1 **Condition-dependent alteration of cellular immunity by secondary symbionts in**
2 **the pea aphid, *Acyrtosiphon pisum*.**

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16 **Abstract**

17 Endosymbionts can fundamentally alter host physiology. Whether such changes are
18 beneficial or detrimental to one or both partners may depend on the dynamics of the
19 symbiotic relationship. Here we investigate the relationship between facultative
20 symbionts and host immune responses. The pea aphid, *Acyrtosiphon pisum*,
21 maintains an obligate primary symbiont, but may also harbour one or more facultative,
22 secondary symbionts. Given their more transient nature and relatively recent adoption
23 of a symbiotic lifestyle compared to primary symbionts, secondary symbionts may
24 present a challenge for the host immune system. We assessed the response of several
25 key components of the cellular immune system (phenoloxidase activity, encapsulation,
26 immune cell counts) in the presence of alternative secondary symbionts, investigating
27 the role of host and secondary symbiont genotype in specific responses. There was no
28 effect of secondary symbiont presence on the phenoloxidase response, but we found
29 variation in the encapsulation response and in immune cell counts based largely on
30 the secondary symbiont. Host genotype was less influential in determining immunity
31 outcomes. Our results highlight the importance of secondary symbionts in shaping
32 host immunity. Understanding the complex physiological responses that can be
33 propagated by host-symbiont associations has important consequences for host
34 ecology, including symbiont and pathogen transmission dynamics.

36 **Key-words**

37 immune cell count; ecological immunity; encapsulation; endosymbiont; host-microbe;
38 symbiosis.

1. Introduction

Establishing endosymbiotic relationships can confer substantial benefits to hosts, including provisioning of nutrients and protection from pathogens (see Su et al. 2013, for a review). Such intimate interactions often result in fundamental changes in host physiology and phenotype either as a direct or indirect response to symbiont presence (Montgomery & McFall-Ngai 1994; Braendle et al. 2003). While some changes are beneficial for the host (i.e. increased survival in the presence of pathogens) others can negatively impact host life history traits such as survival and fecundity under certain ecological conditions, tipping relationships towards the parasitic end of the symbiosis spectrum (Oliver et al. 2006; Simon et al. 2011; Vorburger & Gouskov 2011).

One aspect that is likely altered by the presence of symbionts is the host immune system (Gross et al. 2009; Zheng et al. 2011; Hooper et al. 2012). In order to cultivate a beneficial symbiotic relationship, hosts must respond differently to symbiotic and pathogenic microbes, but this can prove problematic. Microbe-associated molecular patterns (MAMPs), for example, used by hosts to recognise and respond to pathogens, are also commonly found in other microbiota (Chu & Mazmanian 2013), including symbionts. Consequently, symbiosis may select for alterations in the host defences that facilitate establishment and maintenance (see Nyholm & Graf 2012, for review).

Specialised organs or cells within the host can provide a protective environment for symbionts while affording hosts a degree of control over the location and size of symbiont populations (Montgomery & McFall-Ngai 1994; Braendle et al. 2003).

Additionally, reducing or down-regulating the host immune system may facilitate the establishment and maintenance of a stable symbiont population (Gerardo et al. 2010; Douglas et al. 2010). The host immune system can also play a role in regulating

65 symbiont populations (Nakabachi et al. 2005; McFall-Ngai 2007; Anselme et al.
 66 2008; Login et al. 2011). In weevils, for example, an antimicrobial peptide selectively
 67 targets endosymbionts to regulate population growth within the host (Login et al.
 68 2011).
 69 Our mechanistic understanding of the interaction between symbionts and the
 70 host immune system is limited, particularly with regard to facultative, secondary
 71 symbionts, those that are not required for host survival (Hinde 1971; Schmitz et al.
 72 2012). Often less co-evolved with their hosts than primary, obligate symbionts,
 73 secondary symbionts may exhibit less adaptation towards lower virulence, and may be
 74 more likely to be detected by the host immune system. This may select for symbionts
 75 that avoid detection. One secondary symbiont in *Drosophila*, the *Spiroplasma* bacteria,
 76 for example, fails to elicit a host immune response despite freely circulating in the
 77 haemocoel; this is most likely due to it largely lacking a cell wall, and consequently
 78 the associated molecules that commonly trigger host recognition (Hurst et al., 2003).
 79 If detected however, hosts can respond to secondary symbiont presence in several
 80 ways. First, symbiont presence could trigger increased immune investment (Kambris
 81 et al. 2009; Rancès et al. 2012). This could beneficially act to prime the host immune
 82 system, making hosts less vulnerable to subsequent pathogen attacks (Wong et al.
 83 2011; Rancès et al. 2012). Conversely, stimulating the immune system in this manner
 84 could leave hosts more vulnerable to pathogen attack if the functional capacity of
 85 their immune system is consequently exhausted, as has been shown in weevils
 86 (Vigneron et al. 2012). Second, if generic host immune responses triggered by
 87 secondary symbiont presence pose costs to, or limit the establishment and
 88 maintenance of, other, beneficial symbionts, there may be selection for a broadly
 89 reduced immune response in order to facilitate beneficial symbiont presence. While

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90 this strategy profits honest, beneficial symbionts (Haine 2008), it leaves hosts
91 vulnerable to cheating symbionts and pathogens.

92 To investigate how the immune system is altered by presence of secondary
93 symbionts, we used the pea aphid, *Acyrtosiphon pisum*, as a model system. The pea
94 aphid has greatly reduced or altered immune responses compared to other
95 invertebrates studied to date (Altincicek et al. 2008; Gerardo et al. 2010; Laughton et
96 al. 2011). One hypothesis for the loss of immune function is that it facilitates
97 colonisation by symbionts, especially secondary symbionts (Douglas et al. 2010).
98 Aphids maintain a primary symbiont, *Buchnera aphidicola*, that provides them with
99 essential amino acids otherwise lacking in their diet (Douglas 1998). *Buchnera* is
100 vertically transmitted and housed in specialist host cells (bacteriocytes) that may offer
101 protection to the symbiont from extracellular host immune responses. Aphids can also
102 host several different secondary symbionts. Initially acquired via horizontal
103 transmission (Oliver et al. 2010; Henry et al. 2013), these symbionts are transmitted
104 with high fidelity from parent to offspring once established, but can be lost under
105 certain ecological conditions (Russell & Moran 2005; Oliver et al. 2008). Aphid
106 secondary symbionts can occupy a range of host tissues, but are also found
107 extracellularly in the haemocoel, where they can come into contact with host immune
108 cells (haemocytes) (Hinde 1971; Fukatsu et al. 2000). The three secondary symbionts
109 used in this study (*Serratia symbiotica*, *Hamiltonella defensa* and *Regiella*
110 *insecticola*) are widely distributed in pea aphids (Ferrari et al., 2012), and exhibit the
111 same patterns of tissue tropism, being found in secondary bacteriocytes, sheath cells
112 and the haemocoel (Moran et al. 2005). However, these symbionts vary in terms of
113 genetic relatedness (Oliver et al., 2010; Burke & Moran 2011), virulence (Degnan et
114 al. 2009; Weldon et al. 2013; Laughton et al. 2014), and their ability to confer specific

phenotypes on their aphid hosts (e.g. resistance to pathogens and parasitoids, tolerance of heat stress (Oliver et al., 2010)), and as such were chosen to represent a variety of potential influences on host immunity.

Here we look at three components of the innate immune system (phenoloxidase activity, encapsulation, immune cell counts) towards three secondary symbionts, and investigate the role of host and symbiont genotype in specific responses. We expect, given their transient nature relative to primary symbionts and potential for interaction with the host immune system, that the presence of these secondary symbionts will stimulate aphid immune responses. The immune parameters are commonly used to establish immune capacity in insects (Schmid-Hempel, 2005) and were chosen based on previous studies on pea aphid immunity (Laughton et al., 2011; Schmitz et al., 2012). Phenoloxidase is an important enzyme used by the immune system for cuticle sclerotisation, clotting of external wounds and in the encapsulation process (Cerenius & Söderhäll 2004; Cerenius et al. 2008). Although aphids have low phenoloxidase titres, they are capable of inducing an encapsulation response (Laughton et al. 2011), the strength of which may be affected by secondary symbiont presence in the haemocoel. Aphid immune cells can phagocytose bacteria and thus their presence could also influence, and thus be influenced by, symbiosis (Laughton et al. 2011; Schmitz et al. 2012).

2. Materials and Methods

2.1 Aphids and bacterial cultures

The pea aphids (*Acyrtosiphon pisum*) used were clonal females produced parthenogenetically. All contained the primary symbiont, *Buchnera aphidicola*. Aphids were reared on *Vicia faba* (fava bean) at 20 °C and 16 hr light:8 hr dark cycle.

140 All assays used the 5A aphid genotype, maintained as four clonal lines differing only
141 in secondary symbiont profile. The no secondary symbiont line (5A0) was collected
142 in 1999 from Madison, WI. Three different secondary symbiont species were
143 introduced into 5A0 aphids in 2003, producing clonal lines that harbor single
144 infections of *Serratia symbiotica* (5AR), *Hamiltonella defensa* (5AT), or *Regiella*
145 *insecticola* (5AU) (Oliver et al. 2003). For Experiment 2 of the immune cell counts, it
146 was found that the original 5AT line had lost its *Hamiltonella*, so a replacement sister
147 line and its paired 5A0 no secondary symbiont line, both maintained at the University
148 of Georgia under identical rearing conditions, was used. The two 5A0/5AT lines
149 originated from the same mother stock and were identical in host genotype,
150 *Hamiltonella* strain and bacteriophage (see below, Oliver et al., 2009). The
151 replacement 5A0/5AT lines were allowed to establish for five generations before
152 being used for assays.

153 We tested a second aphid genotype (LSR1, collected near Ithaca, New York in
154 1998) in the encapsulation assay to further investigate the role of *Regiella* in the
155 immune response. The LSR1 and 5A aphid genotypes were separated into four
156 different clonal lines: a no secondary symbiont line, and three lines each harboring
157 one of three different strains of *Regiella*: 5.15, Ri, and U. Strain 5.15 was collected
158 from a peach-potato aphid, *Myzus persicae*, in 2003 at Burchus Marsh, Australia, and
159 has previously been shown to protect against parasitoid wasps (Vorburger et al. 2010;
160 Hansen et al. 2012). Strain Ri was found as a natural infection of the LSR1 aphids,
161 and was subsequently artificially cleared to produce the LSR1 no secondary symbiont
162 line (Douglas et al. 2006). Strain U was introduced 10 years previously into the 5A
163 line (see above). To produce a fully factored cross infection of the two aphid
164 genotypes with the three symbiont strains, U was introduced into the LSR1 aphids, Ri

165 into the 5A aphids, and 5.15 introduced into both host genotypes. Transfer of the
166 symbionts was carried out via injection of donor haemolymph at least one year prior
167 to the experiment (Hansen et al. 2012).

168 We used two further aphid genotypes (AS3 and ZA17) in the immune cell
169 count assay to explore the role of *Hamiltonella* in this immune response. Paired clonal
170 lines were used for each genotype, one a no secondary symbiont line and one
171 containing a strain of *Hamiltonella*. The *Hamiltonella* strains used all have an
172 association with a lysogenic bacteriophage APSE, which is instrumental in providing
173 the protective phenotype against parasitoid wasps commonly associated with aphids
174 harbouring the *Hamiltonella* symbiont (see Discussion for more detail, Oliver et al.
175 2009; Weldon et al. 2013). The ZA17 and 5AT *Hamiltonella* strains contained the
176 APSE-2 phage, and AS3 *Hamiltonella* had the APSE-3 phage (Oliver et al. 2009). In
177 this case, *Hamiltonella*-containing genotypes were collected from the wild in Utah
178 (AS3, collected 2007) and Pennsylvania (ZA17, collected 2010), and control lines
179 were created by selectively treating the aphids with antibiotics (Martinez et al. 2014)
180 at least 12 generations before experiments were performed to eliminate possible side
181 effects of the antibiotics. All *Hamiltonella*-containing genotypes were screened in
182 2013 to confirm the phage had been retained during the experimental periods. This
183 included screening historical samples of the original 5AT line, covering a time period
184 from 2008-2012. All samples tested positive for phage presence (Laughton,
185 unpublished data).

186 The phenoloxidase and immune cell count assays used a heat-killed Gram-
187 negative bacterium (NG5b, genus *Enterobacter*), originally isolated from natural
188 infections of stock aphids. When alive, the bacteria cause aphid mortality within
189 several days after oral acquisition or intra-haemocoelic injection (Gerardo et al. 2010;

Laughton, unpublished data); heat-killing bacteria prior to experiments maintains a signal of pathogen introduction without subsequent virulence (Barribeau et al. 2014), and has been shown to stimulate variation in immune responses in aphids and other insects (Laughton et al., 2011; Vogelweith et al., 2013). Bacterial cultures were made by incubating a single colony in 10 mL Luria broth (LB) overnight (36 °C with shaking). Cultures were diluted with LB to an $OD_{600} = 1$ ($\sim 7 \times 10^6$ cfu ml⁻¹) and autoclaved to kill the cells before use. Immune challenges were administered via intra-haemocoelic injection dorsolaterally into the abdomen using a minuten pin (0.15mm diameter) dipped into the heat-killed bacterial suspension. An additional wounding control group comprising of a stab with a sterile pin was excluded as previous results had shown no significant difference in immune cell counts between bacterial challenge and sterile wounding treatment groups (Laughton et al., 2011).

2.2 Phenoloxidase

Using the four clonal 5A lines, aphids were split into two treatment groups: 1) an unchallenged control, and 2) challenged with a heat-killed solution of Gram-negative bacteria (NG5b). Twenty-four hours post-challenge, haemolymph was collected using a crude filter centrifuge technique (Laughton et al. 2011). Briefly, approximately 20 aphids per sample were placed on a filter of cellulose wadding inserted into a 1.5 ml centrifuge tube, the tip of which had been cut off. This tube was placed within a second 1.5 ml centrifuge tube, and the whole apparatus centrifuged (8,000 rcf, 10 min, 4 °C), allowing pooled haemolymph to filter through and collect in the second tube below. The samples were immediately frozen to disrupt the haemocytes (-80 °C, n = 4-5 per line x treatment group). Before use, samples were defrosted on ice and diluted to a 1:10 concentration with phosphate buffered solution

(PBS, 150mM NaCl, 10mM Na₂HPO₄, pH 6.5). Phenoloxidase (PO) activity was assayed following Laughton et al. (2011), measuring its catalytic conversion of L-dopa (3,4-dihydroxy-L-phenylalanine, colourless) to dopachrome (red-brown colour) photometrically (Horowitz & Shen 1952; Barnes & Siva-Jothy 2000). An 8 µl haemolymph sample was added to 56 µl dH₂O and 8 µl PBS in the well of a chilled 96-well plate. L-dopa (8 µl, 4 mg ml⁻¹ in dH₂O) was added and the reaction was allowed to proceed at 30 °C in a spectrophotometer (VersaMax tunable microplate reader, Molecular Devices). Readings were taken every 15 seconds for 1 hour at 490 nm and analysed using Softmax Pro v 4.8 software. Enzyme activity (V_{max}) was measured as the maximum linear rate of substrate conversion.

2.3 Encapsulation

The encapsulation response was tested *in vivo* using Sephadex beads (40-125 µm) as a proxy for parasitoid wasp eggs, an approach that has been employed for other insects (Smilanich et al. 2009). Assays were carried out following Laughton et al. (2011). Red-dyed Sephadex beads were injected into 12-day old aphids above the right siphunculus and removed via dissection after an incubation period of 96 hours. Melanotic encapsulation, as seen in aphids (Laughton et al. 2011), occurs when melanin binds to and forms a capsule layer around the foreign body, effectively entrapping it. Melanin is a dark brown color, allowing the degree of encapsulation to be quantified by measuring the red value of each bead in Photoshop and calculating the percent encapsulation with the formula: $[1 - (\text{experimental red value} / \text{baseline red value})] * 100$. In Experiment 1, we tested the impact of different secondary symbiont species on the encapsulation response using the four clonal 5A lines (n = 4-8 aphids per line). One to twenty-three beads (mean = 7.5) were inserted per aphid. Following

the results of Experiment 1, in Experiment 2 we compared the encapsulation response in two aphid genotype backgrounds (5A and LSR1), comparing the no secondary symbiont lines with clonal lines containing single infections of each of three different *Regiella* strains (5.15, Ri and U). One to six beads (mean = 2.8) were inserted per aphid. Due to time constraints, samples were collected over four replicate experiments (final n = 14-20 aphids per line).

2.4 Immune cell counts

Aphid immune cells, previously identified haemocytes that were shown to have immune function (Laughton et al. 2011; 2014; Schmitz et al. 2012), were collected by haemolymph smear samples from leg bleeds and fixed and stained using a Diff-Quick stain set following Laughton et al. (2011). These samples potentially contained both granulocytes and plasmatocytes, as differentiation between these two cell types was not possible (Laughton et al., 2014). To test the effect of an immune challenge on immune cell counts, in Experiment 1, 11-day old aphids from the four 5A lines were split into two treatment groups: 1) an unchallenged control, and 2) challenged with heat-killed Gram-negative bacteria (NG5b). Haemolymph samples (0.125 µl) were collected 24 hours post-challenge (day 12) from individual aphids (n = 4-5 per line x treatment) and immediately fixed and stained.

Following the results of Experiment 1, in Experiment 2, the assay was repeated using clonal paired lines of three aphid genotypes (5A, AS3 and ZA17), each pair consisting of a no secondary symbiont line and a *Hamiltonella* infected line (the 5A line was in this case replaced with an identical sister line, see Section 2.1). Aphids were raised as before, but were not given an immune challenge. Haemolymph was collected from 12-day old aphids (n = 7-15 per line). The presence of *Hamiltonella* in

some lines appeared to increase haemolymph viscosity, making haemolymph collection more difficult. Therefore, the volume of haemolymph collected was reduced to 0.1 µl per sample, which was collected from the pooled leg bleeds of two aphids.

2.5 Statistical analysis

All data were analysed using the statistical package R v2.13 (R Core Team, 2013). Results from phenoloxidase and encapsulation assays were analysed using ANOVA, with data log transformed where required to conform to the standards of normality. For the encapsulation data, the number of beads per aphid, experimental replicate and host genetic background were included as cofactors. Immune cell count data were analysed using a generalised linear model (glm) with a quasipoisson distribution, again with host genetic background included as a cofactor. Minimal models were derived by sequentially removing terms followed by model comparisons using p-values from likelihood-ratio tests. Terms were retained if their removal significantly reduced the explanatory power of the model (Zuur et al., 2009). Non-significant terms are reported from less parsimonious models when relevant.

3. Results

3.1 Phenoloxidase

The presence of secondary symbionts did not have a significant effect on PO activity (Figure 1, ANOVA, $F_{3, 33} = 2.60$, $p = 0.068$, data log transformed) although there was a trend for elevated levels of PO in the *Hamiltonella* aphids compared to the other lines. There was no significant effect of immune challenge (Figure 1, ANOVA, $F_{1, 33} = 0.26$, $p = 0.614$, data log transformed) on PO activity in the aphids.

Figure 1

Effects of secondary symbiont species and immune challenge on phenoloxidase activity. Four clonal lines of the 5A aphid genotype differing in secondary symbiont infection (no secondary symbiont line vs. single infections with *Serratia*, *Hamiltonella* and *Regiella* symbionts) were assayed for phenoloxidase activity 24 hours after receiving either an immune challenge with heat-killed Gram-negative bacteria, or no immune challenge (control) (error bars ± 1 s.e.m).

3.2 Encapsulation

Experiment 1. When comparing across a single genotype of aphid with alternative symbiont species, there was a significant effect of symbiont line on encapsulation response (ANOVA, $F_{3,18} = 3.81$, $p = 0.028$), with the *Regiella* infected aphids exhibiting a three- to four-fold stronger encapsulation response than either the

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303 no secondary symbiont line or the other secondary symbiont (Figure 2A). There was
304 no significant effect of the number of beads per aphid (ANOVA, $F_{1,18} = 1.84$, $p =$
305 0.191) on the encapsulation response.

306 **Experiment 2.** When assessing encapsulation in aphids of two host genotypes
307 with alternative *Regiella* symbiont strains, the presence of a *Regiella* symbiont
308 significantly increased the encapsulation response (Figure 2B, ANOVA, $F_{1,139} = 5.20$,
309 $p = 0.024$). There was no significant difference in the magnitude of this effect due to
310 the different *Regiella* strains (ANOVA, $F_{2,102} = 2.20$, $p = 0.116$). There was also no
311 significant interaction between host genotype and symbiont background (ANOVA
312 likelihood-ratio test, $F_{3,125} = 1.10$, $p = 0.349$, see Supplementary Material Table S1 for
313 full model output), and no significant effect of the number of beads per aphid
314 (ANOVA, $F_{5,125} = 0.96$, $p = 0.442$), experimental replicate (ANOVA, $F_{3,125} = 1.80$, $p =$
315 0.151) or host genetic background (ANOVA, $F_{1,125} = 0.24$, $p = 0.628$) on
316 encapsulation response.

Figure 2

Effect of secondary symbionts on encapsulation response. (A) Degree of encapsulation in the 5A aphid clonal lines, comparing the no secondary symbiont line with lines containing single infections of *Serratia*, *Hamiltonella* and *Regiella* symbionts. (B) Degree of encapsulation in two aphid genotypes (5A and LSR1), each

split into four clonal lines: no secondary symbiont (control) lines and three lines each containing a single infection with one of three *Regiella* symbiont strains (error bars ± 1 s.e.m).

3.3 Immune cell counts

Experiment 1. When comparing across aphids of the same genotype with alternative symbiont species, there was a highly significant effect of secondary symbiont species on immune cell counts (Figure 3A, glm, quasipoisson, dispersion parameter = 12.98, $F_{3,32} = 50.60$, $p < 0.0001$), with the presence of *Hamiltonella* resulting in a five- (vs. 5A0, 5AU) to thirteen-fold (vs. 5AR) elevation in immune cell numbers compared to the other lines for the control treatment (magnitude of difference decreased slightly when comparing immune-challenged treatment groups). There was a marginally significant effect of immune challenge on immune cell counts (Figure 3A, glm, quasipoisson, $F_{1,31} = 4.15$, $p = 0.050$), with a trend for elevated immune cell counts in immune challenged individuals in all but the no secondary symbiont line.

Experiment 2. When comparing the effects of *Hamiltonella* in different host and symbiont genotype combinations, the presence of *Hamiltonella* symbiont (regardless of strain) did not have a significant effect on the immune cell counts (Figure 3B, glm, quasipoisson, dispersion parameter = 5.30, $F_{1,74} = 3.19$, $p = 0.078$). Host genetic background did not significantly effect the haemocyte count in its own right (glm, quasipoisson, $F_{2,72} = 0.14$, $p = 0.866$), although when the full model was initially considered, the difference in responses between the 5A and ZA17 lines indicated that significant interactions between host and symbiont may be possible

(glm, quasipoisson, likelihood-ratio test, $F_{2,70} = 3.03$, $p = 0.055$, see Supplementary Material Table S2 for full model output).

Figures 3A and 3B highlight a large difference in both the absolute number and magnitude of the response to *Hamiltonella* in the 5AT aphid line between the two experiments (Experiment 1 showed a five-fold increase in immune cell counts due to *Hamiltonella* presence, compared to the 5A0 no secondary symbiont line; Experiment 2 recorded only a two-fold difference between these lines). There was a period of three years between Experiments 1 and 2. During this time, our initial 5AT line was found to have lost its *Hamiltonella*, despite being maintained in a constant environment (Laughton et al. 2014). Such a reduction has been previously seen in other *Hamiltonella*-containing aphid lines (Oliver, pers. comm.). Consequently, we obtained a replacement sister 5AT and paired 5A0 line, identical in terms of aphid genotype, *Hamiltonella* strain, and bacteriophage. qPCR analysis of the replacement 5AT line showed it to have a significantly higher *Hamiltonella* density than the line used in Experiment 1, but fewer immune cells in circulation. Interestingly, the replacement paired 5A0 no secondary symbiont line used in Experiment 2 also had immune cell counts four times lower than the initial 5A0 line used in Experiment 1, suggesting an intrinsic change to the baseline immune cell titres preceding any impact of the secondary symbiont.

Figure 3

Effect of secondary symbionts and immune challenge on immune cell counts. (A)

Immune cell counts in the 5A aphid clonal lines, comparing the no secondary symbiont line with lines containing single infections of *Serratia*, *Hamiltonella* and *Regiella* symbionts 24 hours after receiving either an immune challenge with heat-

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372 killed Gram-negative bacteria, or no immune challenge (control). (B) Immune cell
373 counts in three aphid genotypes (5A, AS3 and ZA17), each split into paired clonal
374 lines comprising a no secondary symbiont line and a *Hamiltonella* infected line. The
375 ZA17 and 5AT *Hamiltonella* strains contained the APSE-2 phage, and AS3
376 *Hamiltonella* had the APSE-3 phage (error bars ± 1 s.e.m).

377

378 4. Discussion

379 Secondary symbionts are capable of modifying some pea aphid immune
380 responses, but the magnitude and direction of the change is dependent on symbiont
381 species.
382 Active PO levels have previously been found to be low in aphids without secondary
383 symbionts (Laughton et al. 2011). Our results further support this finding, with neither
384 bacterial pathogen nor secondary symbiont presence initiating a change in host PO
385 activity, despite the presence of MAMPs on these microorganisms (Burke & Moran,
386 2011), which commonly activate the PO pathway in other insects (Cerenius &
387 Söderhäll 2004). However, PO is required for the production of melanin, seen in the
388 encapsulation response, and its presence in aphid immune cells has been previously
389 reported (Laughton et al. 2011; Schmitz et al. 2012). PO is cytotoxic and must be
390 carefully regulated to minimise autoimmune effects (Cerenius & Söderhäll 2004). If
391 MAMP recognition were to trigger PO activation in aphids, then the constant
392 presence of secondary symbionts in the haemocoel could have significant autotoxic
393 consequences for the host, with active PO being sustained in the absence of pathogens,
394 potentially also damaging the primary symbiont population. As such, aphids may
395 have reduced or altered PO activation sensitivity in order to prevent self-harm.

396 Secondary symbiont presence altered both encapsulation responses and
 397 immune cell counts, although the assays were affected differently by specific
 398 secondary symbiont species. When the host background was kept constant (i.e. in the
 399 5A aphids), encapsulation responses were only ever increased by *Regiella* presence,
 400 and immune cell counts were only ever increased by *Hamiltonella* presence.
 401 Variation in the virulence of different secondary symbiont species may cause
 402 differential activation of immune pathways associated with specific immune
 403 responses (Nakamura et al. 2011; Pinto et al. 2012), with hosts potentially relying on
 404 immune activity to maintain control of their symbiont populations (Laughton et al.
 405 2014). Aphid immune cells have known phagocytic properties, and are seen to contain
 406 secondary symbionts, suggesting a role in symbiont control (Laughton et al. 2011;
 407 Schmitz et al. 2012). *Hamiltonella* in the 5A aphid line has previously been shown to
 408 have high virulence, with a fast rate of replication (Weldon et al. 2013; Laughton et al.
 409 2014), and, despite the symbiotic lifestyle resulting in reduction of the *Hamiltonella*
 410 genome, *Hamiltonella* has retained abundant putative pathogenicity loci in comparison
 411 to the primary *Buchnera* symbiont (Degnan et al. 2009). As such, phagocytic immune
 412 cells may be recruited to help regulate *Hamiltonella* populations in the aphid.
 413 Conversely, *Regiella* in the 5A aphid line has been found to have lower virulence,
 414 including a lower rate of replication, than other secondary symbionts (Laughton et al.
 415 2014) and may not stimulate host immune cell activation to the same extent, allowing
 416 host resources to be focused on defence, such as the encapsulation response.
 417 Interestingly, we saw no evidence of an increased encapsulation response or immune
 418 cell abundance in the 5A aphids with *Serratia*, despite previous work indicating a cost
 419 of infection on aphid fitness (Laughton et al. 2014). However, this result is consistent
 420 with previous transcriptomic comparisons of aphids with and without *Serratia*, in

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421 which aphids with *Serratia* show no increased activation of known immune-related
422 genes (Burke & Moran 2011). *Serratia* is a more recently derived symbiont than
423 *Hamiltonella* or *Regiella* (Burke & Moran 2011), and, with more of its genome intact,
424 may have retained mechanisms commonly employed by pathogens for evading host
425 immune systems (Ratzka et al. 2012; Pel & Pieterse 2013), a particularly likely
426 scenario given that many *Serratia* spp. are pathogenic (Petersen & Tisa 2013).
427 Infection in the 5A line in fact resulted in a lower immune cell count than the no
428 secondary symbiont line, suggesting that *Serratia* may be able to suppress the host
429 immune response (Fytrou et al. 2006). However, further work with alternative host
430 and symbiont genotypes is needed to explore this hypothesis.

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431 Additional immune challenge by a pathogen signal was found to significantly
432 increase immune cell counts in aphids that also harboured secondary symbionts,
433 although the extent of this increase varied depending on symbiont species (Fig. 3A).
434 One possible explanation is that aphids require both a MAMP signal and recognition
435 of mechanical wounding to trigger this immune response (Matzinger, 2012). However,
436 both of these factors were also present in the immune-challenged secondary-symbiont
437 free line, which showed no such cellular increase. Requiring that injected bacteria be
438 alive to trigger the response is also unlikely, as immune gene upregulation in the
439 weevil *Sitophilus zeamais* found similar responses in hosts that were injected with live
440 or dead bacteria (Anselme et al. 2008). However, the weevils used in this study were
441 aposymbiotic; our results could potentially be an example of an established secondary
442 symbiont infection acting to prime the host immune system against subsequent attack.
443 Further experiments incorporating a sterile stab control group, and recording response
444 to a live pathogen would help to identify the key factors responsible for the immune
445 activation seen here.

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446 Our results exhibited significant variation in the immune cell count responses
447 based on the strain of symbiont within a symbiont species. Such genotypic variation is
448 increasingly found to be important in shaping host-symbiont interactions with regards
449 to the benefits that secondary symbionts can confer to their hosts (e.g. Ferrari et al.
450 2007; Castañeda et al. 2010; Vorburger et al. 2010; Oliver et al. 2014). Both *Regiella*
451 and *Hamiltonella* can protect aphids from parasitoid wasp attack, but ability varies
452 based on symbiont strain and host aphid species (Oliver et al. 2003; Vorburger et al.
453 2010; Hansen et al. 2012). In *Regiella*, protection has been linked to differences in the
454 expression of pathogenicity-related factors between symbiont strains (Hansen et al.
455 2012), whereas protective ability from *Hamiltonella* relies on an association of the
456 symbiont with a lysogenic bacteriophage APSE (Oliver et al. 2009; Weldon et al.
457 2013), meaning that variation in aphid host phenotype may depend not only on the
458 strain of *Hamiltonella* it harbors, but also that of the phage housed within. Such
459 differences could have wide-reaching consequences for symbiont and host phenotypes,
460 including potential effects on host immune responses. The *Regiella* and *Hamiltonella*
461 strains used here in the encapsulation and immune cell count assays all contained such
462 variations (e.g. the 5.15 and Ri *Regiella* strains, described by Hansen et al. (2012),
463 vary in pathogenicity-factors, and the *Hamiltonella* strains contained two different
464 APSE bacteriophages; see Section 2.1). The extent of the differences seen in the
465 immune cell counts is therefore perhaps not surprising considering the potential for
466 genetic diversity between strains (Schmitz et al. 2012).

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467 Despite work highlighting the importance of host genotype in the phenotypic
468 outcomes of host-symbiont interactions (Ferrari et al. 2007), here it had little
469 quantifiable effect on variation in immune responses. It is important to note, however,
470 that potentially interesting interactions between host genotype and symbiont genotype

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471 were suggested in the immune cell response. However, as paired clonal lines differed
472 from each other in both host and symbiont strain genotype, it was not possible to
473 uncouple the relative contribution of each partner to the interaction. Mode of infection
474 i.e. whether the secondary symbiont is naturally occurring or artificially introduced,
475 may have a bearing on the responses of aphid genotypes, as could aphid biotype. Our
476 secondary symbionts were collected from several different aphid biotypes (see
477 Methods), but we saw no obvious effects of this in our work. Although Ferrari et al.
478 (2007) also used different biotypes, the aphid clonal lines used were all naturally
479 secondary symbiont-free, a rarer state (Frantz et al., 2009; Ferrari et al., 2012) which
480 may be linked to the variation seen in host responses. Further work increasing the
481 range and combinations of symbiont and host genotypes tested would help to clarify
482 this point and may uncover additional host genotype by symbiont genotype
483 interactions.

484 The impact of secondary symbionts on hosts may also be closely linked to
485 changes in population dynamics over time. While symbionts may be lost from a host
486 if too costly (Oliver et al. 2008), little work has looked at the effect of symbiont
487 density on host phenotype. Within the immune cell counts, we found a significant
488 change in the absolute number and magnitude of the response from the seemingly
489 identical *Hamiltonella*-containing aphid line (5AT) used in Experiments 1 and 2
490 (Figure 3). It should be noted however, that the presence of *Hamiltonella* still
491 produced qualitatively the same host response (i.e. an increase in cell counts), albeit at
492 a reduced level. Natural fluctuations in secondary symbiont population densities could
493 therefore have previously overlooked importance for host vigor and success under
494 varying ecological conditions.

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495 This study highlights the importance of secondary symbionts in shaping host
496 immunity. Our findings indicate that while variation in the magnitude of effect is
497 common, some secondary symbiont species reliably stimulate similar host responses.
498 Further work is needed to identify the mechanisms involved in these triggers and
499 tradeoffs, with previous research hinting at a complex interaction of factors including
500 symbiont replication rate and virulence effects (Oliver et al. 2009; Hansen et al. 2012;
501 Laughton et al. 2014). Understanding these interactions, and the magnitude of genetic
502 variation attributed to secondary symbionts, has important consequences for host
503 health, and symbiont and pathogen transmission dynamics.

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