Intraspecific variation in immune gene expression and heritable symbiont density

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Abstract

Host genetic variation plays an important role in the structure and function of heritable microbial communities. Recent studies have shown that insects use immune mechanisms to regulate heritable symbionts. Here we test the hypothesis that variation in symbiont density among hosts is linked to intraspecific differences in the immune response to harboring symbionts. We show that pea aphids (Acyrthosiphon pisum) harboring the bacterial endosymbiont Regiella insecticola (but not all other species of symbionts) downregulate expression of key immune genes. We then functionally link immune expression with symbiont density using RNAi. The pea aphid species complex is comprised of multiple reproductively-isolated host plant-adapted populations. These 'biotypes' have distinct patterns of symbiont infections: for example, aphids from the Trifolium biotype are strongly associated with Regiella. Using RNAseq, we compare patterns of gene expression in response to Regiella in aphid genotypes from multiple biotypes, and we show that Trifolium aphids experience no downregulation of immune gene expression while hosting Regiella and harbor symbionts at lower densities. Using F1 hybrids between two biotypes, we find that symbiont density and immune gene expression are both intermediate in hybrids. We propose that in this system, Regiella symbionts are suppressing aphid immune mechanisms to increase their density, but that some hosts have adapted to prevent immune suppression in order to control symbiont numbers. This work therefore suggests that antagonistic coevolution can play a role in host-microbe interactions even when symbionts are transmitted vertically and provide a clear benefit to their hosts. The specific immune mechanisms that we find are downregulated in the presence of Regiella have been previously shown to combat pathogens in aphids, and thus this work also highlights the immune system's complex dual role in interacting with both beneficial and harmful microbes.

Author summary

Insects frequently form beneficial partnerships with heritable microbes that are passed from mothers to offspring. Natural populations exhibit a great deal of variation in the frequency of heritable microbes and in the within-host density of these infections. Uncovering the mechanisms underlying variation in host-microbe interactions is key to
understanding how they evolve. We study a model host-microbe interaction: the pea aphid and a heritable bacterium that makes aphids resistant to fungal pathogens. We show that aphids harboring bacteria show sharply reduced expression of innate immune system genes, and that this leads to increased densities of symbionts. We further show that populations of aphids that live on different species of plants vary in differential immune gene expression and in the density of their symbiont infections. This study contributes to our mechanistic understanding of an important model of host-microbe symbiosis and suggests that hosts and heritable microbes are evolving antagonistically. This work also sheds light on how invertebrate immune systems evolve to manage the complex task of combatting harmful pathogens while accommodating potentially beneficial microbes.

Introduction

Most insects harbor heritable microbes that have important effects on host fitness [1–3]. A key aspect of these symbioses is variation. Across species, host taxonomy has been shown to play a role in structuring heritable microbial communities [4–6]. Within species, microbes referred to as facultative symbionts are not found in all individuals, and symbiont frequencies are subject to selection on the relative costs and benefits of harboring microbes [7,8]. In addition to microbiome composition, hosts vary in other aspects of symbioses like the density of microbial infections [9,10]. For example, two closely-related species of Nasonia wasps vary in the density at which they harbor Wolbachia bacteria, and this variation is due to a single gene that somehow suppresses maternal transmission of bacteria [11]. Except for a few examples, little is known about the mechanisms that underlie variation in heritable symbioses or the evolutionary genetics of these interactions [12].

Invertebrate immune systems have been shown to play a direct role in mediating interactions with heritable microbes. In grain weevils, for example, an antimicrobial peptide acts to confine mutualistic symbionts to specialized cells called bacteriocytes [13], and silencing expression of immune pathways allows symbionts to escape host cells [14]. Other studies have found more complex interactions between pathogens, the immune system, and vertically-transmitted symbionts. In Drosophila melanogaster, for example, activation of the Toll and IMD pathways results in an increase in density of Spiroplasma symbionts [15] (and see similar examples in mosquitos [16] and tsetse flies [17]), suggesting in some systems the immune system can promote beneficial symbionts by inhibiting other microbes.

Immune genes are among the fastest evolving in eukaryotic genomes [18–20], and natural populations harbor extensive genetic variation in immune mechanisms [21]. Given the importance of the immune system in regulating insect symbioses, it seems likely that variation in immune mechanisms contributes to variation in symbiont density among hosts. This hypothesis is complicated, however, because our models for the maintenance of genetic variation in immune systems are based on antagonistic coevolution between hosts and pathogenic microbes [22,23]. Heritable symbiont infections are thought to spread through host populations because the fitness interests of hosts and microbes are generally aligned, and many symbionts have been shown to benefit their hosts for example by providing protection from pathogens (reviewed in [24]). But symbionts can impose costs on their hosts (e.g. [25]), and selection may favor the loss of symbionts in certain contexts [26]. In addition, within-host selection might lead to a separation of the fitness interests of hosts and microbes. For example, a mutation in a symbiont genome that increases symbiont density might increase the
likelihood of symbiont transmission but come at the expense of host fitness. Hosts, in turn, could evolve greater control over symbiont numbers in an ongoing arms-race for control over a symbiosis. It is unclear, however, whether the ‘arms-race’ dynamics underlying host-pathogen coevolution also govern the evolutionary interactions between immune systems and beneficial microbes.

The pea aphid (*Acyrthosiphon pisum*) is an important insect-symbiont model system [27]. The pea aphid species complex is composed of multiple reproductively-isolated populations adapted to live on different host plants within the family Fabaceae. These biotypes are genetically differentiated and are estimated to have radiated onto different host plants ~500,000 years ago [28] (but see [29]). In addition to obligate intracellular bacteria called *Buchnera aphidicola*, aphids can harbor several species of facultative symbionts. Multiple studies have found that facultative symbionts are non-randomly distributed across aphid biotypes [30–33]. For example, *Regiella insecticola* (which confers protection against fungal pathogens to its host [34–36]) is strongly associated with aphids from the *Trifolium* spp. (clover) biotype across continents. A number of studies have explored whether the strong association between *Trifolium* biotype aphids and *Regiella* is due to improved host plant use with mixed results [37–40]. Alternatively, this association could be due to the risk of exposure to fungal pathogens (though see [41]), to historical contingency (though see [42]), or to host and/or symbiont genetic mechanisms. Beyond the species level, *Regiella* within pea aphids form two main phylogenetic clades, and biotype has been shown to be a significant factor underlying the distribution of *Regiella* strains among pea aphids [43]. Specifically, the strong association between *Regiella* and aphids from the *Trifolium* biotype is mainly driven by *Regiella* from one specific clade (termed ‘Clade 2’) [43]. This system therefore provides a useful natural laboratory to study host-microbe adaptation across multiple environments within a single host species.

In this study, we show that immune mechanisms play a role in intraspecific variation in the density of a heritable bacterial symbiont. We first demonstrate that pea aphids that harbor *Regiella* (but not all other species of symbionts) sharply downregulate key innate immune genes, and that experimental suppression of the immune gene phenoloxidase via RNAi increases symbiont density. We then measure gene expression across aphids from multiple biotypes and find that aphids from *Trifolium* do not experience immune gene downregulation and harbor symbionts at relatively low density. Finally, by performing an F1 cross between genotypes from two biotypes we find that hybrid aphids show intermediate symbiont densities and immune gene downregulation, shedding light on the role of host genetic variation and the genomic architecture of this variation. We discuss these findings in light of the biology of this system and suggest that antagonistic coevolution between ‘beneficial’ microbes and their hosts can shape host-symbiont associations.

**Results**

**Hosting some symbiont species leads to decreased host immune gene expression**

Aphid lines reproduce parthenogenetically under summer conditions, and facultative bacteria can be introduced into or removed from host lines. We established lines that have the same aphid host genotype (LSR1, collected from *Medicago sativa* [44]) with two different strains of *Regiella*: one from each of the two main phylogenetic clades of *Regiella* found in natural populations of pea aphids [43]. *Regiella* strain LSR was originally collected in Ithaca, NY, USA in 1998 with the LSR1 aphid genotype and is a representative of *Regiella* Clade 1. *Regiella* strain .313 was collected in Gloucestershire, UK, in 2007 from an aphid from the *Trifolium* biotype (genotype 313) and is from Clade 2 [45]. We maintained aphids in the lab for four generations
after symbiont establishment and then measured *Regiella* densities using quantitative PCR (qPCR). We have found previously that Clade 2 *Regiella* establish at higher densities in hosts than do Clade 1 strains, independent of host genotype [46]. Consistent with this previous work, *Regiella* strain .313 established in aphid genotype LSR1 at a significantly higher density (3.5X) than *Regiella* strain LSR ($t = 5.1$, $p = 0.006$, Fig 1A).

We then used RNAseq to measure how harboring *Regiella* influences aphid gene expression by comparing these lines with symbiont-free aphids that were sham-injected. We sequenced cDNA made from mRNA for 4 biological replicates of each line (where a biological replicate represents an independent aphid line injected with symbionts or sham-injected). Overall, harboring *Regiella* strain LSR did not significantly alter expression of any genes in the aphid.
The 20 genes with altered expression included key innate immune system genes (S1 Table). In particular, the two copies of phenoloxidase in the pea aphid genome (referred to here as PO1 and PO2) were downregulated in the presence of Regiella. Also downregulated was a gene called hemocytin, which encodes a protein released by immune cells that plays a role in immune cell aggregation [47,48]. Other differentially expressed genes included a toll-like receptor and a putative lipopolysaccharide recognition protein (S1 Table).

We used qPCR to directly compare expression of two immune genes between lines harboring the two Regiella strains and to confirm our RNAseq results. PO1 was significantly downregulated in lines harboring Regiella strain .313 (Fig 1D and 1E, left panels). Hemocytin was significantly downregulated in aphids harboring either symbiont, and the magnitude of this change was significantly stronger for aphids harboring strain .313 than those with strain.LSR (Fig 1D and 1E, left panels).

Next, we established aphid lines that harbored one of several additional species of aphid facultative symbionts as above, and we looked for changes in PO1 and hemocytin expression. Spiroplasma and Serratia symbiotica did not alter expression of either gene, but two strains of Hamiltonella defensa significantly downregulated PO1 expression but not hemocytin (post-hoc tests, S2 Table and Fig 1D and 1E, right panels). Like Regiella, the specific strain of Spiroplasma (.161) we used was found in previous work to protect against fungal pathogens [49], but Serratia and Hamiltonella have been shown not to influence fungal resistance [36,50,51]. These results therefore suggest that the changes we identify in immune gene expression do not reflect the mechanism by which Regiella confers protection to aphids against fungal pathogens, which is currently unknown. Aphid symbionts in the family Enterobacteriaceae (Regiella, Hamiltonella, and Serratia) live both in hemolymph and inside of the insect cells (reviewed in [52]). In contrast, Spiroplasma is mainly extracellular in most insects, and has not been identified inside of aphid cells [53]. Qualitatively, then, our results do not suggest that differences in immune gene expression among facultative symbiont species are due to localization in different tissues.

Immune gene expression influences symbiont density during development

We studied the function of immune gene expression on Regiella densities using RNA interference (RNAi) [54]. We knocked-down expression of PO1 early in development by injecting ~100ng of dsRNA in salt buffer into 1-day old aphids, and we measured the effects on symbiont density. We used dsRNA for lacZ as a control, which is designed to control for the effects of injection and exposure to buffer and dsRNA, which may have an influence on gene expression. Aphids harbored either Regiella strain.LSR or .313. We then sampled aphids at two time-points: at 72hrs after injection and after aphids had become adults (8 days after injection).

Injection with PO1 dsRNA reduced PO1 expression, on average, by ~60% at 72hrs (2-way ANOVA; Treatment: F = 9.8, p = 0.009; Fig 2A). At this early timepoint, we found no significant difference between aphids harboring Regiella strain.LSR vs strain .313 in PO1 expression (Strain: F = 0.10, p = 0.75; Fig 2A). By the time aphids became adults (8 days after injection), PO1 expression in PO1 dsRNA injected aphids was still reduced by ~60% compared with controls (Treatment: F = 3.6, p = 0.02; Fig 2A). By this later timepoint, aphids harboring the two symbiont strains had diverged in expression as found above (Strain: F = 11.5, p = 0.004; Fig 2A). Note that we dissected out and removed developing embryos only from the adult samples before nucleic acid extraction, so we do not directly compare expression in the 72hrs vs. adult samples, but qualitatively PO1 expression increased during development (Fig 2A).
PO1 knockdown led to a 59% and 2% increase in Regiella strain LSR and .313 density at 72hrs, respectively (2-way ANOVA; Treatment: F = 4.7, p = 0.05; Fig 2B). Regiella density in aphids harboring strain LSR vs .313 also differed significantly (Strain: F = 245, p < 0.0001; Fig 2B), suggesting that strain-level differences in symbiont density are present even at this early developmental timepoint. The increase in Regiella density due to PO1 knock-down persisted to adulthood (Treatment: F = 5.7, p = 0.03, Fig 2B), with PO1 dsRNA injection increasing Regiella density by 48% and 40% in aphids harboring Regiella strains LSR and .313, respectively. As we found above, the density of strain .313 was higher than strain LSR in adult aphids (Strain: F = 145, p < 0.0001; Fig 2B). Together, these results show that knockdown of PO1 increases Regiella density over development.
Symbiont density is not impacted by immune gene knock-down later in development

We performed a similar experiment studying the effect of RNAi on symbiont density, but injected dsRNA into adult aphids rather than 1st instars. We injected ~1μg of dsRNA synthesized from PO1 or hemocytin into adult (9 day old) aphid genotype LSR1 aphids infected with Regiella strain LSR. We measured gene expression and Regiella density at 72hrs after injection. This led to a ~69% and ~82% reduction in expression of PO1 and hemocytin, respectively (t-tests; PO1: t = -2.3, p = 0.05; hemocytin: t = -7.3, p < 0.0001; Fig 1C and 1D). We note that injection with dsRNA from PO1 had no effect on expression of the other copy of phenoloxidase in the aphid genome (PO2: t = -0.91, p = 0.38; Fig 1C), demonstrating that our phenoloxidase RNAi assay is specific to PO1 as designed. Knockdowns had no effect on Regiella density in aphids injected as adults (ANOVA; Treatment: F = 0.80, p = 0.46; Fig 1E).

Immune gene downregulation differs across aphid biotypes

We repeated the RNAseq experiment to study the effects of Regiella on gene expression across multiple aphid biotypes. We used a genotype from the Lotus corniculatus biotype (663, collected in Oxfordshire, UK in 2014 with no original facultative symbionts), a genotype from Ononis spinosa (C133, collected in Berkshire, UK in 2003 that originally harbored Hamiltonella), and a genotype from Trifolium spp. (C317, collected from Trifolium pratense in Gloucestershire, UK in 2003 that originally harbored a Clade 2 Regiella). The genetic distance among these biotypes is variable, with Trifolium and Medicago sativa (LSR1) possibly the most closely related and Ononis the most distant; unlike some biotypes (e.g. Lathyrus pratensis), those included here are not thought to represent incipient species [29]). For each aphid genotype, we compared replicate lines that had each been infected with an independent Regiella Clade 2 (.313) infection or had been sham injected as above after 4 generations (after verifying that the symbiont infection had not been lost using PCR).

In the Lotus corniculatus genotype, harboring Regiella strain .313 had a significant effect on the expression of aphid genes (7 genes differentially expressed at an FDR < 0.05; Fig 3A). Of these 7 genes, five were also downregulated in the experiment described above using genotype LSR1, including PO1 and hemocytin (Fig 3D). There is therefore some degree of conservation in the response to Regiella across genetically distinct aphid lines (Fig 3D). In contrast, zero genes were differentially expressed in response to Regiella in the Ononis biotype line at an FDR of < 0.05 (Fig 3B). Similarly, zero genes differed in expression in response to Regiella in the Trifolium line (Fig 3C).

F1 hybrids have an intermediate symbiont density and level of immune expression

We performed an F1 cross (Fig 4A) between two of the biotype lines in order to better understand the role of host genetic variation in Regiella density and immune gene expression. We crossed the Lotus (663) and Trifolium (C317) lines [55], and generated multiple replicate infections with Regiella strain .313 in each line as above. After four generations, we measured Regiella density using qPCR. Regiella density differed between the parental lines (post-hoc tests S3 Table and Fig 4B), with the Lotus line harboring a significantly higher density of Regiella strain .313 (5.2X higher) than the Trifolium line. Further, the F1 lines harbored Regiella at densities intermediate to the parental lines (post-hoc tests S3 Table and Fig 4B).

We then sampled aphids from this same generation to compare changes in immune gene expression due to Regiella in parental and F1 lines. We selected two F1 lines for this assay with
each aphid genotype serving as the maternal line. We used qPCR to measure expression of both copies of phenoloxidase, hemocytin, and also nitric oxide synthase (NOS; an important innate immune mechanism that was not significantly differentially expressed in any of our RNAseq studies). Confirming our RNAseq findings, harboring *Regiella* led to a decrease in expression of *PO1*, *PO2*, and hemocytin in the *Lotus* genotype, but *Regiella* did not affect gene

![Volcano plots of expression data comparing control vs. *Regiella*-infected aphids. Each expressed gene in the aphid genome is represented by a point. The x-axes show the log₂ fold change of each gene, with points to the right side of each plot indicating increased expression in the presence of symbionts, and points to the left showing decreased expression. The y-axes show the -log₁₀ of the p-values indicating statistical significance of each gene's expression change. Colored points are those where the expression change was found to be statistically significant at FDR < 0.05. Panels A, B, and C show plots for the *Lotus*, *Ononis*, and *Trifolium* genotypes respectively, as shown along the top of the figures.](https://doi.org/10.1371/journal.ppat.1009552.g003)
expression in the *Trifolium* genotype (post-hoc tests S4 Table and Fig 4C). Further, the F1 lines showed significant differences in gene expression that were intermediate to the two parental lines: three immune genes were downregulated in response to *Regiella*, but to a significantly lesser extent than in the *Lotus* genotype (post-hoc tests S4 Table and Fig 4C).
Discussion

We show that some aphids harboring the facultative bacterial symbiont *Regiella insecticola* experience reduced expression of key immune genes, and we link decreased immune gene expression with increases in *Regiella* density. We further find that this mechanism is influenced by host genetic factors: some genotypes harbor *Regiella* at lower densities and do not experience altered gene expression when infected, where other aphid genotypes experience reduced immune gene expression and have higher-density *Regiella* infections. This study shows that intraspecific variation in the immune system affects a heritable symbiosis.

Clade 2 *Regiella* establish in aphids at higher densities than Clade 1 strains [46]. We found no significantly differentially expressed genes in the transcriptome of aphids harboring a Clade 1 (.LSR) *Regiella* strain. Using qPCR we confirmed that the immune downregulation we uncover occurs more strongly in aphids harboring the Clade 2 *Regiella* than the Clade 1 strain, potentially contributing to differences in density among *Regiella* strains. An important question, then, is whether symbionts are suppressing host immune mechanisms in order to reach higher densities in hosts, or if hosts are modifying immune mechanisms in order to accommodate symbionts. Fitness costs to aphids of harboring symbionts (including *Regiella*) have been measured in the laboratory and field [35,56], and we have found previously that higher density Clade 2 *Regiella* strains impose stronger survival costs on hosts than the lower density Clade 1 strains [46]. In addition, the two *Regiella* clades confer protection against specific genotypes of the fungal pathogen *Pandora neoaphidis* [45], and therefore *Regiella* density will be positively correlated with symbiont-mediated protection for some fungal genotypes and negatively correlated for others. Together these results suggest that immune downregulation is not an adaptation on the part of the host in order to accommodate symbionts, but instead some *Regiella* strains suppress immune mechanisms in order to establish at higher densities in hosts. Establishing at a higher density could benefit *Regiella* through improved competitive outcomes with other strains and species of symbionts, or through increased horizontal transmission which occurs on evolutionary [43] and even ecological [57] timescales.

Aphid biotypes harbor facultative symbionts at different frequencies that are to some degree conserved across continents [30–33], and decades of research have gone into explaining these patterns in order to better understand the ecological and evolutionary forces shaping beneficial host-microbe interactions. One particularly clear association is between aphids from the *Trifolium* biotype and *Regiella*—and specifically *Regiella* from Clade 2 [43]. Studies attempting to explain this pattern have considered factors like the potential effects of *Regiella* on host plant use [37–40] and pressure from fungal pathogens on different host plants [41]. We suggest that host genetic effects represent an additional factor shaping the aphid-symbiont frequencies in natural populations. Whether a cause or consequence of the strong association between *Regiella* and *Trifolium*, it seems likely that the immune systems of *Trifolium* aphids are better adapted to harboring this symbiont than biotypes that are not naturally associated with Clade 2 *Regiella*. We found no evidence of differential gene expression in an aphid genotype from the *Trifolium* biotype: zero genes were differentially expressed in response to harboring *Regiella* strain .313 (which was confirmed using qPCR on three immune genes). Together, these results are consistent with a scenario where Clade 2 *Regiella* have evolved increased within-host density that harms host survival, and aphids from the *Trifolium* biotype have adapted to prevent immune suppression to control symbiont numbers. Our findings might more broadly suggest, then, that hosts and beneficial heritable microbes can evolve antagonistically, which has been suggested by other studies in aphids [9,58] and other organisms [59].

The phenoloxidase enzyme is required for the activation of melanogenesis in invertebrates. Against multicellular parasites, melanin is deposited around a foreign object via immune cells...
(hemocytes), and the melanin capsule prevents the growth and reproduction of parasites [60]. PO is also upregulated in response to microbial pathogens in many studies [61,62] and it is thought that because phenoloxidase is cytoplasmic it helps immune cells kill phagocytosed microbes [63]. It is important to note that regulation of phenoloxidase also occurs at the post-translational level [60], and future work is needed to link changes in gene expression in the presence of symbionts to protein levels. Aphid immune cells express phenoloxidase [64], are known to have phagocytic properties [64,65], and have been shown using microscopy to contain facultative symbionts including Regiella [65]. Harboring Regiella (and Hamiltonella but not other species of symbionts) leads to a sharp decrease in the numbers of circulating immune cells (called granulocytes) [65]. One possibility is that PO knockdowns via RNAi in our study disrupted the cellular immune responses aphids use to regulate symbionts during development, but the natural mechanisms Regiella might be using to suppress PO and other immune genes are unknown.

In addition to the effects on symbionts we have uncovered in this study, phenoloxidase has been shown to be an important part of the pea aphid’s response to pathogens (e.g. fungal pathogens [66,67]). Functionally, a recent study found that silencing of PO1 and PO2 via RNAi leads to decreased resistance of pea aphids against pathogenic bacteria and a generalist fungal pathogen [68]. Together, these results show that the same molecular mechanisms are influencing interactions with both beneficial and pathogenic microbes in this system. The protection against specialist fungal pathogens conferred by Regiella might benefit hosts, but changes in gene expression in the presence of some strains of Regiella could trade-off with an increased risk of infection with other pathogens. Recent work on animal immune systems has emphasized the role of immune mechanisms in regulating mutualistic interactions between microbes and hosts. In general, how immune systems evolve to manage the complex task of interacting with distinct microbes with different effects on host fitness is an important question.

Finally, our findings emphasize the importance of host genetic variation in associations with beneficial microbes [69]. We found that hybrids between aphid biotypes harbor symbionts at intermediate densities to their parental lines and only partially differentially express immune genes. The extent to which a host responds to symbiont infection is therefore likely a quantitative trait, much like resistance against pathogens, that is influenced by variation at multiple to-be-determined loci, and subject to natural selection on the relative costs and benefits of symbiosis. This work thus contributes to a growing view of animal microbiomes as complex phenotypes that influence animal fitness, and that are to some extent under host control.

Materials and methods

Pea aphids and symbiont establishment

Pea aphids reproduce parthenogenetically under certain light and temperature conditions (16L:8D at 20°C), allowing us to rear large numbers of genetically identical and developmentally synchronized individuals for use in experiments. Wild-collected lines were cured of their original symbiont infections using antibiotics [37] and maintained asexually in the lab for several years before use in experiments (S5 and S6 Tables specify collection information for aphid genotypes and symbiont strains).

Throughout these experiments we used established protocols to infect aphids with facultative symbionts [70–72]. We inject a small volume of hemolymph from an infected donor aphid into a 1st instar recipient using a glass capillary needle. We then rear these aphids to adulthood and then collect an offspring from late in the birth-order to establish the infected line. When this aphid produces offspring, we extract DNA (using a lysis buffer with proteinase K and an ethanol precipitation [73]) and screen the line for symbionts using PCR with
symbiont-specific primers [43] (S7 Table): (94˚C 2 min, 11 cycles of 94˚C 20s, 56˚C (declining 1˚C each cycle) 50 s, 72˚C 30 s, 25 cycles of 94˚C 2 min, 45˚C 50 s, 72˚C 2 min and a final extension of 72˚C 5 min). Each biological replicate in these experiments (an “aphid line”) originated from a separate symbiont-injection and screening except where noted.

**Measurements of symbiont density using qPCR**

We established aphid lines from the LSR1 genotype with two strains of Regiella: Clade 1 Regiella (strain.LSR), and Clade 2 Regiella (strain .313). These two strains of Regiella each come from one of the two main clades of Regiella found among pea aphids as determined using an established protocol for MLST sequence typing [45,74]. We reared lines that had successfully acquired a Regiella infection under asexual conditions for four generations, at which point we re-screened lines for Regiella infection. We then used qPCR to compare symbiont density between these strains. We removed embryos from groups of 7 adult aphids, and extracted DNA using the Qiagen DNEasy kit under recommended conditions. We used qPCR primers that amplify a conserved region of the Regiella hrpA gene (S7 Table). Amplification of g3PDH was used as an endogenous reference gene that controlled for the relative abundance of host DNA in each sample. Primer concentrations were optimized against a serial dilution of gDNA (400/350nM for hrpA, and 300nM for g3PDH and hrpA, respectively). We calculated -ΔC_T values by -(C_T_hrpA−C_T_g3PDH) and analyzed these values with a t-test. Note that this approach reveals the relative density of symbionts relative to host tissue across different samples, but does not measure the absolute abundance of symbionts. Experimental data from this and all of the experiments described below can be found in S1 Experimental Data.

**Effects of Regiella on host gene expression using RNAseq**

We then measured the effects of harboring Regiella on gene expression using RNAseq. We used the lines established above with either Regiella strain.LSR or .313, and symbiont-free aphids of the same host genotype. For the ‘no symbiont’ treatment, we sham injected aphids (injected aphids with a small volume of hemolymph (0.25 μl) from an uninfected adult donor aphid) and handled aphids in the same way as with symbiont-injected aphids.

For transcriptome sequencing, we collected adult, fourth generation aphids on the first day that each line produced offspring and dissected and removed developing embryos (in order to measure gene expression of the mother without including RNA from her embryos). We stored carcasses in TRIzol (Invitrogen) at -80˚C. Each sample contained ~14 adult carcasses collected from multiple host plants. We extracted RNA using TRIzol-chloroform and an isopropanol precipitation with an ethanol wash. We digested genomic DNA and cleaned the RNA using the Zymo Clean & Concentrate–5 kit with the DNAse I enzyme. RNA quality control was conducted on a bioanalyzer chip, and 12 sequencing libraries (4 biological replicates x 3 treatments) were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (including poly-A selection and 15 rounds of PCR amplification). Libraries were sequenced across one lane of Illumina PE150 sequencing (approximately 20 million reads per library) with a 250-300bp insert per library.

**RNAseq analysis**

We estimated the average insert size of paired-end libraries using Picard Tools v.2.21.3 in java 1.8.0, and mapped reads to the pea aphid reference genome v.2.1 [44] using tophat v.2.1.1 [75]. We counted reads mapped to each annotated gene (using a modified version of pea aphid genome annotation v.2.1 (https://bipaa.genouest.org/sp/acrythosiphon_pisum/) with several duplicated genes removed from the file) using the count function in htsq v.0.9.1 [76] and the
'union' overlap mode (S8 Table). We analyzed read counts using EdgeR v.3.22.3 in R v.3.5.0. Genes with a minimum threshold of aligned reads, determined by the filterByExpr function in edgeR, were retained in the analysis. We fit a quasi-likelihood model to the data using the glmQLFit function, and we tested for statistically significant differential expression of each gene using a quasi-likelihood F-test, interpreting genes with a false discovery rate (FDR) of < 0.05 as differentially expressed in response to Regiella infection.

Immune gene expression across facultative symbiont species via qPCR

We used qPCR to verify our RNAseq results, and to explore how differences in gene expression due to Regiella in key innate immunity genes varied across facultative symbiont species. We used qPCR primers (S7 Table) that amplified 80-120bp of two target genes of interest (PO1: ACYPI004484 and hemocytin: ACYP1003478, which were also found to be differentially expressed in the Lotus genotype, described below). We used four endogenous control genes (Glyceraldehyde 3-phosphate dehydrogenase (g3PDH): ACYPI009769, NADH dehydrogenase: ACYPI009382, β-tubulin: ACYPI001007, and Rpl32: ACYPI000074). Primer concentrations were optimized against a 1:10 serial dilution of gDNA (200ng – 0.2ng gDNA per reaction) to an efficiency of 100 +/- 10% (PO1: 100nM; hemocytin: 100nm; g3PDH: 400/350nM F/R; NADH: 350/300nM F/R; β-tubulin: 400nM; and rpl32: 200nM). Reactions were run on a Bio-RAD CFX96 Real-Time System machine, with an initial step of 95˚C for 3 minutes and 40 cycles of 95˚C for 10s and 60˚C for 30s. Each 20μL reaction included a 1X PCR buffer, Mg²⁺ at 2mM, dNTPs at 0.2mM, EvaGreen at 1X, 0.025 units/μL of Invitrogen taq, and 40ng of cDNA. Three technical replicates were run for each reaction.

We measured expression of these genes in lines with and without symbionts in two separate experiments. First, we collected aphids from the Regiella-infected lines used for the RNAseq above (no symbionts, Clade 1 LSR Regiella, and Clade 2 .313 Regiella). We dissected out and removed embryos, pooled adult carcasses, extracted and cleaned RNA, and DNAse treated samples as above. We synthesized cDNA using the BioRad iScript cDNA synthesis kit under recommended conditions. For each sample we averaged the Cₜ values from the endogenous control genes, and calculated -ΔCₜ values by—(Cₜ target−Cₜ mean endogenous control). We analyzed differences in gene expression between symbiont-free, Clade 1, and Clade 2 lines with one-way ANOVAs on the -ΔCₜ values, and used Tukey HSD tests for pair-wise comparisons among different symbiont backgrounds. We performed separate analyses for the two genes.

In a second experiment, we injected three additional symbiont species into aphids and measured PO1 and hemocytin expression as above. For donor aphids, we used an aphid line harboring Serratia symbiotica, a line harboring Spiroplasma sp. (strain .161), and two strains of Hamiltonella defensa (S6 Table). We only successfully established Serratia from two injection events after multiple attempts, and so the biological replicates of this assay were generated by splitting the lines onto multiple plants after 2 generations before sampling at generation 5; the other lines represent independently injected lines. We maintained sham-injected (symbiont-free) aphids under identical conditions as above. Gene expression was measured and analyzed as above. We note that the Spiroplasma sp. strain used in this experiment has been shown, like Regiella, to be protective against fungal pathogens while the other symbiont species used have not found to confer fungal protection. The two Hamiltonella strains used were collected in the same field and may not represent distinct symbiont genotypes from each other. Data were analyzed as above.

Expression knock-down via RNAi

We designed primers that amplify regions of two target genes (531bp of PO1 and 483bp of hemocytin) with the T7 promoter sequence (TAATACGACTCACTATA) on the 5’ end.
of each primer using the e-RNAi Webservice (https://www.dkfz.de/signaling/e-rnai3/). Primer sequences can be found in S7 Table. We PCR amplified these regions from cDNA under recommended conditions. PCR products were sequenced using Sanger sequencing primed with the T7 promoter sequence to confirm target identity. We then purified 160 μL of PCR product (using NaOAc and EtOH precipitation) and concentrated it to 500ng/μL. We used the MEGA-Script RNAi kit to synthesize dsRNA from PCR amplicons under recommended conditions and a 15hr transcription incubation. We ran dsRNA (at a 1/400 dilution) on a 2% agarose gel to verify that a single band was obtained of the correct size, and we then concentrated the dsRNA product to approximately 3300ng/μL using LiCl and an ethanol precipitation, and eluted the final dsRNA product in MEGAScript buffer. We repeated these protocols to generate dsRNA from lacZ as a control as in [77].

In a first experiment, we injected 1st instar (1-day-old) aphids with approximately 100μg dsRNA from either PO1 or lacZ as a control using a glass capillary needle attached to a syringe on the underside of thorax of each aphid. We used two aphid lines that harbored either Regiella strain.LSR or .313. We collected injected aphids at two time-points after injection: at 72hrs and at day 9 when they had undergone their final molt to the adult stage. For the 72hr samples, groups of 3 whole aphids were pooled in TRIzol and stored at -80˚C for RNA extraction, or were stored in tubes at -20˚C for gDNA extraction. For the adult samples, we dissected out developing embryos and stored adult carcasses individually for RNA or DNA extraction, and we also pooled embryos from three adults and stored them in tubes for DNA extraction.

From the samples stored in TRIzol, we extracted RNA, synthesized cDNA, and measured the expression of PO1 and four endogenous control genes using qPCR as above. We calculated -ΔC_T values as above and analyzed these using two-way ANOVAs (with treatment (lacZ vs. PO1) and Regiella strain (LSR vs. .313) as factors). We conducted post-hoc analyses using Tukey’s HSD tests. The two time-points were analyzed separately. We extracted DNA from the remaining samples using the Qiagen DNEasy kit and measured Regiella densities using qPCR amplification of the hrpA gene as above. We analyzed these data using two-way ANOVAs and Tukey’s HSD tests as above in R v.3.5.1 after testing for model assumptions.

For adult injections, we reared LSR1 aphids with Regiella strain.LSR as above. We then injected adult aphids (9 days old) with 0.3μL of dsRNA (approximately 1μg total). In this experiment we performed knock-downs of two genes: PO1 and hemocytin. We first collected aphids harboring strain.LSR at 72 hours after injection to validate our knock-downs. For each gene, we pooled groups of three aphids into 8 samples, removed developing embryos, extracted RNA from adult carcasses, synthesized cDNA, and measured gene expression as above. We analyzed -ΔC_T values for each gene using t-tests with treatment as the independent variable. In this experiment we also measured expression of PO2 in aphids that had been injected with dsRNA from PO1 to verify that our knock-down was specific to PO1. We then injected adult aphids with approximately 1μg dsRNA, removed and discarded developing embryos after 72hrs, pooled samples into groups of 4 dissected aphids, extracted DNA as above, and measured Regiella densities using qPCR as above. We analyzed -ΔC_T values from this experiment using a one-way ANOVA, and included treatment (lacZ control, PO1, or hemocytin) as a factor in the analysis. We conducted post-hoc analyses using a Tukey’s HSD test to compare levels within treatment.

**RNAseq on aphid genotypes from multiple host-plant associated biotypes**

We selected three aphid genotypes each from a different host-plant associated biotype (Lotus corniculatus, Ononis spinosa, and Trifolium spp.); information on collection location can be found in S5 Table. We established Regiella strain .313 infections in each line as above. Each
Regiella-infected line was established from a different symbiont injection and maintained separately on V. faba plants. In parallel, control aphids were sham injected as above. After 4 generations, we froze seven adult aphids from each line in liquid nitrogen and stored at -80°C. RNA was extracted and purified as above. For the Trifolium biotype, RNAseq libraries and sequencing was conducted as described for the LSR genotype used above. For the Lotus corniculatus and Ononis spinosa biotypes, dual-indexed stranded sequencing libraries were constructed using the NEBNext polyA selection and Ultra Directional RNA library preparation kits. Libraries were sequenced on one lane of Illumina HiSeq 4000 (Paired-end 150bp) generating a target of > 2x 280M reads. We analyzed each genotype separately, comparing libraries with and without a Regiella infection, as above.

F1 crosses
We used two of these biotype lines (C317 from Trifolium and 663 from Lotus) for the F1 genetic cross because we found from RNAseq data that these lines responded differently to Regiella infection. To induce male and female aphids for genetic crosses, we transferred stocks to ‘autumn’ conditions (short day, 13L:11D at 18°C). After 30 days we moved third and fourth instar nymphs onto leaf-plates (a fava bean leaf in 2% agar in Petri dishes) to isolate virgin egg-laying sexual females (oviparae) and males. Oviparae have a characteristic thicker hind tibia, and this feature was used to isolate probable oviparae from males. The male screening was less stringent because virgin males were not needed. We setup each cross by placing the corresponding genotype oviparae and males onto a fava bean seedling, replenishing breeding stocks as they became available. After 24 hours, we treated melanized eggs with 10% calcium propionate to clean off the surface and then transferred eggs using fine-tipped forceps to a small petri dish with Whatman filter paper moistened with sterile water. We sealed the plates with parafilm wrap and left them in autumn conditions for a further 24 hours, after which dishes were transferred to a 2°C incubator to diapause. After 3 months, eggs were removed from the diapause conditions and with fine-tip forceps, rolled against a Kimwipe to reduce any microbial growth. We then transferred diapaused eggs to a new leaf-plate and placed them in ‘autumn’ conditions (as above) until a fundatrix hatched. Each fundatrix was separated and a line was considered stable after two generations. We used this protocol to generate five F1 lines: three with line 663 as the maternal genotype and two with C317 as the maternal genotype.

qPCR measures of gene expression in the F1 panel
We established replicate Regiella strain .313 infections in parental and the five F1 hybrid lines as above. After 4 generations, we collected three adults from each biological replicate, removed embryos via dissection, extracted DNA, and measured symbiont density all as above. We grouped the replicate F1 lines from each direction of the cross together to analyze these data, and used a one-way ANOVA in R version 3.5.0, comparing density among genotypes using a Tukey’s HSD post-hoc test.

We then measured expression of four immune genes aphids with and without Regiella using qPCR in the parental and two of the F1 lines. Four generations after injection, four aphids from each biological replicate from each genotype were removed from plants, embryos were dissected out of adult carcasses and stored in TRIzol at -80C. We extracted RNA and synthesized cDNA as above. For each sample, we measured expression against the four endogenous control genes used above. Here we measured expression of four target genes: two copies of phenoloxidase (PO1: ACYPI04484 and PO2: ACYPI072244), hemocytin (ACYPI003478), and Nitric Oxide Synthase (ACYPI001689). -ΔCt values were analyzed using an ANOVA after testing for model assumptions; post-hoc tests using the multcomp package in R v.3.5.0 were
conducted to compare the specific effect of Regiella on each genotype, and to compare interaction terms between Regiella presence/absence and host genotype. Analysis of expression of each gene was conducted separately.

Supporting information
S1 Table. Significantly differentially expressed genes from the RNAseq experiments. Numbers show log₂ fold changes of expression in aphids with vs. without Regiella. Host genotype and symbiont strain are indicated in the header.

S2 Table. Results of post-hoc tests (Tukey’s HSD) analyzing gene expression of PO1 and Hemocytin (corresponding with Fig 1D and 1E). The top section labeled "Regiella" shows the results of statistical analyses of lines harboring Regiella. The bottom section labeled “Other Symbiont Species” shows the results of statistical analyses of lines harboring one of the Hamiltonella strains, Spiroplasma, or Serratia. Statistical significance at p < 0.05, p < 0.01, and p < 0.001 is indicated with a *, **, or *** respectively.

S3 Table. Results of post-hoc tests (Tukey’s HSD) analyzing Regiella densities in F1 lines. Statistical significance at p < 0.05, p < 0.01, and p < 0.001 is indicated with a *, **, or *** respectively.

S4 Table. Results of post-hoc tests (Tukey’s HSD) analyzing gene expression in F1 lines. Significance at p < 0.05, p < 0.01, and p < 0.001 is indicated with a *, **, or *** respectively.

S5 Table. Collection information for the aphid genotypes used in this study. Reg refers to Regiella insecticola; Ham refers to Hamiltonella defensa.

S6 Table. Collection information for the symbiont strains used in this study.

S7 Table. Primer Sequences.

S8 Table. Sequencing and alignment results.

S1 Experimental Data. Contains the data for each experiment as indicated in the tab headers.

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