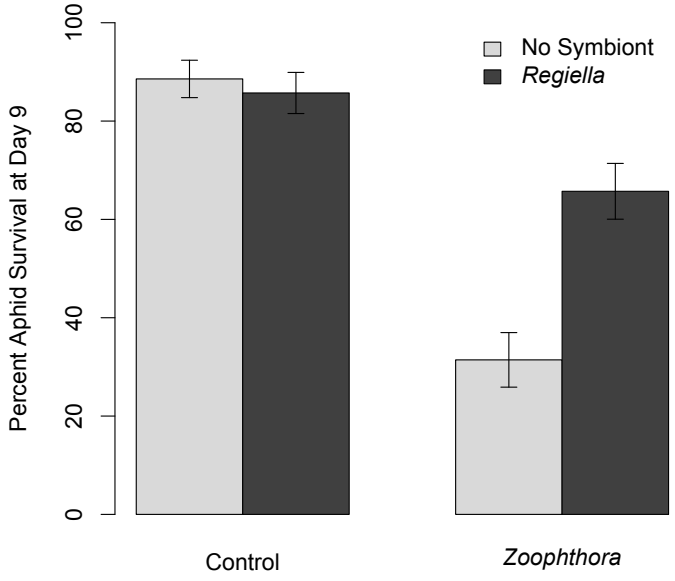
model (a Generalized Linear Model (GLM) with quasibinomial error structure and logit link function) to aphid survival with symbiont, fungal exposure, and genotype as fixed effects. As expected, aphids exposed to fungus had significantly lower survival than control aphids (odds ratio (OR) = 0.082, 95% confidence interval (CI) = [0.037, 0.17], $p = 1.4*10^{-10}$). There was also a significant interaction between exposure and symbiont-infection (OR = 4.4, CI = [1.4, 14], p = 0.012), but no effect of *Regiella* independently (OR = 1.4, CI = [0.52, 3.8], p = 0.52). Survival also differed between the two genotypes (OR = 0.46, CI = [0.25, 0.83], p = 0.0093). These results confirm that aphids are less susceptible to *Pandora* when they harbor the strains of *Regiella* used in our experiments.



Supplementary Figure 5A-1: Results of infection with *Pandora*. Survival of each aphid was recorded 8 days after infection, and is reported as % survival of each group. Lighter bars represent aphids with no secondary symbionts, and

darker bars represent aphids that harbored *Regiella* for each genotype. Bars are +/- standard error.

To ensure that the results of our *Zoophthora* infection were consistent across experimental replicates and across pathogen genotypes, we repeated the infection using a second fungal genotype in the LSR aphid genotype. We used the same infection protocol as described for *Zoophthora* in the main text. Nine days after exposure we recorded the survival of each aphid. We fit a logistic regression model (a Generalized Linear Model (GLM) with quasibinomial error structure and logit link function) to aphid survival with symbiont and fungal exposure as fixed effects. Aphids exposed to ZO again had lower survival than control aphids (OR = 0.059, CI = [0.024, 0.15], $p = 2.5*10^{-9}$). There was again a significant interaction between exposure and symbiont-infection (OR = 5.4, CI = [1.6, 18], p = 0.0076), but no effect of *Regiella* independently (OR = 0.77, CI = [0.28, 2.1], p = 0.62).

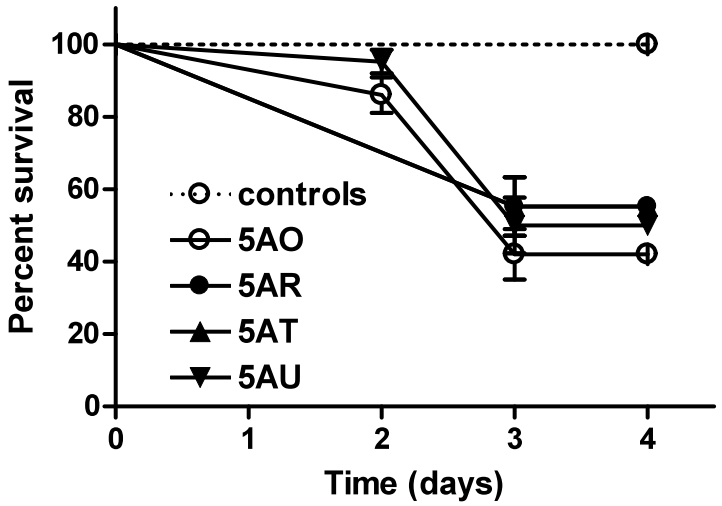


Supplementary Figure 5A-2: Infection with an alternative *Zoophthora* genotype. Survival of each aphid was recorded 9 days after infection, and is reported as % survival of each group. Lighter bars represent aphids with no secondary symbionts, and darker bars represent aphids that harbored *Regiella*. All aphids used were from the LSR genotype. Bars are +/- standard error.

Supplementary Information 5B

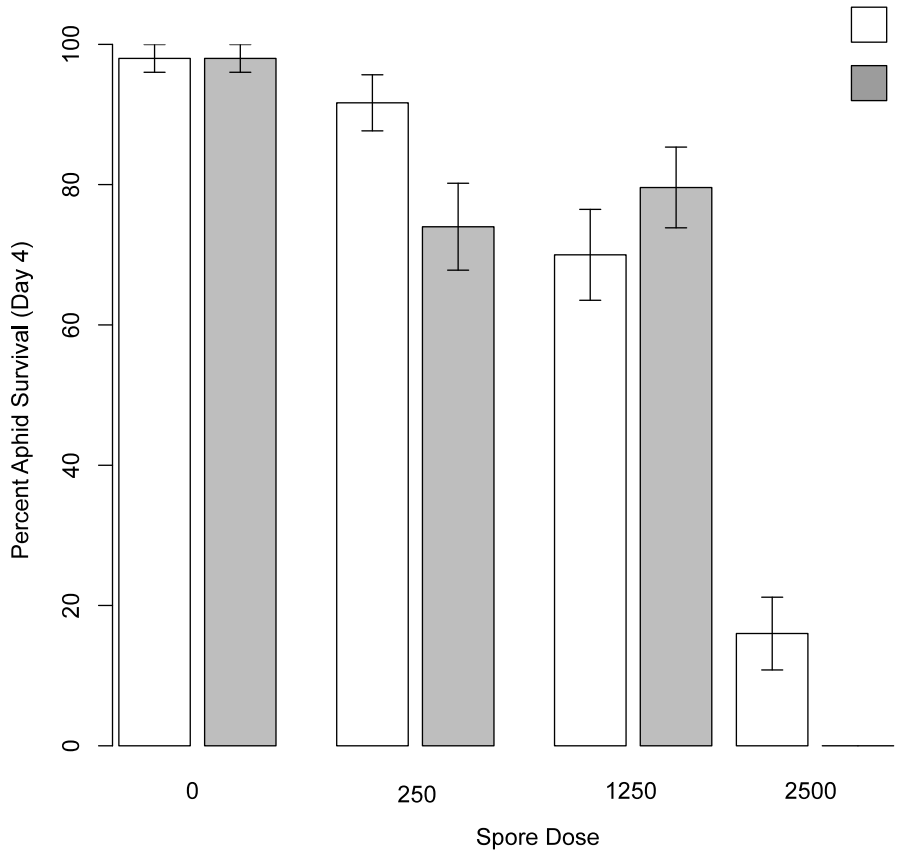
We repeated the *Beauveria* infection with two additional species of aphid secondary symbionts, *Hamiltonella defensa* and *Serratia symbiotica*, to see if these symbionts confer protection to pea aphids against *Beauveria*. All three secondary symbionts were established in genotype 5A at the same time, and have been maintained in the lab under identical conditions for several years. We used a higher spore dose in this trial (approximately 7000 spores). For control treatments, spores were heat-killed by autoclaving (20 min at 121°C and 1.5 bar pressure) prior to use. Aphids (3 per plate) were put on agar plates (10 cm in

diameter) containing a square of tap-water agar with a fresh fava bean leaf inserted into it. Plates were then sealed with parafilm to ensure high humidity over the infection period. Survival of each aphid was recorded 2, 3, and 4 days after infection. We used a cox proportional hazards model (with censoring) in R version 2.11. Minimal models were derived by removing terms followed by model comparison with a likelihood ratio test. Terms were retained in the minimal model if their removal significantly reduced the explanatory power of the model. Exposure to *Beauveria* significantly reduced host survival ($p = 7.7*10^{-3}$). Harboring a symbiont and host genotype had no effect on survival. These results confirm that *Regiella* has no protective effect against *Beauveria* infection, and suggest further that the tested *Hamiltonella and Serratia* genotypes also do not protect aphids against *Beauveria* infection.



Supplementary Figure 5B-1: *Beauveria* infection with other secondary symbionts. Survival of each aphid was recorded 2, 3, and 4 days after infection, and is shown as a survival curve for each group. The graph shows aphids of genotype 5A (n = 208), for which we included lines with all three secondary symbionts (5AO – no symbiont, 5AR – *Serratia symbiotica,* 5AT – *Hamiltonella defensa*, 5AU – *Regiella insecticola*). The dotted line indicates control aphids (n=59).

We repeated the infection with *Beauveria* at four different spore doses and recorded the survival status of each aphid four days after exposure. We fit a logistic regression model (this time with a binomial error structure). The dose of fungal pathogen exposure had a significant impact on aphid survival (OR = 0.91, CI = [0.89, 0.92], $p = 2.0*10^{-16}$), but symbiont and genotype had no effect and were removed from the model.

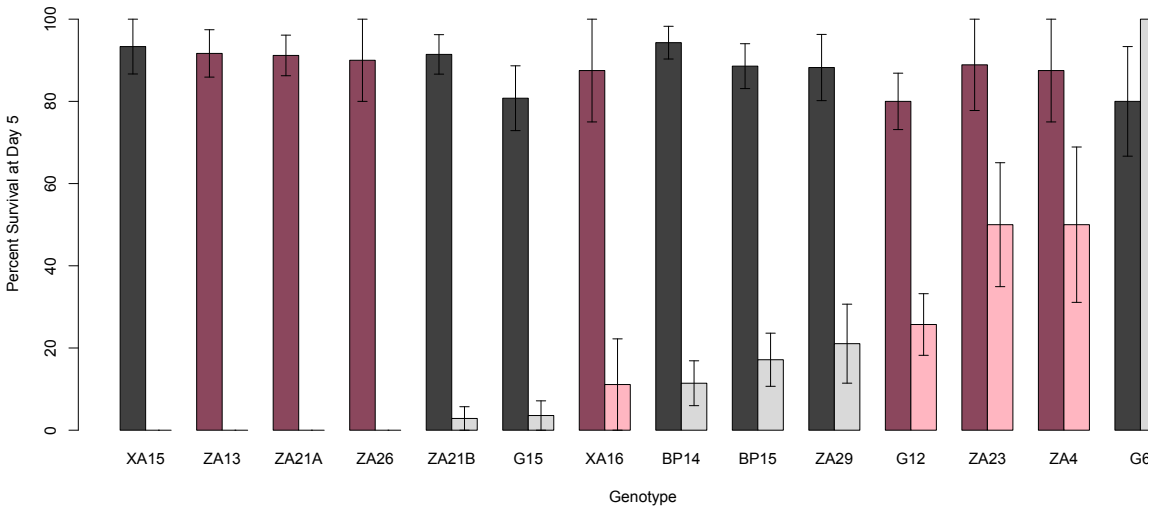


Supplementary Figure 5B-2: Repeat of infection with *Beauveria*. Survival of each aphid was recorded 4 days after infection, and is shown as % survival in each group. White bars represent aphids with no secondary symbionts (n = 198), and grey bars represent aphids that harbored *Regiella* (n = 199), displayed for both genotypes (5A: n = 198, LSR: n = 199). The x-axis shows the dose of spores given to each group. Error bars are +/- standard error.

Supplementary Information 5C

Symbiont genotypes are likely to vary among populations, and this genetic variation is expected to influence the extent to which protective symbionts are able to increase the resistance of their hosts. In the main text, we discuss how in two pairs of host and symbiont, *Regiella* does not confer protection from the generalist fungal pathogen *Beauveria bassiana*. To ensure that these results

were not specific to the two strains we used in the experiment, we assayed several additional wild-caught lines, some with and some without *Regiella*, for symbiont—mediated protection. With this experimental design, we are not controlling for host genotype, as each strain used in the experiment will differ both in terms of symbiont and host genotypic background. However, we are able to determine if multiple wild-collected lines with *Regiella* are, on average, more resistant to *Beauveria* than wild-collected lines without symbionts. We fit a logistic regression model (a Generalized Linear Model (GLM) with binomial error structure and logit link function) to aphid survival with symbiont, fungal exposure, and genotype as fixed effects. Minimal models were derived by removing terms followed by model comparison, and terms were retained in the minimal model if their removal significantly reduced the explanatory power of the model. Exposure to *Beauveria* significantly reduced survival (Binomial GLM, $\chi^2 = 394$, d.f. = 1, $p = 2.2*10^{-16}$), and we found significant variation in survival ($\chi^2 = 23.5$, d.f. = 13, p = 0.036) and in resistance to *Beauveria* among aphid genotypes (Treatment * Genotype interaction, $\chi^2 = 51.1$, d.f. = 13, $p = 1.9*10^{-6}$). Symbiont status had no effect on survival or *Beauveria* resistance, and thus it was removed from the model. Variation in protection among *Regiella* genotypes is an ongoing area of investigation, and clearly more data are needed. However, these results suggest that the lack of protection from *Beauveria* we describe in two genotypes in the main text is not merely a sampling effect.



Supplementary Figure 5C: *Beauveria* infection with wild-collected lines. Survival of each aphid was recorded 5 days after infection. Pink bars (ZA13, ZA21A, ZA26, XA16, G12, ZA23, ZA4) represent aphid lines that harbor *Regiella*, black bars (XA15, ZA21B, G15, BP14, BP15, ZA29, G6) represent symbiont-free genotypes. The darker bars of each color are Control aphids, and the lighter bars are *Beauveria* infected aphids. Error bars are +/- standard error.

CHAPTER 6: IMMUNE COSTS IN THE PRESENCE OF PROTECTIVE MICROBES

INTRODUCTION

Researchers are increasingly considering the importance of ecological context when studying an organism's immune response. A first principle of this field, which has been termed ecological immunology, is that immune responses are costly for hosts, leading to a tradeoff between investment in pathogen defense and other life-history traits (Rolff and Siva-Jothy 2003; Sadd and Schmid-Hempel 2009). However, the tradeoff between immunity and life history traits is context dependent (Moret and Schmid-Hempel 2000; Otti et al. 2012). Exploring the conditions that determine the impact of an immune response on an individual's fitness is critical for understanding how evolution acts on parasite defense.

Recent work has demonstrated the important role that an organism's microbial community can play in mitigating disease (reviewed in Brownlie and Johnson 2009). The human gut, for example, is host to symbiotic bacteria that defend against colonization by opportunistic pathogens (Round and Mazmanian 2009). Other examples include fungi that protect plants from pathogen attack (Arnold et al. 2003), a herpes virus that confers resistance against two pathogenic bacteria of mice (Barton et al. 2007), and *Spiroplasma* bacteria that protect *Drosophila* flies from parasitic nematodes (an advantage that may have led to the spread of *Spiroplasma* through a North American fly population

(Jaenike et al. 2010)). The number of examples of protective associations is rapidly increasing, indicating that symbiont-mediated protection commonly influences disease outcome in a taxonomically diverse set of hosts.

A number of recent studies have looked at interactions between symbionts and host immune systems, and have found that immune responses can play a role in regulating symbiont populations (e.g. (Anselme et al. 2006; Nyholm et al. 2009; McFall-Ngai et al. 2010; Lazzaro and Rolff 2011; Login et al. 2011; Nyholm and Graf 2012). However, extent to which symbiont-mediated protection from a pathogen changes a host's own response when infected remains to be determined. As a first step towards this understanding, we measure immune costs in hosts with and without symbionts to determine whether protective symbionts can modify the costs of immunity. We use the best-understood model of microbial symbiosis, that of pea aphids (*Acyrthosiphon pisum*) and their facultative bacterial symbionts. All aphids harbor the primary symbiont *Buchnera aphidicola*, which is responsible for amino acid synthesis, and individual aphids within a population may also harbor zero to a few facultative, 'secondary' symbionts (Moran et al. 2005a,b). Aphid symbionts are vertically transmitted with high fidelity and live both intracellularly in bacteriocytes and other cells, and extracellularly in the insect's body cavity (Moran and Dunbar 2006). Aphid secondary symbionts confer a number of ecologically relevant traits to their hosts, including defense against parasitic wasps and fungal pathogens (Oliver et al. 2003; 2005; Scarborough et al. 2005; Oliver et al. 2009). Here we focus on the association between pea aphids and the Gram-negative secondary symbiont

Regiella insecticola, which confers substantial protection to the pea aphid against the natural aphid-specific fungal pathogen *Pandora neoaphidis* (Scarborough et al. 2005; Lukasik et al. 2012; Parker et al. 2013).

METHODS

We first established a set of three aphid lineages that varied in symbiont composition but which had the same host genetic background. We used genotype LSR, which was collected with a strain of *Regiella* (LSR-Ri). This line was cleared with antibiotics, producing an uninfected strain (LSR01), and was subsequently infected with a second strain of *Regiella* (LSR 5.15) via intrahemocoellic microinjection with hemolymph transferred from another species of aphid, *Myzus persicae* (Vorburger et al. 2010; Hansen et al. 2012).

In natural populations of pea aphids, *Regiella* (including strain LSR-Ri) protects aphids from the fungal pathogen *Pandora* (Scarborough et al. 2005; Parker et al. 2013; Lukasik et al. 2012), but has no effect on other pathogens and parasites (Oliver et al. 2010). *Regiella* 5.15 in natural populations of *M. persicae* was shown to protect its aphid host from infection with parasitoid wasps (Vorburger et al. 2010). Subsequent genomic analysis revealed substantial genetic differences between strains—the genome of *Regiella* 5.15 contained intact Type 1 and 3 secretion system and other pathogenicity factors not found in LSR-Ri thought to be responsible for the protection seen against wasps (Hansen et al. 2012). We first tested these two strains against the fungal pathogen *Pandora* to see of *Regiella* 5.15 protected pea aphid hosts against fungi.

We exposed the three aphid lineages with different symbiont backgrounds (No *Regiella* symbiont (LSR01), *Regiella* symbiont (LSR-Ri), *Regiella* symbiont (5.15)) to two different treatments (control, fungal pathogen). We used standardized laboratory infection protocols in which aphids were exposed to a shower of spores of a single fungal strain (genotype ARSEF 2588) and then transferred to plants (Parker et al. 2013). Kept in high humidity (< 90%), *Pandora* kills susceptible aphids between 4 and 9 days after exposure (Fig. 1b), and we recorded the survival of each aphid during this period. *Pandora* is transmitted when infected hosts produce sporulating cadavers the release spores into the environment, and we recorded whether each killed aphid produced spores as a second measure of resistance against *Pandora*. Survival data were analyzed using non-parametric survival models using the 'Survival' package in R v. 2.15. A test of proportional hazards was conducted to ensure the data fit model assumptions. Minimal models were derived with model comparisons using ANOVA; terms were removed if their removal did not significantly influence model fit. A Tukey's post-hoc test was conducted between all combinations of factors using the multcomp package in R (Hothorn et al. 2008). We analyzed whether each aphid produced a sporulating cadaver after *Pandora* exposure using a binomial generalized linear model (GLM) in R with symbiont background as a fixed effect. We then screened for immune costs by first creating a heat-killed suspension of *Pandora* and then injecting aphids with pathogen signal. After a fungal infection, five sporulating cadavers were removed from plants at the first sign of infection and dehydrated at 4°C. These cadavers were stored at

4°C for one month, at which point they were crushed up in 200μL Insect Ringer's Solution, and autoclaved for 20 minutes at 121°C. Aphids produce two genetically identical polyphenic morphs, with and without wings, and in our previous work (Chapters 2 & 3) we found that immune costs to *Pandora* were limited to winged aphids. We produced winged aphids by exposed developing aphids to the alarm pheromone (E)-β-farnesene (EBF) (5 μL of 1000 ng/μL EBF every 48 hrs for 10 days). We then reared offspring of these EBF-exposed aphids, born within a 24-hour period, on fava plants for seven days. Aphids were divided randomly into two treatments: a control treatment where they were stabbed ventrally in the thorax with a minutin pin (0.15mm) dipped in autoclaved Ringer's solution (without *Pandora*), and an exposed treatment where they were stabbed with a needle dipped in the autoclaved *Pandora* solution. Aphids were allowed to heal in a Petri dish for 1 hour, and were then reared to adults. We recorded fecundity at 12 days after treatment. Fecundity data were analyzed using a quasipoisson GLM in R with treatment and symbiont background as fixed effects. Minimal models were derived as described above.

We investigated the cellular immune response of aphids from two of the symbiont backgrounds (symbiont free and *Regiella* LSR-Ri). Aphids possess several distinct cell (hemocyte) types, one of which (granulocyte) has a demonstrated role in aphid immunity (Laughton et al. 2011a; Schmitz et al. 2012). We sampled aphids at 72 and 96 hours post *Pandora* infection, and quantified the number of cells in fixed volumes (0.25μL) of hemolymph taken from *Pandora*-infected and control aphids as a measure of cellular immunity

(Sorrentino et al. 2002; Wilson et al. 2003; Amdam et al. 2005; Márkus et al. 2009). Cell count data were analyzed using a quasipoisson GLM with treatment and symbiont background as fixed effects.

RESULTS

Symbiont background (χ^2 = 9.52, 2DF, p = 0.0085), *Pandora* treatment (χ^2 = 201, 1DF, p < 0.0001), and the interaction between symbiont and treatment (χ^2 = 53.1, 2DF, p < 0.0001) all significantly affected pea aphid survival (Figure 1). Post-hoc tests showed that the survival of *Pandora* infected aphids that harbored *Regiella* LSR-Ri was significantly higher than those that did not have symbionts (z = 4.09, p < 0.001) and those that harbored *Regiella* 5.15 (z = 9.26, p < 0.001), but there was no significant difference in survival between symbiont-free aphids and aphids that harbored *Regiella* 5.15 (z = 2.7, p = 0.07). This showed that there was no protective effect of *Regiella 5.15.* In addition, harboring *Regiella* LSR-Ri significantly reduced the number of *Pandora*-exposed aphids that produced a sporulating cadaver (z = -6.10, p < 0.0001), but harboring *Regiella* 5.15 had no effect on sporulation (z = 1.08, p = 0.28) (Figure 6-1).

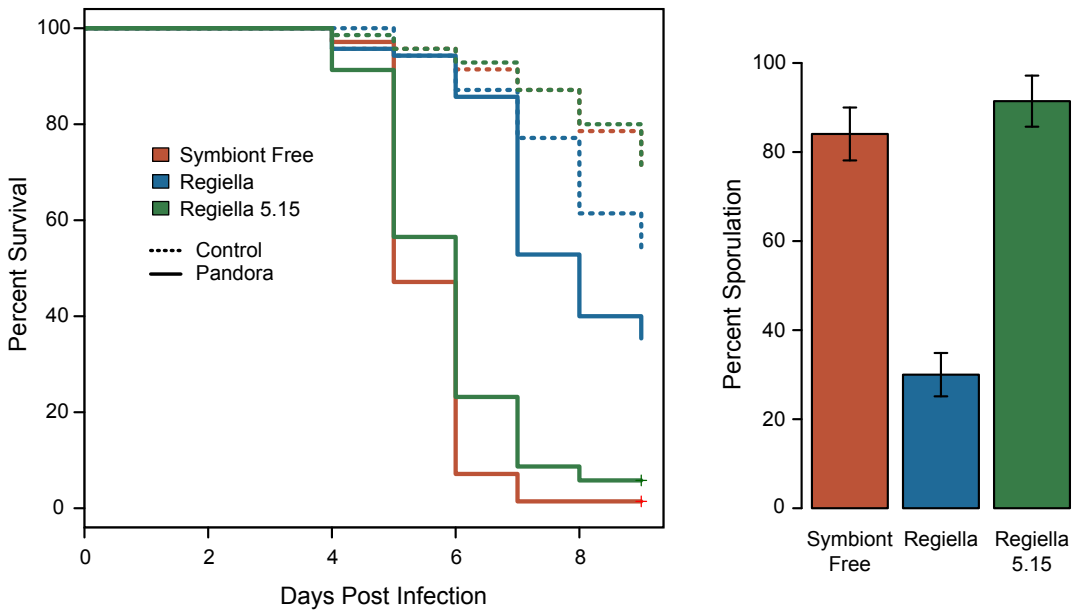


Figure 6-1: Resistance of aphids with different symbiont backgrounds. The left panel shows survival of control (dotted lines) and *Pandora* exposed aphids (solid lines) between 0 and 9 days after treatment. Harboring *Regiella* LSR-Ri significantly increased survival of *Pandora*-exposed aphids relative to aphids harboring *Regiella* 5.15 and symbiont-free aphids. The right panel shows the percent of aphids exposed to *Pandora* that produced a sporulating cadaver. Again, harboring *Regiella* LSR-Ri significantly increased aphid resistance to *Pandora*. Error bars show +/- standard error.

Exposure to heat-killed *Pandora* significantly reduced aphid lifetime fecundity ($F = 9.95$, 2DF, $p = 0.0030$), confirming our previous findings (Chapters 2 & 3) that there is a cost of immunity to *Pandora*. Harboring symbionts had no direct effect on aphid fecundity ($F = 1.79$, 1DF, $p = 0.17$), but there was a significant interaction between symbiont and treatment ($F = 3.38$, 2DF, $p = 0.35$). Post-hoc tests indicated there was a significant difference in the effect of *Pandora* between LSR01 and LSR-Ri ($t = 2.22$, $p = 0.027$), but no difference between LSR01 and LSR5.15 ($t = 0.0$, $p = 0.99$). This indicates that immune costs were limited to symbiont-free aphids and aphids harboring the 5.15 symbiont. Aphids harboring

LSR-Ri, which protected aphids from *Pandora*, did not experience a significant cost of immunity (Figure 6-2).

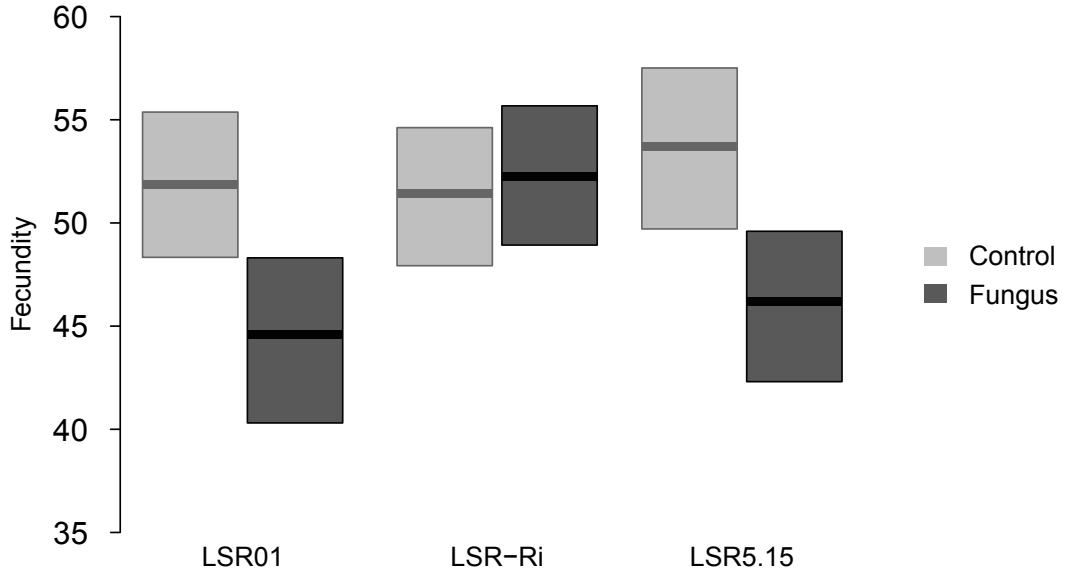


Figure 6-2: Immune costs. The y-axis shows fecundity at 12-days after exposure of aphids from the three symbiont backgrounds. Control aphids are shown in light grey, aphids exposed to heat-killed *Pandora* are shown in dark grey. The boxes represent the 95% confidence intervals for each value. There we significant costs of exposure to *Pandora* in aphids harboring *Regiella* 5.15 and symbiont-free aphids, but no costs in aphids harboring the protective LSR-Ri strain.

There was no significant effect of symbiont background independently on the number of immune cells ($F = 2.1$, 1DF, $p = 0.16$). Treatment significantly increased the number of immune cells ($F = 18$, 1DF, $p < 0.0001$), and there was a significant interaction between treatment and symbiont background ($F = 5.11$, 1DF, $p = 0.029$) indicating that the increase in cells was stronger in aphids that harbored *Regiella* LSR-Ri (Figure 6-3). There was no effect of time point on cell counts. Lastly, post-hoc tests showed that there was no effect of treatment on

symbiont-free (LSR01) aphids (t = 8.6, p = 0.16), suggesting that significant changes in cell numbers were limited to aphids harboring *Regiella*.

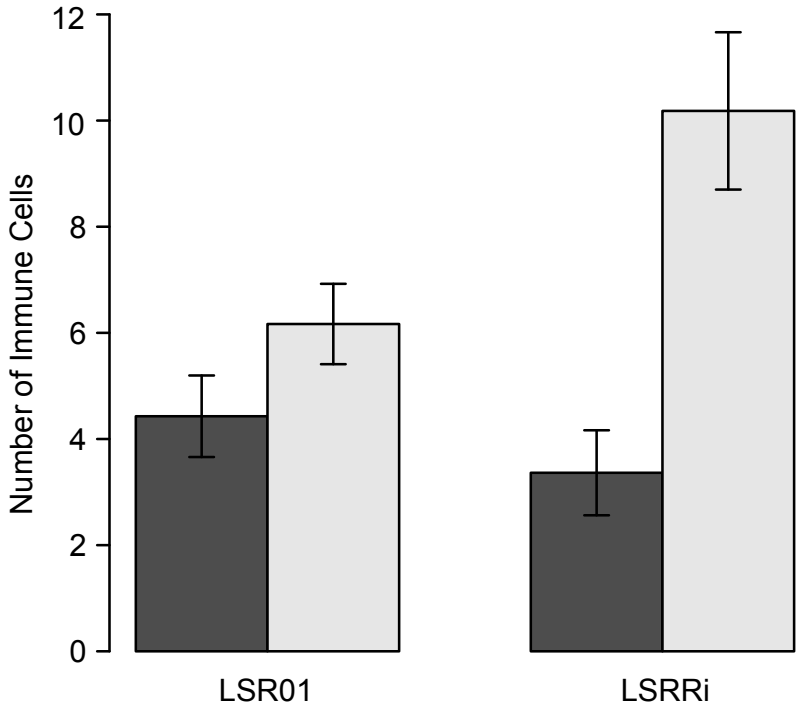


Figure 6-3: Cellular immune response after infection. The y-axis shows the average number of granulocytes in 0.25μL hemolymph. Control aphids are shown in dark grey, *Pandora*-infected aphids are light grey. Bars show +/- standard error.

DISCUSSION

Infections with *Pandora* confirmed that *Regiella* isolated from pea aphids protects its host from fungal infection. We also found that *Regiella* strain 5.15 (from *M. persicae*) did not protect pea aphids from *Pandora* infection. This allowed us to compare the costs of immunity in aphids with protective and non-protective strains of *Regiella*. We found significant costs of exposure to *Pandora* in

symbiont-free aphids, but found no costs in genetically-identical aphids that harbored a protective *Regiella* symbiont. Further, we found costs in aphids harboring a non-protective strain of *Regiella*, suggesting that this reduction in costs is not due solely to harboring a symbiont. Lastly, we found that harboring a protective *Regiella* led to an increase in immune cell titer after *Pandora* challenge.

There are two potential explanations for the patterns of immune costs seen here. First, aphids may be investing less in an immune response when they harbor established protective symbionts. Alternatively, symbionts could be provisioning the host with additional resources that increase immune investment but mask the cost-of-immunity. Recent studies have shown how the presence of bacteria can enhance a host's own immune response (*e.g.* infection with the bacterial symbiont *Wolbachia* in *Drosophila* increases the expression of immune-system genes (Xi et al. 2008)). Our immune cell titer data suggests that this may be the case in aphids as well—that harboring *Regiella* activates the aphid's own cellular immune response to *Pandora*, but clearly more data are needed.

Our results suggest that in addition to being an important determinant of disease outcome, symbiont—mediated protection is a common ecological factor that can influence the expression of immune costs. Recent work has emphasized the importance of host nutrition (Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000; Mckean et al. 2008; Cotter et al. 2010) and predation (Otti et al. 2012) as ecological factors that influence the expression of immune costs. This work expands our understanding of these contexts to protective symbionts,

and suggests that a broad array of ecological factors determine the link between immunity and host fitness.

CHAPTER 7: CONCLUSIONS

For natural selection to act on disease resistance, hosts must vary in their susceptibility to a disease (Gillespie 1975), and so variability in disease resistance is a widely studied phenomenon. Several early studies in aphids contributed to our understanding of this variation: pea aphids were found to vary dramatically in susceptibility to fungal pathogens and parasitoid wasps (Henter and Via 1995; Ferrari and Godfray 2003). This variation was assumed to be due to variation in host genetics, and as a result, aphids were used as an example of the tremendous genetic variation in susceptibility among individuals in wild populations. Eventually, however, it was discovered that symbiotic bacteria conferred protection to aphids against these parasites (Oliver et al. 2003; Scarborough et al. 2005; Oliver et al. 2005; 2009; Vorburger et al. 2010; Parker et al. 2013; Lukasik et al. 2012), leaving unclear the roles that host immune responses and variation in host genotype in play aphid parasite defense.

Since this discovery, significant work has been done to characterize the aphid immune response to pathogens. Altincicek et al. (2008) found no detectable lysozyme-like activity of pea aphids to live bacterial infection, and no homologs of antimicrobial peptides. Similarly, Gerardo et al. (2010) found using genome annotation that aphids are missing many genes thought to be critical for recognition, signaling, and killing of microbes. Subsequent work has shown that aphids have relative few immune cells, have a limited cellular immune response

(Laughton et al. 2011a), and are susceptible to *e. coli* that are harmless to other insects (Altincicek et al. 2011).

These two groups of findings, that aphids are protected from pathogens by symbionts and also have a limited immune repertoire, together suggested to some researchers that the reason that aphids have a reduced immune system is because reliance on protection from symbionts relaxed selection on host-derived mechanisms of resistance (Altincicek et al. 2008). More generally, symbiont-mediated protection is a common phenomenon influencing host-pathogen dynamics in a range of taxa (Currie et al. 2003; Barton et al. 2007; Haine 2008; Brownlie and Johnson 2009; Jaenike et al. 2010). What remains unclear is the relative importance of host and symbiont defenses in these systems.

In chapter three I describe several immunological mechanisms that aphids may be using to defend against their primary aphid-specific fungal pathogen *Pandora neoaphidis*. Cellular immune assays showed changes in granulocyte titer upon fungal infection, implicating hemocytes in fungal defense. Similarly, sequencing of the mRNA transcripts of infected vs. control aphids uncovered a number of putative immune genes with potential roles in cellular immunity, and using quantitative PCR I demonstrated that several of these genes are upregulated upon *Pandora* infection. In chapter four I then look for variation in *Pandora* resistance among genotypes that were collected without symbionts, and found substantial variation due to aphid genotype in the absence of symbionts.

Together, these findings suggest that reliance on symbiont-mediated immunity has not led to relaxed selection on aphid fungal defenses, and

therefore does not support the hypothesis that aphids have a reduced immune system because of their reliance on symbionts for protection. In general, this work shows that even in systems where symbiont-mediated protection is an important factor influencing host-pathogen dynamics, natural selection can still maintain host mechanisms for combating infection and variation in resistance.

This work on variation in susceptibility highlights some interesting questions for future work concerning the nature of this variation. In addition to variation in resistance, I also demonstrate that aphid genotypes vary in their ability to recover reproductive fitness during *Pandora* infection, a processes termed 'fecundity tolerance'. One form of fecundity tolerance that has been highlighted in recent work (Vale and Little 2012) has been termed 'fecundity compensation,' where individuals shift their reproductive efforts earlier in their reproductive period in response to infection (at a cost to future reproduction). Several studies in aphids have highlighted the importance of fecundity compensation as a response to infection (Altincicek et al. 2008; Barribeau et al. 2010), especially in light of other reduced defenses. Future work in this system should determine the relative roles of host resistance mechanisms, symbiont-mediated protection, and mechanisms of tolerance such as fecundity compensation as a way of gaining a comprehensive understanding of how the immune system and non-immunological defenses interact in an evolutionary context (Parker et al. 2011).

Chapters five and six also contribute to our understanding of variation in pathogen susceptibility. In chapter five I show that the protection conferred to aphids by *Regiella* extends to two species of aphid specific fungal pathogens, but

not to a generalist insect fungal pathogen. Different processes underlie evolution between hosts and specialist vs. generalist pathogens. In particular, pathogen specialization can lead to co-evolution with hosts, where interactions between hosts and parasites generates selection for adaptations, and counter-adaptations (Agrawal and Lively 2002). Hosts and generalist pathogens may not experience this close co-evolution if pathogens can easily find susceptible hosts, and so the findings of chapter five may suggest that close coevolution between aphids, symbionts, and fungal pathogens is needed for symbiont-mediated protection to arise against a particular pathogen.

Similarly, in chapter six, I demonstrate that strains of *Regiella* isolated from different species of aphids (*A. pisum* and *M. persicae*) vary in protection conferred to hosts. Clearly more data are needed using additional strains of *Regiella*, but this finding raises the possibility that variation among symbiont genotypes is an additional factor contributing to variation in host susceptibility to pathogens. How evolution acts on traits determined by both host and symbiont genetic traits is unknown, and in my future work, I plan to use this system to ask how natural selection acting on disease resistance shapes associations between aphids and *Regiella*. Specifically, I plan to use RNAseq to determine how *Regiella* gene expression varies in different host genotypic backgrounds. Variation in this expression would suggest that natural selection can act on pathogen resistance by altering the relationships between hosts and their symbionts.

Inherent to the discussion so far is the idea of costs. The hypothesis that aphids have a reduced immune system due to protection from symbionts relies on the implicit assumption that these host-derived immune mechanisms are costly, and therefore should be selected against in the absence of pressure from *Pandora*. Indeed, the idea that immune responses are costly for hosts is fundamental to our understanding of host-pathogen coevolution. Because an organism's resources are limited, they must be allocated in a way that maximizes fitness. Maximizing costly parasite defenses might not be the best evolutionary strategy for an organism if this comes at the expense of growth, maintenance, and fecundity. The internal constraints caused by costs, combined with the variation in parasite prevalence found in nature, leads to variation among individuals in defense traits.

Despite the importance of immune costs for our understanding of a diverse array of observations in host-pathogen coevolution (e.g. why disease prevalence varies across ecological gradients (Altizer et al. 2011; Møller et al. 2011); how parasite virulence evolves (Gandon and Michalakis 2000; Mackinnon and Read 2004; de Roode et al. 2011)), studies frequently report no changes in host fitness after eliciting an immune response (Williams et al. 1999; Labbé et al. 2010). Some have suggested that studying costs of tolerance in addition to costs of resistance may account for these studies (Baucom and de Roode 2011).

Another possibility is that the costs of resistance are dependent on ecological context, and that studies fail to measure immune costs because they are not incorporating ecological factors influencing the expression of costs into their

experimental design. Previous work has emphasized the important role that host nutritional status (e.g. starvation vs. abundant resources) plays in the expression of costs (Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000; Mckean et al. 2008; Cotter et al. 2010). My work demonstrates that additional ecological factors play a role in the expression of costs. In chapter three, I show that immune costs to *Pandora* are limited to winged aphids; a polyphenic trait produced in response to environmental cues of stress or pathogen exposure. There are a number of host pathogen systems where individuals are of different polyphonic morphs (Amdam et al. 2005), and this work suggests that polyphenism is an important and previously unrecognized context for the expression of immune costs. Similarly, in chapter six, I demonstrate that aphids that harbor the protective symbiont *Regiella* do not experience a fecundity cost of *Pandora* exposure. This result suggests that host microbial community is a common, but previously unrecognized ecological context for the expression of immune costs. Together these two results expand our understanding of how ecological factors contribute to the expression of immune costs. A study that searched for immune costs in pea aphids, but only focused on unwinged individuals, might have incorrectly assumed that there were no costs of immunity in this system. Similarly, a study focusing only in aphids harboring *Regiella* might have reached similar incorrect conclusions. The factors determining the link between immunity and fitness will vary between systems, but these results highlight the importance of considering ecological factors when studying immune costs.

In summary, this thesis contributes to our understanding of two aspects of host-pathogen evolutionary biology. First, I show that aphid resistance to fungal pathogens is determined by a complex suite of factors, including polyphenic morph, host genotype, harboring secondary symbionts, and symbiont genotype. These findings demonstrate the complexity of the factors generating variation in host defenses. Second, I show that the expression of costs of immunity to fungal pathogens in an aphid are determined by its polyphenic morph and by its microbial community, indicating that several ecological factors determine when immune responses to pathogens affect host fitness. Together these findings emphasize the need for ecological context in studies of host-pathogen interactions.

REFERENCES

Agrawal, A., and C. M. Lively. 2002. Infection genetics: gene-for-gene versus matching-alleles models and all points in between. Evolutionary Ecology Research 4:79–90.

Ahmed, A. M., S. L. Baggott, R. Maingon, and H. Hurd. 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. Oikos 97:371–377.

Akbari, A., G. Marthinsen, J. T. Lifjeld, F. Albregtsen, L. Wennerberg, N. C. Stenseth, and K. S. Jakobsen. 2008. Improved DNA fragment length estimation in capillary electrophoresis. Electrophoresis 29:1273–1285.

Allen, D. E., and T. J. Little. 2011. Identifying energy constraints to parasite resistance. J Evolution Biol 24:224–229.

Altincicek, B., B. ter Braak, A. M. Laughton, K. I. Udekwu, and N. M. Gerardo. 2011. Escherichia coli K-12 pathogenicity in the pea aphid, *Acyrthosiphon pisum*, reveals reduced antibacterial defense in aphids. Developmental and Comparative Immunology 35:1091–1097.

Altincicek, B., J. Gross, and A. Vilcinskas. 2008. Wounding-mediated gene expression and accelerated viviparous reproduction of the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 17:711–716.

Altizer, S., and A. K. Davis. 2010. Populations of monarch butterflies with different migratory behaviors show divergence in wing morphology. Evolution 64:1018-1028.

Altizer, S., R. Bartel, and B. A. Han. 2011. Animal migration and infectious disease risk. Science 331:296–302.

Amdam, G. V., A. L. T. O. Aase, S. C. Seehuus, M. Kim Fondrk, K. Norberg, and K. Hartfelder. 2005. Social reversal of immunosenescence in honey bee workers. Exp. Gerontol. 40:939–947.

Anselme, C., A. Vallier, S. Balmand, M. O. Fauvarque, and A. Heddi. 2006. Host PGRP Gene Expression and Bacterial Release in Endosymbiosis of the Weevil *Sitophilus zeamais*. Appl Environ Microb 72:6766–6772.

Antonovics, J., M. Boots, D. Ebert, B. Koskella, M. Poss, and B. M. Sadd. 2012. The origin of specificity by means of natural selection: Evolved and nonhost resistance in host-pathogen interactions. Evolution 67:1–9.

Armitage, S. A. O., J. J. W. Thompson, J. Rolff, and M. T. Siva-Jothy. 2003. Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. J Evolution Biol 16:1038–1044.

Arnold, A. E., L. C. Mejía, D. Kyllo, E. I. Rojas, Z. Maynard, N. Robbins, and E. A. Herre. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences 100:15649–15654.

Artacho, P., C. C. Figueroa, P. A. Cortes, J. C. Simon, and R. F. Nespolo. 2011. Short-term consequences of reproductive mode variation on the genetic architecture of energy metabolism and life-history traits in the pea aphid. Journal of Insect Physiology 57:986–994.

Ayres, J. S., and D. S. Schneider. 2008. A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. Plos Biol 6:2764–2773.

Balog, A., M. Mehrparvar, and W. W. Weisser. 2013. Polyphagous predatory rove beetles (Coleoptera: Staphylinidae) induce winged morphs in the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae). Eur J Entomol 110:153–157.

Barrett, L. G., and M. Heil. 2012. Unifying concepts and mechanisms in the specificity of plant-enemy interactions. Trends Plant Sci. 17:282–292.

Barribeau, S. M., D. Sok, and N. M. Gerardo. 2010. Aphid reproductive investment in response to mortality risks. BMC Evol Biol 10:251.

Barton, E. S., D. W. White, J. S. Cathelyn, K. A. Brett-McClellan, M. Engle, M. S. Diamond, V. L. Miller, and H. W. Virgin. 2007. Herpesvirus latency confers symbiotic protection from bacterial infection. Nature 447:326–U7.

Baucom, R. S., and J. C. de Roode. 2011. Ecological immunology and tolerance in plants and animals. Funct Ecol 25:18–28.

Baverstock, J., H. Roy, S. Clark, P. Alderson, and J. Pell. 2006. Effect of fungal infection on the reproductive potential of aphids and their progeny. Journal of Invertebrate Pathology 91:136–139.

Baverstock, J., K. E. Baverstock, S. J. Clark, and J. K. Pell. 2008. Transmission of *Pandora neoaphidis* in the presence of co-occurring arthropods. Journal of Invertebrate Pathology 98:356–359.

Baverstock, J., P. Alderson, and J. Pell. 2005a. *Pandora neoaphidis* transmission and aphid foraging behaviour. Journal of Invertebrate Pathology 90:73–76.

Baverstock, J., P. G. Alderson, and J. K. Pell. 2005b. Influence of the aphid pathogen *Pandora neoaphidis* on the foraging behaviour of the aphid parasitoid *Aphidius ervi*. Ecol Entomol 30:665–672.

Bender, W., P. Spierer, and D. S. Hogness. 1983. Chromosomal walking and jumping to isolate DNA from the Ace and rosy loci and the bithorax complex in *Drosophila melanogaster*. J Mol Biol 168:17–33.

Best, A., A. White, and M. Boots. 2008. Maintenance of host variation in tolerance to pathogens and parasites. P Natl Acad Sci Usa 105:20786–20791.

Best, A., S. Webb, A. White, and M. Boots. 2011. Host resistance and coevolution in spatially structured populations. P Roy Soc B-Biol Sci 278:2216–2222.

Blanchet, S., O. Rey, and G. Loot. 2010. Evidence for host variation in parasite tolerance in a wild fish population. Evolutionary Ecology 24:1129–1139.

Bonte, D., H. Van Dyck, J. M. Bullock, A. Coulon, M. Delgado, M. Gibbs, V. Lehouck, E. Matthysen, K. Mustin, M. Saastamoinen, N. Schtickzelle, V. M. Stevens, S. Vandewoestijne, M. Baguette, K. Barton, T. G. Benton, A. Chaput-Bardy, J. Clobert, C. Dytham, T. Hovestadt, C. M. Meier, S. C. F. Palmer, C. Turlure, and J. M. J. Travis. 2012. Costs of dispersal. Biol Rev Camb Philos Soc 87:290–312.

Boots, M., and M. Mealor. 2007. Local interactions select for lower pathogen infectivity. Science 315:1284–1286.

Boughton, R. K., G. Joop, and S. A. O. Armitage. 2011. Outdoor immunology: methodological considerations for ecologists. Funct Ecol 25:81–100.

Brisson, J. A., and D. L. Stern. 2006. The pea aphid, *Acyrthosiphon pisum*: an emerging genomic model system for ecological, developmental and evolutionary studies. Bioessays 28:747–755.

Brownlie, J. C., and K. N. Johnson. 2009. Symbiont-mediated protection in insect hosts. Trends Microbiol 17:348–354.

Chadwick, W., and T. J. Little. 2005. A parasite-mediated life-history shift in *Daphnia magna*. Proc Biol Sci 272:505–509.

Chapuisat, M., A. Oppliger, P. Magliano, and P. Christe. 2007. Wood ants use resin to protect themselves against pathogens. P Roy Soc B-Biol Sci 274:2013–2017.

Chen, C., and M. Feng. 2005. Epizootiological modeling of *Pandora neoaphidis* mycosis transmission in *Myzus persicae* colonies initiated by primarily infected alates. Appl Environ Microb 71:4104–4107.

Chen, C., and M.-G. Feng. 2006. Experimental simulation of transmission of an obligate aphid pathogen with aphid flight dispersal. Environmental Microbiology 8:69–76.

Chen, C., and M.-G. Feng. 2004. Sitobion avenae alatae infected by Pandora neoaphidis: their flight ability, post-flight colonization, and mycosis transmission to progeny colonies. Journal of Invertebrate Pathology 86:117–123.

Christophides, G., D. Vlachou, and F. Kafatos. 2004. Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. Immunol Rev 198:127–148.

Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37:D141–5.

Corby-Harris, V., K. E. Habel, F. G. Ali, and D. E. L. Promislow. 2007. Alternative measures of response to *Pseudomonas aeruginosa* infection in *Drosophila melanogaster*. J Evolution Biol 20:526–533.

Cotter, S. C., E. Topham, A. J. P. Price, and R. M. Kilner. 2010. Fitness costs associated with mounting a social immune response. Ecol Lett 13:1114–1123.

Currie, C. R., B. Wong, A. E. Stuart, T. R. Schultz, S. A. Rehner, U. G. Mueller, G.-H. Sung, J. W. Spatafora, and N. A. Straus. 2003. Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299:386–388.

de Roode, J. C., C. L. Fernandez de Castillejo, T. Faits, and S. Alizon. 2011. Virulence evolution in response to anti-infection resistance: toxic food plants can select for virulent parasites of monarch butterflies. J Evolution Biol 24:712–722.

Dionne, M. S., and D. S. Schneider. 2008. Models of infectious diseases in the fruit fly Drosophila melanogaster. Disease Models and Mechanisms 1:43–49.

Dwyer, G. 1991. The roles of density, stage, and patchiness in the transmission of an insect virus. Ecology 72:559–574.

Evans, J. D., K. Aronstein, Y. P. Chen, C. Hetru, J. L. Imler, H. Jiang, M. Kanost, G. J. Thompson, Z. Zou, and D. Hultmark. 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol Biol 15:645–656.

Feder, D., C. B. Mello, E. S. Garcia, and P. Azambuja. 1997. Immune responses in *Rhodnius prolixus*: influence of nutrition and ecdysone. Journal of Insect Physiology 43:513–519.

Feng, M.-G., C. Chen, and B. Chen. 2004. Wide dispersal of aphid-pathogenic Entomophthorales among aphids relies upon migratory alates. Environmental Microbiology 6:510–516.

Feng, M.-G., C. Chen, S.-W. Shang, S. H. Ying, Z.-C. Shen, and X.-X. Chen. 2007. Aphid dispersal flight disseminates fungal pathogens and parasitoids as

natural control agents of aphids. Ecol Entomol 32:97–104.

Ferrari, J., and H. Godfray. 2003. Resistance to a fungal pathogen and host plant specialization in the pea aphid. Ecol Lett 6:111–118.

Ferrari, J., C. B. Muller, A. R. Kraaijeveld, and H. C. Godfray. 2001. Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. Evolution 55:1805–1814.

Fineblum, W. L., and M. D. Rausher. 1995. Tradeoff Between Resistance and Tolerance to Herbivore Damage in a Morning Glory. Nature 377:517–520.

Fournier, A., F. Widmer, and J. Enkerli. 2010. Assessing winter-survival of *Pandora neoaphidis* in soil with bioassays and molecular approaches. Biological Control 54:126–134.

Gandon, S., A. Buckling, E. Decaestecker, and T. Day. 2008. Host-parasite coevolution and patterns of adaptation across time and space. J Evolution Biol 21:1861–1866.

Gandon, S., and Y. Michalakis. 2000. Evolution of parasite virulence against qualitative or quantitative host resistance. Proc Biol Sci 267:985–990.

Gandon, S., Y. Capowiez, Y. Dubois, Y. Michalakis, and I. Olivieri. 1996. Local adaptation and gene-for-gene coevolution in a metapopulation model. Proc Biol Sci 263:1003–1009.

Gerardo, N. M., B. Altincicek, C. Anselme, H. Atamian, S. M. Barribeau, M. de Vos, E. J. Duncan, J. D. Evans, T. Gabaldon, M. Ghanim, A. Heddi, I. Kaloshian, A. Latorre, A. Moya, A. Nakabachi, B. J. Parker, V. Perez-Brocal, M. Pignatelli, Y. Rahbé, J. S. Ramsey, C. J. Spragg, J. Tamames, D. Tamarit, C. Tamborindeguy, C. Vincent-Monegat, and A. Vilcinskas. 2010. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. Genome Biol. 11:R21.

Gillespie, J. 1975. Natural-selection for resistance to epidemics. Ecology 56:493–495.

Govind, S. 2008. Innate immunity in *Drosophila*: Pathogens and pathways. Insect Sci. 15:29–43.

Graham, A. L., D. M. Shuker, L. C. Pollitt, S. K. J. R. Auld, A. J. Wilson, and T. J. Little. 2011. Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Funct Ecol 25:5–17.

Grell, M. N., A. B. Jensen, P. B. Olsen, J. Eilenberg, and L. Lange. 2011. Secretome of fungus-infected aphids documents high pathogen activity and weak host response. Fungal Genet. Biol. 48:343–352.

Groeters, F. R., and H. Dingle. 1989. The cost of being able to fly in the milkweed oleander aphid, *aphis nerii* (homoptera, aphididae). Evolutionary Ecology 3:313–326.

Guershon, M., and A. Ayali. 2012. Innate phase behavior in the desert locust, *Schistocerca gregaria*. Insect Science 19:649–656.

Haine, E. R. 2008. Symbiont-mediated protection. Proc Biol Sci 275:353–361.

Haine, E. R., Y. Moret, M. T. Siva-Jothy, and J. Rolff. 2008. Antimicrobial Defense and Persistent Infection in Insects. Science 322:1257–1259.

Hajek, A. E., and I. Delalibera. 2010. Fungal pathogens as classical biological control agents against arthropods. BioControl 55:147–158.

Hamilton, C., B. T. Lejeune, and R. B. Rosengaus. 2011. Trophallaxis and prophylaxis: social immunity in the carpenter ant *Camponotus pennsylvanicus*. Biology Letters 7:89–92.

Hamilton, W., and M. ZUK. 1982. Heritable true fitness and bright birds - A role for parasites. Science 218:384–387.

Hansen, A. K., C. Vorburger, and N. A. Moran. 2012. Genomic basis of endosymbiont-conferred protection against an insect parasitoid. Genome Res 22:106–114.

Hanski, I., M. Saastamoinen, and O. Ovaskainen. 2006. Dispersal-related life-history trade-offs in a butterfly metapopulation. Journal of Animal Ecology 75:91–100.

Hatano, E., G. Kunert, and W. W. Weisser. 2010. Aphid wing induction and ecological costs of alarm pheromone emission under field conditions. PLoS ONE 5:e11188.

Hatano, E., J. Baverstock, G. Kunert, J. K. Pell, and W. W. Weisser. 2012. Entomopathogenic fungi stimulate transgenerational wing induction in pea aphids, *Acyrthosiphon pisum* (Hemiptera: Aphididae). Ecol Entomol 37:75–82.

Henter, H. J., and S. Via. 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.

Hibbett, D. S., M. Binder, J. F. Bischoff, M. Blackwell, P. F. Cannon, O. E. Eriksson, S. Huhndorf, T. James, P. M. Kirk, R. Lücking, H. Thorsten Lumbsch, F. Lutzoni, P. B. Matheny, D. J. McLaughlin, M. J. Powell, S. Redhead, C. L. Schoch, J. W. Spatafora, J. A. Stalpers, R. Vilgalys, M. C. Aime, A. Aptroot, R. Bauer, D. Begerow, G. L. Benny, L. A. Castlebury, P. W. Crous, Y.-C. Dai, W. Gams, D. M. Geiser, G. W. Griffith, C. Gueidan, D. L. Hawksworth, G. Hestmark,

K. Hosaka, R. A. Humber, K. D. Hyde, J. E. Ironside, U. Kõljalg, C. P. Kurtzman, K.-H. Larsson, R. Lichtwardt, J. Longcore, J. Miądlikowska, A. Miller, J.-M. Moncalvo, S. Mozley-Standridge, F. Oberwinkler, E. Parmasto, V. Reeb, J. D. Rogers, C. Roux, L. Ryvarden, J. P. Sampaio, A. Schüßler, J. Sugiyama, R. G. Thorn, L. Tibell, W. A. Untereiner, C. Walker, Z. Wang, A. Weir, M. Weiss, M. M. White, K. Winka, Y.-J. Yao, and N. Zhang. 2007. A higher-level phylogenetic classification of the Fungi. Mycol Res 111:509–547.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biom J 50:346–363.

Hufbauer, R. 2002. Aphid population dynamics: does resistance to parasitism influence population size? Ecol Entomol 27:25–32.

Inouye, B. D., P. Tiffin, and R. Harrison. 2003. Measuring tolerance to herbivory with natural or imposed damage: a reply to Lehtilä. Evolution 57:681–682. BioOne.

International Aphid Genomics Consortium. 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. Plos Biol 8:e1000313.

Jackson, M. A., C. A. Dunlap, and S. T. Jaronski. 2010. Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl 55:129–145.

Jacot, A., H. Scheuber, and M. Brinkhof. 2004. Costs of an induced immune response on sexual display and longevity in field crickets. Evolution 58:2280–2286.

Jaenike, J., R. Unckless, S. N. Cockburn, L. M. Boelio, and S. J. Perlman. 2010. Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. Science 329:212–215.

Karlsson, B., and A. Johansson. 2008. Seasonal polyphenism and developmental trade-offs between flight ability and egg laying in a pierid butterfly. Proc Biol Sci 275:2131–2136.

Klemola, T., N. Klemola, T. Andersson, and K. Ruohomäki. 2007. Does immune function influence population fluctuations and level of parasitism in the cyclic geometrid moth? Popul Ecol 49:165–178.

Koga, R., T. Tsuchida, and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. Proc Biol Sci 270:2543–2550.

Konrad, M., M. L. Vyleta, F. J. Theis, M. Stock, S. Tragust, M. Klatt, V. Drescher, C. Marr, L. V. Ugelvig, and S. Cremer. 2012. Social transfer of pathogenic fungus promotes active immunisation in ant colonies. Plos Biol 10:e1001300.

Korb, J., and S. Katrantzis. 2004. Influence of environmental conditions on the expression of the sexual dispersal phenotype in a lower termite: implications for the evolution of workers in termites. Evol Dev 6:342–352.

Koskela, T., S. Puustinen, V. Salonen, and P. Mutikainen. 2002. Resistance and tolerance in a host plant-holoparasitic plant interaction: genetic variation and costs. Evolution 56:899–908.

Kover, P. X., and B. A. Schaal. 2002. Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. Proceedings of the National Academy of Sciences 99:11270–11274.

Kraaijeveld, A. R., and H. C. J. Godfray. 2008. Selection for resistance to a fungal pathogen in *Drosophila melanogaster*. Heredity 100:400–406.

Kraaijeveld, A. R., E. C. Limentani, and H. C. Godfray. 2001. Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. Proc Biol Sci 268:259–261.

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

Krug, P. J., D. Gordon, and M. R. Romero. 2012. Seasonal polyphenism in larval type: rearing environment influences the development mode expressed by adults in the sea slug *Alderia willowi*. Integr. Comp. Biol. 52:161–172.

Kunert, G., S. Otto, U. S. Röse, J. Gershenzon, and W. W. Weisser. 2005. Alarm pheromone mediates production of winged dispersal morphs in aphids. Ecol Lett 8:596–603.

Kurokawa, T., I. Yao, S. Akimoto, and E. Hasegawa. 2004. Isolation of six microsatellite markers from the pea aphid, *Acyrthosiphon pisum* (Homoptera, Aphididae). Mol Ecol Notes 4:523–524.

Labbé, P., P. F. Vale, and T. J. Little. 2010. Successfully resisting a pathogen is rarely costly in *Daphnia magna*. BMC Evol Biol 10:355.

Laughton, A. M., J. R. Garcia, B. Altincicek, M. R. Strand, and N. M. Gerardo. 2011a. Characterisation of immune responses in the pea aphid, *Acyrthosiphon pisum*. Journal of Insect Physiology 57:830–839.

Laughton, A. M., M. Boots, and M. T. Siva-Jothy. 2011b. The ontogeny of immunity in the honey bee, *Apis mellifera* L. following an immune challenge. Journal of Insect Physiology 57:1023–1032.

Lavine, M. D., G. Chen, and M. R. Strand. 2005. Immune challenge differentially affects transcript abundance of three antimicrobial peptides in hemocytes from the moth *Pseudoplusia includens*. Insect Biochemistry and Molecular Biology

35:1335–1346.

Lazzaro, B. P., and J. Rolff. 2011. Immunology. Danger, microbes, and homeostasis. Science 332:43–44.

Lazzaro, B. P., B. Sceurman, and A. Clark. 2004. Genetic basis of natural variation in *D. melanogaster* antibacterial immunity. Science 303:1873–1876.

Lee, K. P., J. S. Cory, K. Wilson, D. Raubenheimer, and S. J. Simpson. 2006. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proc Biol Sci 273:823–829.

Lee, K. P., S. J. Simpson, and K. Wilson. 2008. Dietary protein-quality influences melanization and immune function in an insect. Funct Ecol 22:1052–1061.

Lefèvre, T., A. J. Williams, and J. C. de Roode. 2011. Genetic variation in resistance, but not tolerance, to a protozoan parasite in the monarch butterfly. Proc Biol Sci 278:751–759.

Lefèvre, T., L. Oliver, M. D. Hunter, and J. C. de Roode. 2010. Evidence for trans-generational medication in nature. Ecol Lett 13:1485-1493.

Lehtilä, K. 2003. Precision of herbivore tolerance experiments with imposed and natural damage. Evolution 57:677–80.

Lemaitre, B., and J. Hoffmann. 2007. The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743.

Lemaitre, B., J. M. Reichhart, and J. A. Hoffmann. 1997. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. P Natl Acad Sci Usa 94:14614–14619.

Little, T., and A. Kraaijeveld. 2004. Ecological and evolutionary implications of immunological priming in invertebrates. Trends Ecol Evol 19:58–60.

Lively, C. M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. The American Naturalist 153:S34–S47.

Lively, C. M., and M. F. Dybdahl. 2000. Parasite adaptation to locally common host genotypes. Nature 405:679–681.

Login, F. H., S. Balmand, A. Vallier, C. Vincent-Monegat, A. Vigneron, M. Weiss-Gayet, D. Rochat, and A. Heddi. 2011. Antimicrobial peptides keep insect endosymbionts under control. Science 334:362–365.

Lukasik, P., M. van Asch, H. Guo, J. Ferrari, H. Charles J Godfray, and W. van der Putten. 2012. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. Ecol Lett 16:214–8.

Mackinnon, M. J., and A. F. Read. 2004. Immunity promotes virulence evolution in a malaria model. Plos Biol 2:E230.

Maynard, C. L., C. O. Elson, R. D. Hatton, and C. T. Weaver. 2012. Reciprocal interactions of the intestinal microbiota and immune system. Nature 489:231–241.

Márkus, R., B. Laurinyecz, E. Kurucz, V. Honti, I. Bajusz, B. Sipos, K. Somogyi, J. Kronhamn, D. Hultmark, and I. Andó. 2009. Sessile hemocytes as a hematopoietic compartment in *Drosophila melanogaster*. P Natl Acad Sci Usa 106:4805–4809.

McFall-Ngai, M., S. V. Nyholm, and M. G. Castillo. 2010. The role of the immune system in the initiation and persistence of the *Euprymna scolopes--Vibrio fischeri* symbiosis. Semin Immunol 22:48–53.

Mckean, K. A., C. P. Yourth, B. P. Lazzaro, and A. G. Clark. 2008. The evolutionary costs of immunological maintenance and deployment. BMC Evol Biol 8:76.

Miller, M. R., A. White, and M. Boots. 2005. The evolution of host resistance: tolerance and control as distinct strategies. J Theor Biol 236:198–207.

Moran, N. A., and H. E. Dunbar. 2006. Sexual acquisition of beneficial symbionts in aphids. P Natl Acad Sci Usa 103:12803–12806.

Moran, N., and A. Telang. 1998. Bacteriocyte-associated symbionts of insects - A variety of insect groups harbor ancient prokaryotic endosymbionts. Bioscience 48:295–304.

Moran, N., J. Russell, R. Koga, and T. Fukatsu. 2005a. Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. Appl Environ Microb 71:3302–3310.

Moran, N., P. Degnan, S. Santos, H. Dunbar, and H. Ochman. 2005b. The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. P Natl Acad Sci Usa 102:16919–16926.

Moret, Y., and M. Siva-Jothy. 2003. Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. P R Soc B 270:2475–2480.

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

Myers, J. H., J. S. Cory, J. D. Ericsson, and M. L. Tseng. 2011. The effect of food

limitation on immunity factors and disease resistance in the western tent caterpillar. Oecologia 167:647–655.

Møller, A. P., L. Z. Garamszegi, J. M. Peralta-Sánchez, and J. J. Soler. 2011. Migratory divides and their consequences for dispersal, population size and parasite-host interactions. J Evolution Biol 24:1744–1755.

Nielsen, C., and A. E. Hajek. 2005. Control of invasive soybean aphid, *Aphis glycines* (Hemiptera: Aphididae), populations by existing natural enemies in New York State, with emphasis on entomopathogenic fungi. Environ Entomol 34:1036–1047.

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

Nyholm, S. V., and J. Graf. 2012. Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. Nature Reviews Microbiology 10:815–827.

Nyholm, S. V., J. J. Stewart, E. G. Ruby, and M. J. McFall-Ngai. 2009. Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes.* Environmental Microbiology 11:483–493.

Oliver, K. M., P. H. Degnan, G. R. Burke, and N. A. Moran. 2010. Facultative Symbionts in Aphids and the Horizontal Transfer of Ecologically Important Traits. Annu Rev Entomol 55:247–266.

Oliver, K. M., P. H. Degnan, M. S. Hunter, and N. A. Moran. 2009. Bacteriophages Encode Factors Required for Protection in a Symbiotic Mutualism. Science 325:992–994.

Oliver, K., J. Russell, N. Moran, and M. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. P Natl Acad Sci Usa 100:1803–1807.

Oliver, K., N. Moran, and M. Hunter. 2005. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. P Natl Acad Sci Usa 102:12795–12800.

Otti, O., I. Gantenbein-Ritter, A. Jacot, and M. W. G. Brinkhof. 2012. Immune response increases predation risk. Evolution 66:732–739.

Owen, J. C., and F. R. Moore. 2008a. Relationship between energetic condition and indicators of immune function in thrushes during spring migration. Canadian Journal of Zoology 86:638–647.

Owen, J. C., and F. R. Moore. 2008b. Swainson’s thrushes in migratory

disposition exhibit reduced immune function. Journal of Ethology 26:383–388.

Papierok, B., and A. E. Hajek. 1997. Fungi: Entomophthorales. *in* L. A. Lacey, ed. Manual of Techniques in Insect Pathology. Academic Press.

Parker, B. J., B. D. Elderd, and G. Dwyer. 2010. Host behaviour and exposure risk in an insect-pathogen interaction. The Journal of animal ecology 79:863–870.

Parker, B. J., C. J. Spragg, B. Altincicek, and N. M. Gerardo. 2013. Symbiont-Mediated Protection against Fungal Pathogens in Pea Aphids: a Role for Pathogen Specificity? Appl Environ Microb 79:2455–2458.

Parker, B. J., S. M. Barribeau, A. M. Laughton, J. C. de Roode, and N. M. Gerardo. 2011. Non-immunological defense in an evolutionary framework. Trends Ecol Evol 26:242–248.

Pell, J. K., J. Eilenberg, A. E. Hajek, and D. C. Steinkraus. 2001. Biology, ecology and pest management potential of Entomophthorales. Pp. 71–153 *in* T. M. Butt, C. Jackson, and N. Magnan, eds. Fungi as biocontrol agents: progress, problems and potential. CAB International, Wallingford.

Pell, J. K., J. J. Hannam, and D. C. Steinkraus. 2010. Conservation biological control using fungal entomopathogens. BioControl 55:187–198.

Pinheiro, J. C., and D. M. Bates. 2000. Mixed-Effects Models in S and S-PLUS. Springer-Verlag, New York.

Plantegenest, M., J. S. Pierre, C. A. Dedryver, and P. Kindlmann. 2001. Assessment of the relative impact of different natural enemies on population dynamics of the grain aphid *Sitobion avenae* in the field. Ecol Entomol 26:404–410.

Råberg, L., D. Sim, and A. F. Read. 2007. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science 318:812–814.

Råberg, L., J. Å. Nilsson, P. Ilmonen, M. Stjernman, and D. Hasselquist. 2000. The cost of an immune response: vaccination reduces parental effort. Ecol Lett 3:382–386.

Roff, D. 1977. Dispersal in dipterans: its costs and consequences. The Journal of animal ecology 443–456.

Rolff, J., and M. T. Siva-Jothy. 2003. Invertebrate ecological immunology. Science 301:472–475.

Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal

immune responses during health and disease. Nat Rev Immunol 9:313–323.

Roy, B. A., and J. W. Kirchner. 2000. Evolutionary dynamics of pathogen resistance and tolerance. Evolution 54:51–63.

Russell, J., A. Latorre, B. Sabater-Munoz, A. Moya, and N. Moran. 2003. Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol Ecol 12:1061–1075.

Sadd, B. M., and P. Schmid-Hempel. 2009. Principles of ecological immunology. Evol Appl 2:113–121.

Sadd, B., and P. Schmid-Hempel. 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. Current Biology 16:1206–1210.

Sammataro, D., U. Gerson, and G. Needham. 2000. Parasitic mites of honey bees: Life history, implications, and impact. Annu Rev Entomol 45:519–548.

Scarborough, C., J. Ferrari, and H. Godfray. 2005. Aphid protected from pathogen by endosymbiont. Science 310:1781–1781.

Schlenke, T. A., J. Morales, S. Govind, and A. G. Clark. 2007. Contrasting infection strategies in Generalist and specialist wasp parasitoids of Drosophila melanogaster. PLoS Pathog 3:1486–1501.

Schmid-Hempel, P. 2003. Variation in immune defence as a question of evolutionary ecology. P R Soc B 270:357–366.

Schmitz, A., C. Anselme, M. Ravallec, C. Rebuf, J.-C. Simon, J.-L. Gatti, and M. Poirie. 2012. The Cellular Immune Response of the Pea Aphid to Foreign Intrusion and Symbiotic Challenge. PLoS ONE 7:e42114.

Schulenburg, H., C. Boehnisch, and N. K. Michiels. 2007. How do invertebrates generate a highly specific innate immune response? Mol Immunol 44:3338–3344.

Scott, J. J., D.-C. Oh, M. C. Yuceer, K. D. Klepzig, J. Clardy, and C. R. Currie. 2008. Bacterial protection of beetle-fungus mutualism. Science 322:63.

Serbielle, C., S. Moreau, F. Veillard, E. Voldoire, A. Bézier, M.-A. Mannucci, A.-N. Volkoff, J.-M. Drezen, G. Lalmanach, and E. Huguet. 2009. Identification of parasite-responsive cysteine proteases in *Manduca sexta*. Biol. Chem. 390:493–502.

Sheldon, B., and S. Verhulst. 1996. Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 11:317–321.

Simms, E. L., and J. Triplett. 1994. Costs and benefits of plant responses to

disease: resistance and tolerance. Evolution 1973–1985.

Simms, E. L., and M. D. Rausher. 1987. Costs and benefits of plant resistance to herbivory. Am Nat 570–581.

Simpson, S. J., G. A. Sword, and N. Lo. 2011. Polyphenism in insects. Curr Biol 21:R738–49.

Singer, M. S., K. C. Mace, and E. A. Bernays. 2009. Self-Medication as Adaptive Plasticity: Increased Ingestion of Plant Toxins by Parasitized Caterpillars. PLoS ONE 4:e4796.

Siva-Jothy, M., and J. Thompson. 2002. Short-term nutrient deprivation affects immune function. Physiol Entomol 27:206–212.

Smith, C. D., A. Zimin, C. Holt, E. Abouheif, R. Benton, E. Cash, V. Croset, C. R. Currie, E. Elhaik, C. G. Elsik, M.-J. Fave, V. Fernandes, J. Gadau, J. D. Gibson, D. Graur, K. J. Grubbs, D. E. Hagen, M. Helmkampf, J.-A. Holley, H. Hu, A. S. I. Viniegra, B. R. Johnson, R. M. Johnson, A. Khila, J. W. Kim, J. Laird, K. A. Mathis, J. A. Moeller, M. C. Munoz Torres, M. C. Murphy, R. Nakamura, S. Nigam, R. P. Overson, J. E. Placek, R. Rajakumar, J. T. Reese, H. M. Robertson, C. R. Smith, A. V. Suarez, G. Suen, E. L. Suhr, S. Tao, C. W. Torres, E. van Wilgenburg, L. Viljakainen, K. K. O. Walden, A. L. Wild, M. Yandell, J. A. Yorke, and N. D. Tsutsui. 2011a. Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). P Natl Acad Sci Usa 108:5673–5678.

Smith, C. R., C. D. Smith, H. M. Robertson, M. Helmkampf, A. Zimin, M. Yandell, C. Holt, H. Hu, E. Abouheif, R. Benton, E. Cash, V. Croset, C. R. Currie, E. Elhaik, C. G. Elsik, M.-J. Fave, V. Fernandes, J. D. Gibson, D. Graur, W. Gronenberg, K. J. Grubbs, D. E. Hagen, A. S. I. Viniegra, B. R. Johnson, R. M. Johnson, A. Khila, J. W. Kim, K. A. Mathis, M. C. Munoz Torres, M. C. Murphy, J. A. Mustard, R. Nakamura, O. Niehuis, S. Nigam, R. P. Overson, J. E. Placek, R. Rajakumar, J. T. Reese, G. Suen, S. Tao, C. W. Torres, N. D. Tsutsui, L. Viljakainen, F. Wolschin, and J. Gadau. 2011b. Draft genome of the red harvester ant *Pogonomyrmex barbatus*. P Natl Acad Sci Usa 108:5667–5672.

Sorrentino, R. P., Y. Carton, and S. Govind. 2002. Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Dev. Biol. 243:65–80.

Sternberg, E. D., T. Lefevre, J. Li, C. L. F. de Castillejo, H. Li, M. D. Hunter, and J. C. de Roode. 2012. Food plant derived disease tolerance and resistance in a natural butterfly-plant-parasite interactions. Evolution 66:3367–3376.

Strand, M. R. 2008. The insect cellular immune response. Insect Science 15:1–14.

Svensson, E. I., and L. Råberg. 2010. Resistance and tolerance in animal enemy-victim coevolution. Trends Ecol Evol 25:267–274.

Teixeira, L., A. Ferreira, and M. Ashburner. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. Plos Biol 6:e2.

Tiffin, P., and B. D. Inouye. 2000. Measuring tolerance to herbivory: accuracy and precision of estimates made using natural versus imposed damage. Evolution 54:1024–1029.

Tinsley, M. C., S. Blanford, and F. M. Jiggins. 2006. Genetic variation in *Drosophila melanogaster* pathogen susceptibility. Parasitology 132:767.

Todesco, M., S. Balasubramanian, T. T. Hu, M. B. Traw, M. Horton, P. Epple, C. Kuhns, S. Sureshkumar, C. Schwartz, C. Lanz, R. A. E. Laitinen, Y. Huang, J. Chory, V. Lipka, J. O. Borevitz, J. L. Dangl, J. Bergelson, M. Nordborg, and D. Weigel. 2010. Natural allelic variation underlying a major fitness trade-off in *Arabidopsis thaliana*. Nature 465:632–636.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn, and L. Pachter. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7:562–578.

Triggs, A., and R. J. Knell. 2011. Interactions between environmental variables determine immunity in the Indian meal moth *Plodia interpunctella*. Journal of Animal Ecology 81:386–394.

Tryselius, Y., and D. Hultmark. 1997. Cysteine proteinase 1 (CP1), a cathepsin L-like enzyme expressed in the *Drosophila melanogaster* haemocyte cell line mbn-2. Insect Mol Biol 6:173–181.

Tsuchida, T., R. Koga, H. Shibao, T. Matsumoto, and T. Fukatsu. 2002. Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. Mol Ecol 11:2123–2135.

Vale, P. F., and T. J. Little. 2012. Fecundity compensation and tolerance to a sterilizing pathogen in *Daphnia*. J Evolution Biol 25:1888–1896.

Valtonen, T. M., A. Kleino, M. Rämet, and M. J. Rantala. 2010. Starvation reveals maintenance cost of humoral immunity. Evolutionary Biology 37:49–57. Springer.

Van den Heuvel, J., H. Hummelen, M. Verbeek, A. Dullemans, and F. VanderWilk. 1997. Characteristics of *Acyrthosiphon pisum* virus, a newly identified virus infecting the pea aphid. Journal of Invertebrate Pathology 70:169–176.

Vass, E., and A. J. Nappi. 1998. The effects of dietary yeast on the cellular immune response of *Drosophila melanogaster* against the larval parasitoid, *Leptopilina boulardi*. J Parasitol 84:870–872.

Vega, F. E., M. S. Goettel, M. Blackwell, D. Chandler, M. A. Jackson, S. Keller, M. Koike, N. K. Maniania, A. Monzón, and B. H. Ownley. 2009. Fungal entomopathogens: new insights on their ecology. Fungal Ecology 2:149–159. Elsevier.

Vorburger, C., and A. Gouskov. 2011. Only helpful when required: a longevity cost of harbouring defensive symbionts. J Evolution Biol 24:1611–1617.

Vorburger, C., C. Sandrock, A. Gouskov, L. E. Castañeda, and J. Ferrari. 2009. Genotypic variation and the role of defensive endosymbionts in an all-parthenogenetic host-parasitoid interaction. Evolution 63:1439–1450.

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

Waldenström, J., S. Bensch, S. Kiboi, D. Hasselquist, and U. Ottosson. 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol Ecol 11:1545–1554.

Williams, T. D., J. K. Christians, J. J. Aiken, and M. Evanson. 1999. Enhanced immune function does not depress reproductive output. Proc Biol Sci 266:753–757. The Royal Society.

Wilson, A., B. Massonnet, J. Simon, N. Prunier-Leterme, L. Dolatti, K. Llewellyn, C. Figueroa, C. Ramirez, R. Blackman, A. Estoup, and P. Sunnucks. 2004. Cross-species amplification of microsatellite loci in aphids: assessment and application. Mol Ecol Notes 4:104–109.

Wilson, A., H. Dunbar, G. Davis, W. Hunter, D. Stern, and N. Moran. 2006. A dual-genome microarray for the pea aphid, *Acyrthosiphon pisum*, and its obligate bacterial symbiont, *Buchnera aphidicola*. BMC Genomics 7:50.

Wilson, K., R. Knell, M. Boots, and J. Koch Osborne. 2003. Group living and investment in immune defence: an interspecific analysis. Journal of Animal Ecology 72:133–143.

Woolhouse, M., J. Webster, E. Domingo, B. Charlesworth, and B. Levin. 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32:569–577.

Xi, Z., L. Gavotte, Y. Xie, and S. L. Dobson. 2008. Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its *Drosophila* host. BMC Genomics 9:1.

Ye, Y. H., S. F. Chenoweth, and E. A. McGraw. 2009. Effective but Costly, Evolved Mechanisms of Defense against a Virulent Opportunistic Pathogen in *Drosophila melanogaster*. PLoS Pathog 5(4): e1000385.

Zou, Z., J. D. Evans, Z. Lu, P. Zhao, M. Williams, N. Sumathipala, C. Hetru, D. Hultmark, and H. Jiang. 2007. Comparative genomic analysis of the *Tribolium* immune system. Genome Biol. 8:R177.