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Life-history strategy determines constraints on immune function

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Abstract

1) Determining the factors governing investment in immunity is critical for understanding host-pathogen ecological and evolutionary dynamics. Studies often consider disease resistance in the context of life-history theory, with the expectation that investment in immunity will be optimized in anticipation of disease risk. Immunity, however, is constrained by context-dependent fitness costs. How the costs of immunity vary across life-history strategies has yet to be considered.

2) Pea aphids are typically unwinged but produce winged offspring in response to high population densities and deteriorating conditions. This is an example of polyphenism, a strategy used by many organisms to adjust to environmental cues. The goal of this study was to examine the relationship between the fitness costs of immunity, pathogen resistance, and the strength of an immune response across aphid morphs that differ in life-history strategy but are genetically identical.

3) We measured fecundity of winged and unwinged aphids challenged with a heat-inactivated fungal pathogen, and found that immune costs are limited to winged aphids. We hypothesized that these costs reflect stronger investment in immunity in anticipation of higher disease risk, and that winged aphids would be more resistant due to a stronger immune response. However, producing wings is energetically expensive. This guided an alternative hypothesis—that investing resources into wings could lead to a reduced capacity to resist infection.

4) We measured survival and pathogen load after live fungal infection, and we characterized the aphid immune response to fungi by measuring immune cell concentration and gene expression. We found that winged aphids are less resistant and mount a weaker immune response than unwinged aphids, demonstrating that winged aphids pay higher costs for a less effective immune response.

5) Our results show that polyphenism is an understudied factor influencing the expression of immune costs. More generally, our work shows that in addition to disease resistance, the costs of immunity vary between individuals with different life-history strategies. We discuss the implications of these findings for understanding how organisms invest optimally in immunity in light of context-dependent constraints.

INTRODUCTION

Despite the ubiquity of pathogens and parasites in nature, hosts vary extensively in how well they defend themselves against infection. This variation is attributed in part to costs associated with defense—activating and maintaining immunological mechanisms comes at the expense of other components of host fitness. On an evolutionary scale, immunity is costly because resistance traits can be inherited with linked deleterious mutations, or can be negatively pleiotropic such that protective

alleles produce deleterious effects on other traits (Kraaijeveld & Godfray 1997; Valtonen *et al.* 2010). On an ecological scale, deploying immune mechanisms is costly both because immune responses can cause autoimmune damage (Sadd & Siva-Jothy 2006; Graham *et al.* 2010), and because mounting an immune response is energetically costly. Individuals are faced with a limited pool of resources that must fuel the immune system among other fitness-related traits (Hamilton & Zuk 1982; Simms & Rausher 1987). When resources are depleted, for instance through nutritional constraints, immunity suffers (Feder *et al.* 1997; Vass & Nappi 1998; Siva-Jothy & Thompson 2002; Lee *et al.* 2006; Myers *et al.* 2011; Brunner, Schmid-Hempel & Barribeau 2014).

Given these costs, natural selection is expected to optimize investment in immunity in anticipation of environmental conditions. This idea is illustrated by studies that associate disease resistance with life-history strategy. Species with rapid growth and short life spans, for example, invest little in immunological defenses compared with species with longer life spans. For example, 'slow-living' species of Neotropical birds have higher natural antibody titers compared to 'fast-living' species, suggesting that they invest more in developmentally costly adaptive immune mechanisms (Lee *et al.* 2008). Long-lived species are thought to invest more heavily in immunity because they have a higher likelihood of pathogen exposure (Cronin et al. 2010; Johnson et al. 2012), though see (Martin, Weil & Nelson 2007). Similarly, the higher rate of parasitism associated with sociality is thought to lead social species to invest more in immunity compared with solitary species (Møller et al. 2001). Similar patterns are found within species, where developmentally-plastic life-history strategies have been shown to influence disease resistance. Many organisms respond to ecological cues with dramatic shifts in morphology through a form of plasticity referred to as polyphenism (Simpson, Sword & Lo 2011). As these morphological and behavioral changes also lead to shifts in other classic life-history traits, such as fecundity (Miner et al. 2005), polyphenism can be viewed as a type of life-history strategy. In locusts, for example, high population densities lead to a switch from a solitary non-dispersing morph to a swarming migratory morph (Guershon & Ayali 2012), and these migratory-morph locusts are more resistant to a pathogenic fungus (Wilson et al. 2002). Similarly, the Egyptian cotton leafworm exhibits a density-dependent polyphenism related to cuticle color. The darker, high-

density morph (which is smaller and in poorer body condition) is more resistant to fungal pathogen infection, likely because of increased phenoloxidase activity (Wilson *et al.* 2001; Cotter *et al.* 2004). These studies are consistent with the framework that individuals in high-density populations should invest more in disease resistance when parasite transmission is density-dependent, a phenomenon referred to as 'density-dependent prophylaxis' (Wilson & Reeson 1998).

Because immunity and other aspects of host fitness draw from a common pool of resources, it may seem reasonable to predict a simple relationship between immunity and fitness measures—that a stronger immune response will increase resistance but come at a greater cost to the host. However, empirical work has demonstrated that the expression of immune costs is dependent on ecological context. For example, immune induction of bumblebees decreases survival only under conditions of starvation (Moret & Schmid-Hempel 2000). Thus in certain contexts, the pool of resources available to an organism is sufficient to produce an immune response without negatively influencing survival or fecundity, but under other conditions immunity trades-off with other fitness traits. The context dependency of immune costs may explain why empirical studies frequently fail to measure fitness effects of immunity (Zuk & Stoehr 2002). However, we do not yet understand how context-dependent immune costs constrain optimal investment in immunity across life-history strategies.

Here we study the costs, efficacy, and strength of the immune response in winged/dispersing and wingless/sedentary morphs of the pea aphid (*Acyrthosiphon pisum*). Throughout the summer, pea aphids produce genetically-identical offspring that are normally unwinged. In response to signals of crowded and deteriorating conditions, however, adult female aphids induce a proportion of their embryos to begin developing wings (Sutherland 1969; Brisson & Stern 2006; Grantham *et al.* 2016) through a signaling cascade that involves biogenic amines and the molt hormone ecdysone (Vellichirammal, Madayiputhiya & Brisson 2016). Nymphs emerge with developing wing buds that quickly degrade in unwinged morphs but develop in winged morphs until becoming fully functional at adulthood (approximately 9 days after birth), when winged aphids disperse to new environments (Brisson 2010). This system allows us to study immune costs in individuals that differ in life-

history strategy but are genetically identical. Our hypothesis was that winged aphids are provisioned with a stronger immune system to bolster resistance to disease in response to signals of unfavorable, crowded environments, and that the costs of immunity would be higher in winged aphids. However, the production of wings and the associated musculature requires resources, and previous work has demonstrated that wing production trades off with reproductive investment, development time, and body size (Johnson 1963; Dixon & Howard 1986; Groeters & Dingle 1989; Brisson 2010). This guided an alternative hypothesis—that the energy invested in wing production could lead to a reduced capacity to invest in disease resistance. Under this scenario, winged aphids would be less resistant to infection and would mount a weaker immune response. We tested between these hypotheses by measuring lifetime fecundity, survival, pathogen load, and cellular and humoral immune measures after challenge with a natural fungal pathogen.

METHODS

Study organism: We maintained pea aphids asexually on fava bean (*Vicia faba*) plants in 16 hr light: 8 hr dark at 20°C. All aphids used in a given experiment were born within a 24hr period to reduce ontogenetic differences among individuals. We exposed developing aphids to the pheromone (E)-*β*-farnesene (EBF) at a dose that causes them as adults to give birth to approximately 50% winged and 50% unwinged offspring (5 μL of 1000 ng/μL EBF every 48 hrs for 10 days) (Barribeau, Sok & Gerardo 2010; Barribeau, Parker & Gerardo 2014a). This protocol allowed us to produce both winged and wingless offspring under identical, 'ideal' conditions: both morphs were born from the same mothers and reared at the same densities on the same host plants. For the experimental work described below (cost of exposure experiments and assays of resistance) we used three genotypes: LSR1-01, 5AO, and G6 (Table S1). For the immunological and genomic studies we used genotype LSR1-01. None of the aphid lines used in the study harbor secondary bacterial symbionts (Parker, Garcia & Gerardo 2014).

Cost of Exposure Assays: Our protocol was designed to assess the cost of immune activation without pathogen virulence (Mckean et al. 2008) or immune manipulation (Barribeau *et al.* 2014b) in winged vs. unwinged aphids. To do so, we challenged aphids with a heat-killed inoculum of an ecologically relevant pathogen, *Pandora neoaphidis* (Van Veen *et al.* 2008). We measured the fitness of control aphids (stabbed with a sterile needle) relative to those given the heat-killed pathogen (stabbed with needle coated with heat-killed fungal spores and mycelia). To make the heat-killed fungal solution, we first grew an isolate of *P. neoaphidis* (genotype ARSEF 2588, USDA ARS Collection of Entomopathogenic Fungal Cultures) for 2 weeks on SDAEY plates at 20°C (Papierok & Hajek 1997). We then added approximately 1 cm^2 of mycelium to 250µl Ringers solution, and then autoclaved (121°C for 20 minutes) and homogenized this solution with a pestle. We exposed six 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, Gross & Vilcinskas 2008; Barribeau *et al.* 2010). In previous work, we found no differences in fecundity between non-stabbed and sterile-stabbed aphids (Barribeau *et al.* 2014a). We then allowed the aphids 30 minutes to recover in a clean Petri dish before we put them individually onto fava bean plants in cup cages. We performed three replicates of the experiment, carried out several weeks apart, each using a different host genotype—LSR1-01 (n = 209 aphids), 5AO (n = 214), and G6 (n = 104). Every 48 hours we counted the number of offspring produced by each aphid; 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 individuals stopped reproducing. We analyzed these data using generalized linear models (GLMs), with a quasi-corrected Poisson distribution and log link function, in R (v 2.11). We included morph, treatment, and genotype as factors. Minimal models were derived by the step-wise removal of terms in the reverse order of the model, followed by model comparisons using ANOVA and F-tests. Terms were retained if their removal significantly reduced the explanatory power of the model (at $p < 0.05$). We then performed multiple comparison tests using the multcomp package in R (Hothorn, Bretz & Westfall 2008).

Live infection: We characterized the susceptibility of winged and unwinged aphids to live *Pandora* infection by exposing aphids to a *Pandora* 'spore shower' (based on Hajek & Papierok 2012), the natural route of *Pandora* infection. *Pandora* strain ARSEF 2588 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 (now sporulating) fungus was inverted over hollow tubes with aphids at the bottom of the chamber. This exposure results in a dose of approximately 5 spores / mm^2 based on our previous work (Parker *et al.* 2014)**.** Fungal plates were rotated among treatment groups to ensure that each treatment received an equal inoculation dose by controlling for variation among culture plates, and control aphids were handled similarly but were not exposed to spores. We exposed winged (n = 150) and unwinged (n = 169) aphids of the same three genotypes used above (5A0, n = 131; LSR, n = 138; G6, n = 50). As *Pandora* kills hosts between five and eight days after exposure (Parker *et al.* 2014), we recorded survival and whether each aphid had produced a sporulating cadaver on the ninth day after pathogen exposure. We analyzed these data using a GLM with a binomial distribution (and logit link function). For the survival data, we included morph, treatment, and genotype as factors in the model, and for the sporulation data we included morph and genotype (as no control aphids produced a sporulating cadaver). We performed model comparisons as above, using ANOVA and χ^2 tests.

Quantifying pathogen load: We measured *Pandora* titers using quantitative PCR to measure the pathogen load of infected aphids. A live infection was performed as described above. Aphids were flash frozen in liquid nitrogen at 24, 48, 72, and 96 hours after infection, and then stored at -80°C. At each time point, we collected two biological replicates from three genotypes of both winged and unwinged aphids. Each biological replicate contained five aphids collected from the same bean plant. To extract DNA, aphids were washed in ethanol and then homogenized in Bender buffer (with Proteinase K), followed by protein precipitation with potassium acetate and DNA precipitation using ethanol. Primers for the *Pandora* 18S ribosomal RNA gene (Accession: EU267189.1) were designed using Primer Express 3.0 (Supplementary Information H), and primer 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 plasmidPrep Mini Spin Kit under recommended conditions. The cloned fragment was sequenced with the 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 x 10^6 copies), with three technical replicates. The threshold cycle (Ct) was averaged across technical replicates, and *Pandora* 18S copy number was determined using Applied Biosystems Step One Plus software. To analyze these data we used a quasi-corrected Poisson-distributed GLM as above, with morph, genotype, and day as factors.

Cellular Immunity Assays: We counted circulating immune cells as a measure of the aphid cellular immune response. Hemolymph was collected from leg wounds from aphids until 0.25µl was obtained, and samples were then smeared onto a slide. Hemolymph was then fixed and stained using a Diff-Quik stain set (Dade Behring). Previous work on aphid immunity has identified distinct immune cells (haemocytes) (Laughton et al. 2011; Schmitz et al. 2012), some of which display phagocytic properties (Vilcinskas & Götz 1999; Schmitz *et al.* 2012) and therefore may play a role in the aphid immune response to fungal pathogens (Hajek & St Leger 1994). We then counted the number of circulating immune cells under a light microscope. Using both winged and unwinged aphids, we performed two experiments using this basic protocol. In the first experiment, we stabbed aphids (n = 51 total) with a heat-killed *Pandora* solution as above, with a sterile stab control and a no-stab control, and collected hemolymph 24 hours after exposure. In the second experiment we performed a live infection as described above, and collected hemolymph from infected and uninfected aphids (n = 69 total) at 48 and 96 hours after exposure. We analyzed cell counts using a quasi-corrected Poisson-distributed GLM as described above. Note that in a pilot study we found that winged aphids had about half as much hemolymph as unwinged aphids, but had a higher concentration of circulating cells. We therefore do not make direct comparisons between morphs using cell concentrations, and instead show relative changes in cell titer as a result of *Pandora* exposure between morphs.

Identifying candidate immune genes with RNAseq: We used RNA sequencing of winged aphids to compare the transcriptional response of control vs. *Pandora*-infected aphids. Winged aphids (genotype LSR1-01), which were produced using EBF-exposure as above, were infected using a combination of spores from three fungal strains (ARSEF 2773, 2853, 2588). RNA was extracted and then pooled from aphids 48 and 72 hours after pathogen exposure using Trizol following the manufacturer's specifications. For both treatments (control, *Pandora* infected), RNA from 10 aphids (collected from two host plants) from the two time-points was pooled (20 aphids total), and libraries were constructed using the Illumina TruSeq kit. cDNA libraries were then multiplexed across four lanes on an Illumina HiSeq machine, producing ~250 million reads per treatment. Reads were quality trimmed using the ea-utils fastq-mcf (http://code.google.com/p/ea-utils/wiki/FastqMcf), and were mapped to pea aphid genome assembly version 2 using Tophat v. 2.0.3 (Trapnell et al. 2012; Kim et al. 2013). Transcripts were assembled using cufflinks v. 2.0.1 (Trapnell et al. 2012). Differential expression of transcripts was determined using cuffdiff, and transcripts were assigned to genes based on published annotations from the pea aphid genome project (International Aphid Genomics Consortium 2010).

qPCR assays of candidate immune gene expression: We used quantitative PCR to examine the expression patterns of genes identified through RNAseq as potentially important in the aphid response to *Pandora* infection. We included both winged and unwinged aphids, produced through EBF-exposure as above, across multiple time points. We infected aphids (LSR1-01 genotype) with *Pandora* as above (with strain ARSEF 2588), and flash-froze aphids in liquid nitrogen at 12, 24, and 48 hours after exposure. We tested three biological replicates (5 aphids each collected from one host plant per replicate) of each combination of morph, treatment, and time point. Aphids were stored at -80°C until RNA extraction using Trizol. Genomic DNA contamination was reduced using the Invitrogen Turbo DNA-free kit, 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 using Primer Express 3.0 (see primer table in the Supplemenary Information). Primer concentrations were optimized to 100 +/- 5% efficiency, and *A. pisum* Ef1α was used as the endogenous control (Wilson et al. 2006). We included

nine of the genes identified from the RNAseq experiment, and also included Cathepsin L, which was not differentially expressed in our RNAseq analysis as a negative control. For each gene, we subtracted the critical threshold value (Ct) for the endogenous control from the target gene for each sample (ΔCt). We analyzed differential expression of each gene at each time-point using ANOVA after testing for normality to compare ΔCt values for control and infected aphids from both morphs. A significant interaction effect between morph and treatment indicated that the magnitude of a change in expression of the target gene differed between morphs.

RESULTS

Exposure to heat-killed fungal solution reduced lifetime fecundity in winged aphids but had no effect on unwinged aphids (treatment x morph interaction; Table 1, Figure 1) across three different genotypes (Table 1; Figure SA; Supplementary Information A). We confirmed that heat-killed *Pandora* did not affect aphid survival (Figure SB; Supplementary Information B; Barribeau *et al.* 2014a).

Winged aphids were significantly more likely to die from fungal infection than unwinged aphids (Table 1, Figure 2A) across multiple genotypes (Table 1, Figure SC; Supplementary Information C). Winged aphids were also significantly more likely to produce a sporulating cadaver than unwinged aphids (Table 1, Figure 2B) across genotypes (Figure SC; Supplementary Information C). *Pandora* is transmitted after it produces a sporulating cadaver, and the success or failure of spore production is thus essential to pathogen transmission. *Pandora's* disproportionate success on winged hosts is not driven solely by differences in the ability of spores to penetrate the host cuticle as injection with spores rather than surface exposure produced similar results (Figure SD; Supplementary Information D). *Pandora* grew logarithmically during the first four days of infection, and winged aphids had higher pathogen loads than unwinged aphids across aphid genotypes, and pathogen load differed across genotypes (Table 1, Figure 2C). Together these results indicate that winged aphids are more susceptible than unwinged aphids to *Pandora* infection.

Winged aphids exhibited significantly depleted circulating immune cells after exposure to heat-killed *Pandora* compared to control stab and unchallenged aphids, but we found no significant changes in cell concentration in unwinged aphids (Table 1, Figure 3A). We found the same pattern using a live *Pandora* infection—96 hours after exposure winged aphids exhibited significantly depleted immune cells, while cell number in unwinged aphids remained unchanged (Table 1, Figure 3B). Neither morph showed reduced cell counts 48 hrs after a live *Pandora* infection (Figure SE; Supplementary Information E), giving a coarse-grained indication of the temporal scale of immune activation and depletion in response to this pathogen.

We verified that the aphids used for transcriptome sequencing were successfully infected with fungus and showed the same patterns of resistance as above (Figure SF; Supplementary Information F). We found evidence of expression in at least one of the two libraries (winged control, winged fungus) of 38,227 unique transcripts using whole transcriptome sequencing. Quality trimming the reads using ea-utils fastq-mcf tool improved the percentage of reads that mapped to the reference genome (Table S2; Supplementary information G). We identified 1,668 significantly differentially expressed transcripts, including multiple putative immune-related genes. These included phenoloxidase, lysozyme-i, C-type lectin, serine proteases, and a number of cathepsins. We also detected significant down-regulation of cuticle proteins, which may be biologically important as *Pandora* penetrates the host cuticle. Similar to other transcriptomic studies of invertebrates responses to fungal infections (Xia et al. 2013), we also found differential expression of genes that may be involved in detoxification, such as Cytochrome p450 and Peroxidasin, and in DNA repair, such as Cop 9 signalosome. A complete list of transcripts and expression values is included as Supplementary File 2.

Using qPCR, we found that several putative immune genes were upregulated 48 hours after infection, and that phenoloxidase, several cathepsins, and legumain were upregulated in response to *Pandora* more strongly in unwinged than winged aphids (Figure 4, Supplementary Information I). Earlier time-points did not show significant patterns of differential expression. We also measured expression at 72-hrs after infection for phenoloxidase, and found that the upregulation in response to fungal

infection at the 48-hr timepoint had subsided by 72 hrs (Figure SJ; Supplementary Information J).

Discussion

We found strong fecundity costs of an immune response to a natural fungal pathogen in winged, but not in genetically-identical unwinged aphids. Two hypotheses could potentially explain this pattern. First, winged aphids may be mounting a stronger immune response than unwinged aphids. Winged aphids are produced in response to signals of crowding and other ecological conditions that could increase the probability of pathogen exposure (Sutherland 1969; Brisson & Stern 2006). Winged aphids may invest more heavily in immunity in anticipation of this risk, and consequently experience greater immune costs than unwinged aphids. Under this scenario, we would expect winged aphids to be less susceptible to *Pandora* infection than unwinged aphids. Second, relatively higher costs of immunity in winged aphids could be the result of greater energetic demands—the production of wings and associated musculature requires host resources and leads to lower lifetime fecundity in aphids (Figure 1, (Groeters & Dingle 1989) and in other animals (Chapman, Reynolds & Wilson 2015). As a result, winged aphids may be energetically limited to the point that an immune response negatively influences fecundity. Under this scenario, we would expect winged aphids to be equally or more susceptible to live *Pandora* infection than unwinged aphids.

We characterized the susceptibility of both morphs to live *Pandora* infection to test between these two hypotheses. We found that winged aphids had lower survival after pathogen exposure and produced more sporulating cadavers. Winged aphids also had higher pathogen loads than unwinged aphids as measured by qPCR. These results demonstrate that winged aphids are more susceptible to fungal infection than unwinged aphids and are consistent with the hypothesis of energetic limitation. We note that an alternative interpretation of our live infection assay could be that winged aphids simply disperse from a crowded area before they get exposed to fungal spores. Under this scenario, winged aphids would invest less in immunity in anticipation of lower overall disease risk, which is consistent with our findings of higher susceptibility to fungal pathogen infection. However, winged offspring are produced when unwinged adult mothers experience crowded conditions. Winged

offspring experience these same high density conditions during their juvenile instars, as their wings (which are developing during the juvenile phase) do not become functional until adulthood. We therefore think it is likely that winged aphids are experiencing higher disease risk from crowding during development than unwinged aphids, but future work is needed on the disease dynamics of this system.

We then used cellular immune assays and measures of gene expression to characterize the pea aphid immune response to *Pandora*. We counted circulating immune cells under a light microscope and found that unwinged aphids maintained cell titers during the course of fungal infection but that cell titers were depleted in winged aphids. This result was consistent across both a heat-killed fungal challenge and with a live fungal infection. We also found a slight decrease in cell concentration in sterile stab vs. control aphids of both morphs (although this change was not significant), perhaps due to recruitment of circulating haemocytes to the wound site. A number of studies have interpreted changes in hemocyte counts as evidence for differences in immune activity, but patterns have not been consistent across systems. For example, higher numbers of hemocytes in solitary vs. gregarious lepidopterans was interpreted as greater investment in immunity in solitary species (Wilson et al. 2003), and increases in immune cell numbers have been reported as indicating a response to parasitic wasp infection in Drosophila (Sorrentino, Carton & Govind 2002; Márkus *et al.* 2009). Conversely, depleted cell counts have been found in some insect systems several days after fungal infection (Hung & Boucias 1992; Gillespie, Burnett & Charnley 2000). Similarly, studies have shown decreases in cell titers as a consequence of resource allocation. For example, when worker bumblebees transition from nursing to foraging they decrease hemocyte titer (Amdam et al. 2005). We argue that the patterns seen in our data are reflective of stronger investment in immunity in unwinged aphids, but we acknowledge the difficulties of interpreting changes in immune cell titers in the absence of other measures of an immune response.

We therefore also looked at changes in expression of putative immune genes in response to fungal infection. RNA sequencing of winged aphids revealed upregulation of canonical immune genes, including phenoloxidase and several cathepsins (which are proteases that have been shown to be expressed in

hemocytes and to have lysozymal activity against bacteria and viruses in other invertebrates (Tryselius & Hultmark 1997; Nishikori *et al.* 2009; Serbielle *et al.* 2009; Hamilton, Lejeune & Rosengaus 2011; Liu *et al.* 2013)) in response to fungal infection. A similar study using proteomics to study the response of grain aphids (*Sitobion avenae*) to *Pandora* also found expression of phenoloxidase and cathepsins (Grell et al. 2011), suggesting that these mechanisms may be conserved across aphid species. Using qPCR, we assayed expression of several of these genes across multiple time-points during fungal infection in winged and unwinged aphids. At 48hrs post-infection, phenoloxidase, legumain, and three cathepsins were more strongly upregulated in unwinged than in winged aphids. Together our investigation of the pea aphid immune response to *Pandora* suggests that unwinged aphids are mounting a stronger immune response than winged aphids. In addition, our work demonstrates that aphids mount an immune response to fungal infection using several canonical insect immune mechanisms, which is of interest given that aphids have a reduced immune gene repertoire compared to other insects (Gerardo et al. 2010).

By measuring survival, fecundity, and the immune responses of different morphs after pathogen exposure, we describe the relationship between the strength, effectiveness, and cost of an immune response (Graham et al. 2011). Consistent with our alternative hypothesis, increased immune costs were associated with decreased resistance and a weaker immune response. We propose that reduced investment in immunity is a necessary developmental response to the limited resources available for winged aphids given their energetic investment in wings and the associated musculature. In other words, winged aphids are energetically constrained because they invest in wing production, and as a result have insufficient resources to produce an immune response without negatively influencing lifetime fecundity. However, there is an additional, adaptive explanation that should be considered. Because winged aphids are produced in response to crowded conditions, they may be programmed to invest less in immunity because they are more likely to experience greater competition for resources. These limitations may have shaped epigenetic programming of resource use away from non-dispersal traits like disease resistance.

While the evolutionary and ecological costs of having and using an immune system are likely universal, the relative costs to an individual depend on ecological context. The fitness costs associated with immunity have informed our understanding of the evolution and maintenance of variation among individuals in pathogen susceptibility (Schmid-Hempel 2003; Sadd & Schmid-Hempel 2009) and how pathogen virulence evolves (Gandon & Michalakis 2000; Mackinnon & Read 2004; de Roode *et al.* 2011). Condition-dependent immune costs have been observed in other systems, primarily under conditions of starvation (Kraaijeveld & Godfray 1997; Moret & Schmid-Hempel 2000; Mckean *et al.* 2008; Cotter *et al.* 2010). Determining the conditions under which immunity impacts host fitness is thus critical for our understanding of host-pathogen interactions. Our results show that polyphenism is an important and understudied factor influencing the expression of immune costs. Many organisms, from seasonal forms of lepidopterans and different castes of social hymenopterans to the cannibalistic and non-cannibalistic morphs of tiger salamanders, similarly use developmental plasticity to survive in heterogeneous environments (Pfennig & Collins 1993; Simpson *et al.* 2011). We expect that variable immune costs across morphs influence host-parasite ecology and evolution across the large number of taxonomically-diverse organisms that exhibit polyphenism.

More generally, our results have implications for understanding how immune investment is constrained by ecological context. Several studies have shown that life-history strategies influence disease resistance within and between species, and suggest that natural selection optimizes immune investment in anticipation of disease risk. However, because immune costs are context-dependent, we need a better understanding of how immune costs vary across life-history strategies to understand how costs constrain optimal investment in immunity. One possibility is that in many cases resistance cannot be tailored to disease risk across life-history strategies because investment in immunity is constrained by context-dependent costs. Our expectation is that winged aphids experience a higher risk of disease because they are produced in response to crowded conditions. Several studies have measured high rates of fungal infection in wild-collected winged aphids, and these studies highlight the importance of winged aphids in the spread of fungal pathogens (Feng, Chen & Chen 2004; Feng *et al.* 2007). It is therefore surprising that winged aphids invest less in immunity, and this system could represent a scenario where

immune costs cause individuals to deviate from optimal immune investment. However, additional data on the probability of pathogen exposure of aphid morphs under natural conditions is needed to test these assumptions.

Lastly, our findings have implications for our understanding of life-history strategies associated with dispersal. Winged aphids are important for the movement of aphid genotypes. Like aphids, many species exhibit physiological differences associated with migration and dispersal, especially as a result of the increased energetic demands of movement (Karlsson & Johansson 2008; Bonte *et al.* 2012). If dispersal influences the association between immunity and fitness similarly in other taxa, the increased susceptibility of dispersing animals could be an important driver of disease dynamics. This may also have evolutionary implications as 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, thereby influencing host-pathogen coevolution.

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DATA ACCESSIBILITY: Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.4kp7s (Parker et al. 2017). Raw reads from RNA sequencing were uploaded to the NCBI short read archive (SRA) with accession number SRP093772.

AUTHORS' CONTRIBUTIONS STATEMENT: BJP, SMB, and NMG designed the research and wrote the paper; BJP, SMB, and AML performed the research; BJP, SMB, and LHG analyzed the data. The authors declare no conflicts of interest.

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FIGURE AND TABLE LEGENDS

Table 1) Results of the statistical analyses.

Figure 1) Fecundity cost of exposure to heat-killed *Pandora*: Lifetime fecundity of unwinged aphids (left panel) and winged aphids (right panel) 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* (dark grey). Boxes show the bootstrapped 95% confidence estimates of lifetime fecundity.

Figure 2) Susceptibility of morphs to live *Pandora* infection: A. Survival: Percent survival 8 days after *Pandora* infection of unwinged (left) and winged (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. Percent Sporulation.** The percent of aphids that produced a sporulating cadaver after *Pandora* infection of unwinged (left) and winged (right) aphids. No control aphids sporulated. Bars show +/- standard error. **C. Pathogen load:** log number of copies of *Pandora* 18S (in 50ng DNA) measured with qPCR for winged (dotted line, open circles) and unwinged (solid line, solid circles) aphids at 1-4 days after exposure.

Figure 3) Cell counts: Relative concentration of immune cells in adult aphids (number of cells in 0.25μL hemolymph 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:** Mean relative immune cell concentration ± SE in 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* (dark grey) 24 hr after exposure. **B. Cellular immunity after live infection:** Mean relative immune cell concentrations ± SE of control (light grey) and *Pandora* exposed (dark grey) aphids 96 hours after exposure.

Figure 4) Immune gene expression: Mean relative fold change ± SE 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). Asterisks indicate statistically significant differences, as determined by the interaction effect between morph and treatment at each time-point (* < 0.05, ** < 0.01, *** < 0.001). The ACYPI gene IDs are shown just under the gene names. **A.** The top figure shows expression for nine genes found to be significantly differentially expressed in the transcriptome. **B.** The bottom figure shows expression for Cathepsin L, which was not found to be statistically significantly differentially expressed in the transcriptome.

SUPPLEMENTARY FILES

Supplementary File 1: Contains Supplementary Information A – J, including supplementary figures SA – SJ and supplementary tables S1 – S2.

Supplementary File 2: Contains complete list of significantly differentially expressed genes, with ACYPI annotations, from the transcriptomics work.

Table 1: Results of the statistical analyses.

	Test Statistic	d.f.	*p*
Cost of Exposure			
Morph	F = 113	1	**p < 0.0001**
Treatment	F = 5.33	1	**p = 0.02**
Genotype	F = 69.6	2	**p < 0.0001**
Morph * Treatment	F = 6.87	1	**p = 0.009**
Morph * Genotype	F = 11.2	2	**p < 0.0001**
Treatment * Genotype	F = 1.25	2	p = 0.28
Morph * Treatment * 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 9 days after live infection			
Morph	χ^2 = 13.3	1	**p < 0.0001**
Treatment	χ^2 = 64.7	1	**p < 0.0001**
Genotype	χ^2 = 17.0	2	**p = 0.0002**
Morph * Treatment	χ^2 = 7.08	1	**p = 0.0080**
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
Sporuation by 9 days after live infection			
Morph	χ^2 = 27.6	1	**p < 0.0001**
Genotype	χ^2 = 20.1	2	**p < 0.0001**
Morph * Genotype	χ^2 = 5.66	2	p = 0.06
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 (96hrs)			
Morph	F = 2.09	1	p = 0.16
Treatment	F = 8.75	1	**p = 0.0066**
Morph * Treatment	F = 15.6	1	**p = 0.00056**

Figure 1

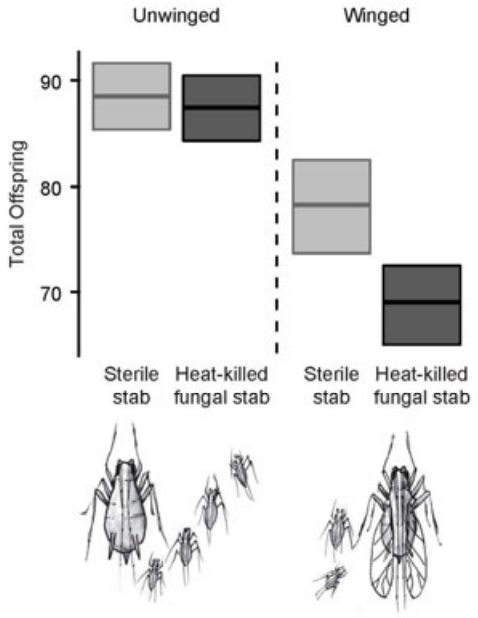

Figure 2

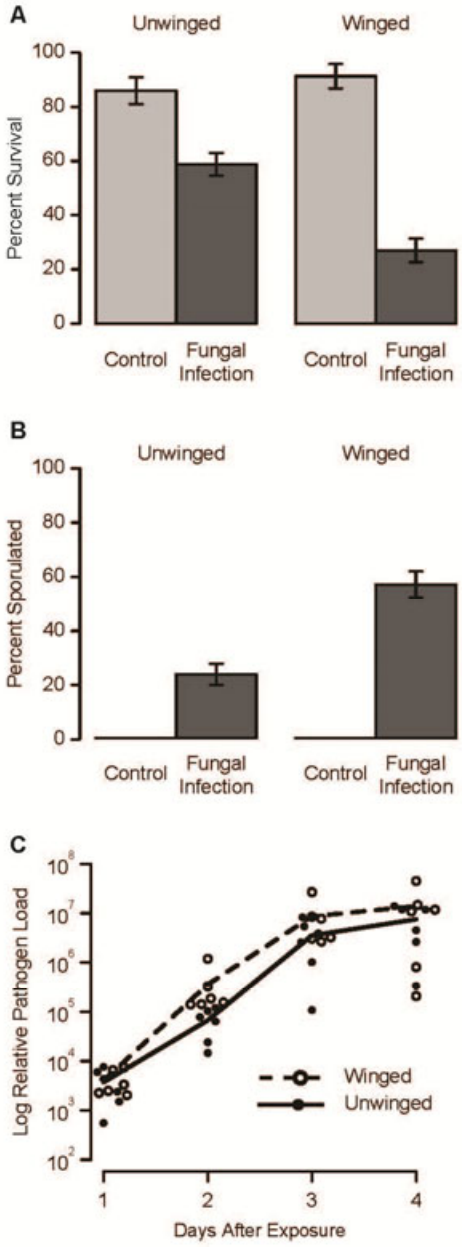

Figure 3

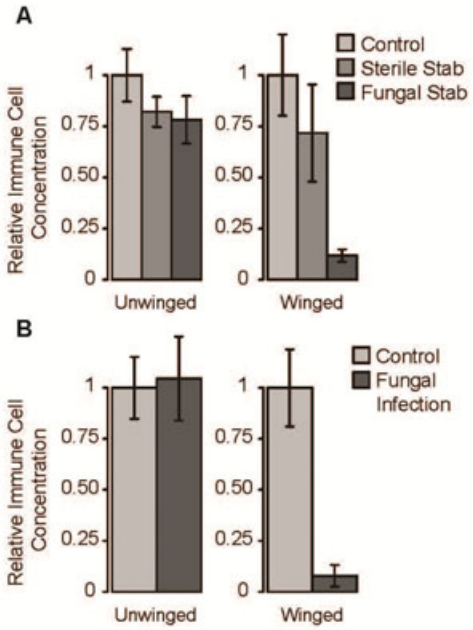

Figure 4