 Costs and benefits of a superinfection of facultative symbionts in aphids

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Symbiotic associations between animals and inherited micro-organisms are widespread in nature. In many cases, hosts may be superinfected with multiple inherited symbionts. *Acrithosiphon pisum* (the pea aphid) may harbour more than one facultative symbiont (called secondary symbionts) in addition to the obligate primary symbiont, *Buchnera aphidicola*. Previously we demonstrated that, in a controlled genetic background, *A. pisum* infected with either *Serratia symbiotica* or *Hamiltonella defensa* (called R- and T-type in that study) were more resistant to attack by the parasitoid *Aphidius ervi*. Here, we examined the consequences of *A. pisum* superinfected with both resistance-conferring symbionts. We found that an *A. pisum* line co-infected with both *S. symbiotica* and *H. defensa* symbionts exhibits even greater resistance to parasitism by *A. ervi* than either of the singly infected lines. Despite this added benefit to resistance, superinfections of *S. symbiotica* and *H. defensa* symbionts appeared rare in our survey of Utah *A. pisum* symbionts, which is probably attributable to severe fecundity costs. Quantitative polymerase chain reaction estimates indicate that while the density of *H. defensa* is similar in singly and superinfected hosts, *S. symbiotica* densities increased dramatically in superinfected hosts. Over-proliferation of symbionts or antagonistic interactions between symbionts may be harmful to the aphid host. Our results indicate that in addition to host–symbiont interactions, interactions among the symbionts themselves probably play a critical role in determining the distributions of symbionts in natural populations.

**Keywords:** endosymbiont; *Wolbachia*; multiple infection; parasitoid; proteobacteria

1. INTRODUCTION

Symbiotic associations between animals and inherited micro-organisms are widespread in nature (e.g. Buchner 1965; Douglas 1989; Werren & Windsor 2000; Terry et al. 2004). For example, molecular diagnostic surveys have revealed that just two bacterial symbionts, *Wolbachia* and *Cardinium*, are found in nearly 30% of certain arthropod taxa (Werren & Windsor 2000; Weeks et al. 2003; Zchori-Fein & Perlman 2004). It is also clear that many invertebrate hosts are superinfected, i.e. infected with multiple inherited symbionts (e.g. Buchner 1965; Unterman et al. 1989; Fukatsu & Nikoh 2000; Subandiyah et al. 2000; Dubilier et al. 2001; Sandström et al. 2001; von Dohlen et al. 2001; Zchori-Fein & Brown 2002; Distel et al. 2002; Weeks et al. 2003).

In general, we know little about interactions among symbionts within the superinfected hosts. Most research in this area seems to have focused on symbionts that cause cytoplasmic incompatibility (CI). In CI systems, symbionts in males depress the fitness of females of a different infection status by engineering the failure of fertilization, in this way increasing the relative fitness of females with the same infection status. In the case of symbionts causing CI, superinfections are generally predicted to invade in populations of single infections (Frank 1998). Outside of CI systems, however, the interactions of multiple symbionts in producing a host phenotype have scarcely been explored. Among the inherited symbionts that invade by providing benefits to hosts, such as nutritional supplementation (see Douglas (1998) for a review), coexistence of multiple lineages is likely to be maintained when hosts receive a net benefit that exceeds any extra costs of superinfection. Regardless of symbiont type, several factors should limit the diversity of symbionts in a single host, including competition among symbionts, increased virulence and bottlenecks experienced by symbionts during vertical transmission (Mira & Moran 2002). On the other hand, there are mechanisms that may allow for the coexistence of multiple symbionts, e.g. selective tissue tropism, and differential growth dynamics (Ijichi et al. 2002) or complementarity with regard to the host needs.

The pea aphid (*Acrithosiphon pisum*) harbours several inherited secondary symbionts (SS) in addition to *Buchnera aphidicola*, the obligate primary symbiont. While *Buchnera* is necessary for successful aphid reproduction (Prosser & Douglas 1991), the role of these SS in *A. pisum* has only just begun to be explored. Already, we can attribute a diverse array of interesting phenotypes to these symbionts. For example, *Regiella insecticola* (called U-type in that study) has recently been implicated in host-plant specialization in Japanese *A. pisum* (Tsuchida et al. 2004), and *Serratia symbiotica* (called PASS in that study) has been implicated in thermal tolerance in North American *A. pisum* (Montllor et al. 2002). Further, and most relevant to the current study, isolates of *S. symbiotica* and *Hamiltonella defensa* have been shown to confer partial resistance to an important natural enemy, the parasitoid...
Aphidius ervi, by causing mortality to the developing wasp larvae (Oliver et al. 2003). The pea aphid symbiont system may be manipulated in such a way that the effect of symbiont infections may be compared in a common host genetic background. Here, we compared clonal lineages of *A. pisum* that were singly and doubly infected with *S. symbiotica* and *H. defensa*. We studied the interactions of the two symbionts in producing the parasitoid-resistance phenotype. We focused primarily on their effects on host fitness in the presence and absence of parasitoids, in an attempt to describe the costs and benefits of multiple infections under varying conditions. We also estimated the densities of primary and secondary symbionts in singly and doubly infected aphids in order to examine the interactions among symbionts. Finally, we screened over 100 field-collected *A. pisum* individuals for SS, estimating the frequencies of single and double infections. Interestingly, we note that our experimental findings can be used to explain the patterns of single and double infections in the field. Thus, we have identified a force that probably shapes the diversity of symbiotic flora harboured by single aphids.

2. MATERIAL AND METHODS

(a) *Acrystosiphon pisum symbionts*

In addition to the obligate association with *Buchnera*, *A. pisum* (Insecta: Hemiptera: Aphididae) harbour five types of SS at intermediate frequencies: three distinct gammaproteobacterial lineages, *S. symbiotica*, *H. defensa* and *R. insecticola*, previously given the provisional labels R-type (or PASS), T-type (or PABS) and U-type (or PAUS), respectively (Unterman et al. 1989; Chen et al. 1996; Fukatsu et al. 2000; Darby et al. 2001; Sandström et al. 2001; Moran et al. 2005b), a *Richettsia* (Alphaproteobacteria; Chen et al. 1996) and a *Spiroplasma* (Mollicutes; Fukatsu et al. 2001). In this study, we examine just two of these symbionts, *S. symbiotica* and *H. defensa*, which are occasionally found in the same *A. pisum* individuals in field populations. Here, we use superinfection to refer to an aphid lineage infected with two secondary symbionts: *H. defensa* and *S. symbiotica* (i.e. not to one SS plus *Buchnera*).

(b) *Study organisms*

*Acrystosiphon pisum*, a polyphagous pest of herbaceous legumes (Eastop 1966), was accidentally introduced to North America from Europe around 1870 (Mackauer 1968). This aphid is cyclically parthenogenetic, allowing clonal lineages to be maintained indefinitely in the laboratory. Each clonal lineage of *A. pisum* used in this experiment consisted of descendants of a single parthenogenetic female kept in cages in a walk-in growth chamber (see Oliver et al. (2003) for aphid and wasp collection information). All aphid clones were maintained on *Vicia faba* (lava bean) at 20 ± 1 °C, and 16L: 8D.

*Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) was introduced to North America in efforts to control aphid populations from Europe, and is an important natural enemy of *A. pisum* in North America (Angalet & Fuester 1977). This wasp is a solitary endoparasitoid. The adult female lays an egg inside an aphid nymph. The wasp larva then develops within the living aphid, eventually killing the host. When the host viscera have been entirely consumed, the aphid cuticle transforms into a characteristic ‘mummy’, usually about 8 days after oviposition. The wasp pupates within the mummy, and a free-living adult *A. ervi* emerges. The wasp culture was maintained in the laboratory at 20 ± 1 °C and 16L: 8D, on an *A. pisum* clone found to be free of secondary symbionts.

(c) Establishing experimental lineages

We used a micro-injection technique, modified from Chen & Purcell (1997), to experimentally manipulate SS infection status allowing us to study the effects of particular SS in a common genetic background (see Oliver et al. (2003) for details). In this study, we used the singly infected *S. symbiotica* and *H. defensa* lines established in Oliver et al. (2003), and created a new lineage co-infected with the same isolates of *S. symbiotica* and *H. defensa*. To verify that all experimental lineages were of the same nuclear genotype, we performed a diagnostic fingerprinting technique (intersequence simple repeats or ISSR; Abbot 2001; Sandström et al. 2001). We also regularly screened the experimental lineages with diagnostic polymerase chain reaction (PCR) to verify SS composition (Sandström et al. 2001). The diagnostic PCR primers used for *H. defensa* were T1279F and 35R and the *S. symbiotica* diagnostic primers were R1279F and 35R (Russell et al. 2003; Russell & Moran 2006). Diagnostic PCR was conducted at 10 μl volumes using a standard reaction mix (Moran et al. 1999) and PCR conditions as in Sandström et al. (2001).

(d) The resistance phenotype in superinfected aphids

Previously, we reported that successful parasitism was reduced by 42% in an *A. pisum* line harbouring *H. defensa* and 23% in a line infected with *S. symbiotica* compared to an uninfected control line of the same aphid genotype (Oliver et al. 2003). Given the benefit conferred by each SS in singly infected hosts, we were interested in the resistance phenotype of this same *A. pisum* genotype simultaneously infected with both resistance-conferring SS. Here, we compared the rates of successful parasitism of each of the three artificially inoculated lineages (S. symbiotica-only, H. defensa-only and superinfection with both SS) of *A. pisum* against an uninfected control lineage. Thirty second instar *A. pisum* were placed on a potted *V. faba* plant in a cup cage 20–24 h prior to wasp introduction. Just prior to the experiment, wasps were given an oviposition experience by exposing them to uninfected aphids. Females with an oviposition experience were then individually assigned at random to the control or one of the experimental lineages. We removed wasps from arenas after 6 h. Arenas were incubated at 20 ± 1 °C and 16L: 8D (see Oliver et al. (2003) for details). We examined the arenas after 10 days and counted the numbers of mummies and surviving aphids to determine susceptibility to parasitism. Trials were conducted in two blocks. Differences between blocks were not significant and data are pooled. We analysed the proportion of successfully parasitized aphids in a logistic regression framework. All statistical analyses were performed using JMP-IN 4.0 software.

We conducted parasitism assays eight months (approximately 20 generations) after the artificial inoculation procedure to minimize any potential effects resulting from the procedure. Koga et al. (2003) reported detrimental effects on aphids after artificial infection with *S. symbiotica*, but these effects had attenuated by eight months post-infection.
(e) **Direct fitness benefits to resistance-conferring infections**

We previously reported that the timing of resistance in *S. symbiotica*-infected aphids occurs at 4–5 days after wasp oviposition (Oliver et al. 2003). We have subsequently conducted serial dissections to follow the course of developing *A. ervi* in *A. pism* infected with *H. defensa*, and found a similar late expression of resistance (K. M. Oliver 2004 unpublished data). Given this late timing of resistance, which corresponds to a relatively large parasitoid in the aphid haemocoel, we wondered if there could be direct benefits to infection with resistance-conferring SS. To determine if parasitized aphids with resistance-conferring SS produce more offspring than parasitized uninfected controls, we performed fecundity assays of singly parasitized aphids in each of our three artificially inoculated lineages and an uninfected control. This experiment was first performed with the two singly infected lineages (*S. symbiotica* and *H. defensa*) compared with an uninfected control. A second experiment was conducted using the same protocol, adding only the superinfected lineage. In both trials, individual third instar aphids were singly parasitized by *A. ervi* in a Petri dish and then placed in groups of four aphids onto a single *V. faba* plant in a cup cage (see above) and incubated at 20 ± 1 °C and 16L : 8D. The cup cages were examined at day 6 (after parasitism) and then every 4 days. The total number of offspring and the number of surviving parasitized adults from each container were recorded at each time period. Offspring were removed at each time point to avoid confusion with later born progeny. Results for single infections and uninfected controls did not differ significantly between trials and data are pooled. In addition to depositing an egg, female *A. ervi* also injects venom that degenerates aphid ovaries, resulting in host castration (Digilio et al. 2000). Our frequent observations of females producing no progeny may have been caused by host castration. The zeros in the data caused by these females resulted in non-normal fecundity distributions. We therefore use non-parametric Wilcoxon rank sum tests to analyse these data. These fecundity trials were conducted 20 months (approximately 50 generations) after the creation of the superinfected line.

(f) **Fitness costs to superinfected aphids**

Aphids infected with more than one symbiont may suffer costs associated with increased densities or due to the competitive interactions among symbionts. Casual observations in the laboratory (K. M. Oliver) suggested that *A. pism* doubly infected with *S. symbiotica* and *H. defensa* performed poorly relative to uninfected and singly infected aphids. To address the severity of this cost, we performed fecundity assays of unparasitized superinfected and uninfected (no SS) *A. pism* to quantify fecundity costs. For each replicate, four fourth instar aphids were placed on a single *V. faba* plant in a cup cage and incubated at 20 ± 1 °C and 16L : 8D. Offspring were counted every 4 days after the onset of reproduction. We also examined generation time, defined here as the time from birth to adulthood (time to first reproduction) and we measured fresh weights at adulthood of these two lines. Newborn nymphs were removed within 2 h of birth and placed on *V. faba* plants in cohorts of approximately ten aphids and maintained at 20 ± 1 °C and 16L : 8D. Eight days later, we began monitoring cages every 4 h and removed and immediately weighed reproducing aphids. These fecundity trials were conducted at 20 and 35 months after the creation of superinfected line.

(g) **Survey of *A. pism* SS collected from alfalfa in Northern Utah**

We surveyed *A. pism* SS collected from alfalfa near Logan, Utah (USA) in August 2003, using diagnostic PCR. Approximately 40 aphids were collected, at distances of 60 m apart, from each of three alfalfa fields. The three alfalfa fields were all within 5 km of one another. Aphids were collected and stored in 100% ethanol until DNA extraction (Engels et al. 1990). There were no differences among fields with regard to SS frequencies and the data shown are pooled. All the clones were screened for known *A. pism* SS (*S. symbiotica*, *H. defensa*, *R. insecticola*, *Rickettsia* and *Spiroplasma*). Each aphid was also screened for *Buchnera* as a positive control to ensure that DNA extractions were successful. Clones testing negative for all known SS were screened with universal primers designed to amplify bacteria other than *Buchnera* (Sandström et al. 2001). Thirty clones, chosen haphazardly, were screened for the presence of *Wobachia* (O’Neill et al. 1992), *Cardinium* (Zchori-Fein & Perlman 2004) and *Arsenophonus*, a symbiont reported from aphids and other insects (e.g. Russell et al. 2003). Diagnostic PCR primers were as follows: *R. insecticola*, U1279F and 35R; *Arsenophonus*, ARS1015F and 35R (Russell & Moran 2005); *Spiroplasma*, TKSSSp and 10F (Fukatsu et al. 2001; Russell et al.; 2003); and *Rickettsia* (PAR4F GGCTCAGAAC GACCGCTATC and PAR1213R CACCCTTTGGCTT CCTCTCTG). The reaction conditions for *R. insecticola*, *Rickettsia* and *Arsenophonus* consisted of a cycle of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min, repeated 35 times and then 72 °C for 6 min. Reaction conditions for *Spiroplasma* consisted of a cycle of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min, repeated 35 times and then 72 °C for 6 min.

(h) **Densities of *S. symbiotica* and *H. defensa* symbionts in singly versus doubly infected aphids**

To determine if SS numbers are regulated independently in superinfected aphids, we performed real-time quantitative PCR (QPCR) using a Lightcycler (Roche Molecular Biochemicals, Indianapolis IN, USA) to estimate the number of bacterial chromosomes in aphids singly and doubly infected with *S. symbiotica* and *H. defensa* SS. Aphids at age 72 ± 4 h were stored at −80 °C until DNA extraction from single individuals (Engels et al. 1990). We used primers designed to amplify a 244 bp fragment of the putative single copy *gyrB* gene from *S. symbiotica* (*gyrBR*-type-402R CGGTGAACAGCTACATGGAA and *gyrBR*-type-158F GCCGACCAATAATTTAGCAT) and a 201 bp fragment of *gyrB* gene from *H. defensa* (*gyrBT*-type-484F TTTCCT GAAATCCATCGTTCC and *gyrBT*-type-685R CAAACG CAACGATCAAGAAA). Neither of these primer combinations amplifies DNA from *Buchnera*. As a negative control, we performed QPCR with the *S. symbiotica* *gyrBR* primers on aphids infected with only *H. defensa* to ensure that primers were SS-specific (and vice versa). Another negative control consisting of the reaction mixture and water instead of DNA accompanied each Lightcycler run. The 20 µl QPCR reactions contained 0.5 µM of each primer, 1×SYBR Green MasterMix (Roche) and 10 pM of template DNA. Touchdown QPCR Reaction conditions were as follows: one cycle of 93 °C for 10 min, followed by 40 cycles of 94 °C for 5 s, 68 °C for 15 s (−1 °C every three cycles down to 55 °C)
and 72 °C for 5 s. At the end of each run, a melting curve analysis was performed, which allowed us to confirm the identity and specificity of amplified products. Separate standard curves for S. symbiotica and H. defensa gyrB were generated using two independent serial dilutions (each) of a TOPO TA plasmid vector (Invitrogen, Carlsbad, CA, USA), one containing a single fragment of the S. symbiotica gyrB gene and the other containing a single fragment of the H. defensa gyrB gene. We calculated the relative DNA concentrations of each sample with aphid EF1-alpha primers (Oliver et al. 2003) and calibrated SS densities accordingly. The symbiont densities were estimated approximately 20 months after creation of the superinfected line.

Koga et al. (2003) reported that S. symbiotica suppressed Buchnera densities. Developing wasp larvae depend upon the production of nutrients by Buchnera (Falabella et al. 2000); therefore, the degree of Buchnera suppression may correlate with parasitoid survival with lower Buchnera levels resulting in poorer parasitoid performance. To determine if Buchnera densities are affected by infection status, we performed QPCR to compare relative densities of Buchnera in aphids singly infected with S. symbiotica and H. defensa and aphids superinfected with both. We used primers designed to amplify a 176 bp fragment of the HS70 (=dnaK) gene from Buchnera (HS70F2 ATGGGTTAAATTTGCTTATTG and HS70R2 ATAGCTTGACGTTTAGG). We calculated relative DNA concentrations using aphid EF1-alpha primers to calibrate relative densities.

3. RESULTS

(a) What is the resistance phenotype of aphids superinfected with S. symbiotica and H. defensa? Acyrthosiphon pisum superinfected with both S. symbiotica and H. defensa are more resistant to parasitism than those harbouring either S. symbiotica or H. defensa (figure 1). The reduction in successful parasitism ranged from 29% for the S. symbiotica-only line, 52% for the H. defensa-only line and 61% for the superinfected line. All the treatments are significantly different from one another. The regression equation comparing successful parasitism in the singly and superinfected lines with the uninfected control is $Y = -0.007 + 0.345S_{sym} + 0.74H_{def} + 0.95$ Super (S. symbiotica-line $X^2 = 12.7, p = 0.0004$, H. defensa-line $X^2 = 63.7, p < 0.0001$, Superinfected-line $X^2 = 103.2, p = 0.0004$).

We found that levels of mortality were similar (roughly 15%) between the parasitized uninfected and singly infected lines (ANOVA, $F_{2,45} = 1.38, p = 0.26$). Mortality, however, was much higher in the parasitized superinfected line (30%) compared to the uninfected and singly infected lines. Despite the increased mortality in parasitized superinfected aphids, this line produced an average of nearly three times as many surviving aphids as the uninfected control line (ANOVA, $F_{1,34} = 8.6, p = 0.006$). This indicates that superinfection results in not just fewer mummies, but also fewer aphids surviving attack. Fewer mummies could lead to an indirect benefit of infection if fewer parasitoids emerge to attack clone-mates, but more aphids surviving parasitoid attack raises the possibility of a direct benefit to infection.

(b) Are there direct fitness benefits to harbouring single or multiple resistance-conferring SS isolates?

Despite the late timing of resistance found in the H. defensa-only line of aphids, we found direct fitness benefits of infection—measured as cumulative fecundity—in the presence of parasitism. Third instar nymphs infected with H. defensa produced considerably more offspring, when parasitized compared to parasitized uninfected controls (0–18 days post-parasitism, Wilcoxon rank sum test, $p = 0.0003$; table 1). Aphids with S. symbiotica produced similar numbers of offspring, 0–18 days post-parasitism, to the uninfected lineage (Wilcoxon rank sum test, $p = 0.48$; table 1). We also found no direct fitness benefit associated with superinfected aphids despite increased survivorship of parasitized aphids. Doubly infected A. pisum produced comparable number of offspring relative to uninfected aphids in the presence of parasitism (Wilcoxon rank sum test, $p = 0.68$; table 1).

(c) Costs associated with double infections

We found severe fecundity costs associated with superinfection in the absence of parasitism. At 12 days post-reproductive onset, the mean number of offspring produced by cages of uninfected aphids was 210, compared to only 38 produced by superinfected aphids (ANOVA, $F_{1,26} = 120.7, p < 0.0001$; table 2). Mortality at 12 days post-reproductive onset was much higher in superinfected

Figure 1. Back-transformed logistical regression estimates of reductions in successful parasitism of Acyrthosiphon pisum by Aphidius ervi in singly and superinfected lines relative to the uninfected control line. Numbers above columns represent the total numbers of aphids counted as alive or parasitized (**p<0.001).
aphids (36/64 alive = 56%) compared to uninfected aphids (61/64 alive = 95%). Cumulative fecundity per surviving aphid at 12 days was also dramatically lower in superinfected aphids compared to uninfected aphids (ANOVA, \( F_{1,26} = 93.8, p < 0.0001 \); table 2).

We also found that generation time was longer in superinfected aphids compared to uninfected controls (Wilcoxon rank sum \( X^2 = 0.002 \); table 2). Fresh weights at adulthood were also significantly lower in superinfected aphids compared to uninfected controls. Superinfected aphids weighed 3.15 g at adulthood compared to 3.95 g for uninfected aphids (ANOVA, \( F_{1,85} = 50.2, p < 0.0001 \); table 2).

**Are there differences in SS density between singly and doubly infected aphids?**

The numbers of *H. defensa* gyrB gene copies per aphid were similar in singly and superinfected *A. pismum* (figure 2; ANOVA of log density per µl: \( F_{1,18} = 0.007, p = 0.93 \)). The average number of *H. defensa* gyrB gene copies in both the *H. defensa*-only line and superinfected lines was approximately 145 000 (\( n = 10 \)). The number of *S. symbiotica* gene copies per aphid, however, was 20-fold greater in superinfected aphids compared to singly infected aphids (figure 2; ANOVA of log density per µl: \( F_{1,21} = 66.5, p < 0.0001 \)). The number of gyrB gene copies in the *S. symbiotica*-only line averaged 9600 per aphid (\( n = 11 \)), but jumped to 211 000 copies in superinfected *A. pismum* (\( n = 12 \)). Qualitatively, it is interesting to note that the resistance rank (\( S. symbiotica < H. defensa < superinfection \)) corresponds broadly to total SS densities; increasing densities lead to increased resistance.

Relative *Buchnera* levels differed significantly among the two single infections and superinfection (ANOVA, \( F_{2,20} = 4.5, p = 0.03 \)). Levels were highest in the *H. defensa*-only infected line (relative value = 1.0), intermediate in the superinfected line (relative value = 0.95) and lowest in the *S. symbiotica*-only line (relative value = 0.78). Reduced *Buchnera* levels did not correlate with reduced parasitoid survival.

**Distributions of *A. pismum* SS in northern Utah**

Table 2 shows the prevalence of SS associated with 120 pea aphid clones collected from alfalfa in northern Utah. All genotypes tested positive for the aphid primary symbiont *Buchnera*. A sub-sample of 30 aphids was screened for *Wolbachia, Cardinium* and *Arsenophonus*; none were detected. In clones that tested negative for all described SS of *A. pismum*, we screened with universal bacterial primers that amplify bacteria other than *Buchnera*, but we did not detect any other bacteria. We found that *A. pismum* superinfected with both *S. symbiotica* and *H. defensa* were less prevalent in our survey than expected by chance (\( G^2 \) likelihood ratio test, \( p = 0.002 \); table 3). Other superinfections occurred within expected prevalence ranges: PAR + *S. symbiotica* (\( G^2 \) likelihood ratio test, \( p = 0.60 \)) and PAR + *H. defensa* (\( G^2 \) likelihood ratio test, \( p = 0.21 \); table 3).

**4. DISCUSSION**

In this study, we found that the same clone lineage of *A. pismum* co-infected with both *S. symbiotica* and *H. defensa* exhibits even greater resistance to parasitism by *A. ervi* than clones singly infected with either (figure 1). The greater resistance phenotype may be the result of total higher densities of SS that we found in superinfected aphids (figure 2), or it could reflect the combined action of distinct defensive mechanisms of each symbiont type. The added benefit to superinfection appears surprising, however, in light of our survey of *A. pismum* SS in northern Utah, which found a lower prevalence of aphids superinfected with *S. symbiotica* and *H. defensa* SS than expected (table 3). The lower than expected prevalence of these double infections may occur if superinfections are unstable, resulting in a winnowing to single infection status. In a previous study, an *A. pismum* lineage that was

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**Table 1. Fecundities (0–18 days post-parasitism) of parasitized *A. pismum*. Wilcoxon rank sum tests comparing fecundities per arena (equal to four aphids) for each artificially inoculated lineage to the uninfected control lineage.**

<table>
<thead>
<tr>
<th>infection status</th>
<th>uninfected</th>
<th><em>H. defensa</em> only</th>
<th><em>S. symbiotica</em> only</th>
<th>superinfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>34</td>
<td>34</td>
<td>34</td>
<td>12</td>
</tr>
<tr>
<td>mean; median fecundity</td>
<td>28.0; 0</td>
<td>87.9; 73.5</td>
<td>34.2; 10.0</td>
<td>13.7; 7.0</td>
</tr>
<tr>
<td>Wilcoxon rank sum test</td>
<td>N/A</td>
<td>( p = 0.003 )</td>
<td>( p = 0.48 )</td>
<td>( p = 0.68 )</td>
</tr>
</tbody>
</table>

**Table 2. Comparison of fecundity, generation time and fresh weights of unparasitized superinfected versus uninfected *A. pismum*.**

<table>
<thead>
<tr>
<th>assay</th>
<th>infection status</th>
<th>uninfected</th>
<th>superinfected</th>
<th>test</th>
</tr>
</thead>
<tbody>
<tr>
<td>cumulative fecundity (0–12 days post-reproductive onset) per cage (4 aphids per cage)</td>
<td>210 (( n = 14 ))</td>
<td>38 (( n = 14 ))</td>
<td>ANOVA ( p &lt; 0.0001 )</td>
<td></td>
</tr>
<tr>
<td>cumulative fecundity per aphid (0–12 days post-reproductive onset)</td>
<td>24.3</td>
<td>7.0</td>
<td>ANOVA ( p &lt; 0.0001 )</td>
<td></td>
</tr>
<tr>
<td>generation time (h) median, mean</td>
<td>211, 209 (( n = 37 ))</td>
<td>220, 214 (( n = 52 ))</td>
<td>Wilcoxon rank sum ( X^2 = 0.002 )</td>
<td></td>
</tr>
<tr>
<td>mean fresh weights at adulthood</td>
<td>3.95 g</td>
<td>3.15 g</td>
<td>ANOVA ( p &lt; 0.0001 )</td>
<td></td>
</tr>
</tbody>
</table>
naturally superinfected with \textit{R. insecticola} and \textit{H. defensa} was observed to undergo spontaneous loss of \textit{R. insecticola} (Sandström et al. 2001). However, our superinfected \textit{A. pism} lineage has been maintained in the laboratory for a minimum of 80 generations.

Instead, the rarity of multiple infections with \textit{S. symbiotica} and \textit{H. defensa} is almost certainly a consequence of severe fitness costs, manifested as dramatically lower cumulative fecundities, longer generation times and lower weights at adulthood, associated with superinfections compared to uninfected controls (table 2). Laboratory fitness assays of single infections with either \textit{S. symbiotica} or \textit{H. defensa} range from slight costs to benefits (Chen et al. 2000; Russell & Moran 2006; K. M. Oliver, unpublished data). Koga et al. (2003) reported costs associated with \textit{S. symbiotica} shortly after infection, but the detrimental effects had attenuated by eight months after infection. Our experiments were carried out in the period between eight months and 3 years after the creation of the superinfected line so costs associated with novel infection were probably minimal. Even in the presence of parasitism, there appears to be no direct fitness benefit to superinfection (table 1). This contrasts with our finding that there are clear benefits to infection in the presence of parasitism with single infections of \textit{H. defensa} (table 2). Given the severity of the cost associated with superinfection, it may be surprising that we find them at all. One explanation is that the cost of superinfection may not be as severe in other host backgrounds. Likewise, different isolates of \textit{S. symbiotica} and \textit{H. defensa} may differ in degree (or kind) in regard to effects exerted on hosts, such that certain pairs of \textit{S. symbiotica} and \textit{H. defensa} isolates may not be as detrimental to hosts. Another possibility is that we are sampling doomed transient superinfections resulting from frequent horizontal transfer events in the field. While \textit{A. pism} SS are primarily transmitted vertically, phylogenetic evidence indicates that occasional horizontal transfer is necessary to explain current SS distributions in natural populations (Russell et al. 2003). Different SS species and isolates may rise to high frequencies due to particular benefits conferred as single infections. These benefits may reflect different ecological pressures, including parasitism by \textit{A. ervi} as well as other natural enemies and also including abiotic factors such as heat. For example, a major benefit conferred by \textit{S. symbiotica} infection is the improved tolerance to heat (Montllor et al. 2002; Russell & Moran 2006); whereas \textit{H. defensa} isolates confer varying levels of defence against parasitoids as well as some effects on heat tolerance (Russell & Moran 2006; Oliver et al. 2005). Spatial and temporal heterogeneity in ecological challenges are expected to maintain polymorphism in symbiont types among aphid lineages. If alternative symbionts are maintained at high densities as single infections, the opportunity for superinfection through horizontal transfer will be maximized (although mechanisms in nature are not yet identified). This situation could result in repeated regeneration of superinfections despite major deleterious effects.

QPCR estimates of SS density indicate that numbers of \textit{H. defensa} do not differ between singly and superinfected lineages (figure 2). This finding is similar to reports that \textit{Wolbachia} densities are regulated independently in superinfected hosts (Rousset et al. 1999; Ikeda et al. 2003; Mouton et al. 2003, 2004). \textit{S. symbiotica} densities, in contrast, increased 20-fold in superinfected aphids compared to aphids infected with \textit{S. symbiotica} only. The severe fecundity cost found in superinfected aphids may

![Figure 2](http://rspb.royalsocietypublishing.org/)

**Figure 2.** Comparison of SS densities in singly versus superinfected \textit{A. pism}. (a) Densities of \textit{H. defensa} in singly infected aphids (light bar) and superinfected aphids (dark bar); (b) the densities of \textit{Serratia symbiotica} in singly infected aphids (light bar) and superinfected aphids (dark bar).

**Table 3.** Frequency of \textit{A. pism} SS collected from North Utah. \(n=120\).

<table>
<thead>
<tr>
<th>symbiont</th>
<th>frequency (%)</th>
<th>expected (%)</th>
<th>(G^2) likelihood ratio test</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninfected</td>
<td>32.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{S. symbiotica}</td>
<td>18.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{H. defensa}</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{R. insecticola}</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Rickettsia} (PAR)</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{S. symbiotica} + \textit{H. defensa}</td>
<td>0.8</td>
<td>7.3</td>
<td>(p=0.002)</td>
</tr>
<tr>
<td>\textit{H. defensa} + PAR</td>
<td>0.8</td>
<td>2.3</td>
<td>(p=0.21)</td>
</tr>
<tr>
<td>\textit{S. symbiotica} + PAR</td>
<td>1.7</td>
<td>1.1</td>
<td>(p=0.60)</td>
</tr>
</tbody>
</table>
be the result of this over-proliferation of \textit{S. symbiotica} in doubly infected hosts. Alternatively, this cost in the superinfected line could result from hostile interactions between SS or some combination of over-proliferation and antagonistic interactions. A correlation between virulence and bacterial density has been documented for \textit{Wölbachia} infecting \textit{Drosophila} and \textit{Asobara} (McGraw et al. 2002; Mouton et al. 2004). There may be competing selective pressures between maintaining sufficiently high densities to ensure vertical transmission, while reducing fitness costs to hosts (Mouton et al. 2004).

We also used QPCR to estimate relative densities of the primary aphid symbiont, \textit{Buchnera}, to determine if \textit{Buchnera} suppression in SS-infected lines correlated positively with increased levels of resistance to parasitism. Although \textit{Buchnera} densities did vary significantly, they did not do so in a pattern consistent with the hypothesis that lower \textit{Buchnera} levels result in poorer parasitoid survival. Superinfection may still result in reduced host quality for wasps and the greatly reduced fecundity found in superinfected unparasitized aphids supports this idea.

Recently, it was reported that \textit{H. defensa} is associated with a bacteriophage, APSE-2, which contains intact homologues of a eukaryotic toxin (CdtB or cytolethal distending toxin) that may target wasp tissue (Moran et al. 2005a). A toxin such as CdtB, which likely relies on bacterial invasion of host tissue for delivery, may be more effective in poorer-quality hosts, such as superinfected aphids, which may contain wasps with a compromised ability to fight off microbial attackers.

To invade a host population, vertically transmitted micro-organisms must cause their hosts to produce more infected daughters than are produced by uninfected females (Bull 1983; Wilf & O’Neill 1997). In the presence of parasitism, \textit{H. defensa} confers resistance to \textit{A. ervi} and infected aphids produce more offspring than uninfected aphids, indicating that these symbionts can invade and persist in host populations. Despite the added resistance conferred by superinfection with \textit{S. symbiotica} and \textit{H. defensa}, greatly reduced fecundities would prevent invasions of the superinfection into \textit{A. pism} populations. Given that the interference interactions among bacterial clones are known to be common within the Gammaproteobacteria, evidence of competition between symbionts should perhaps not be surprising. Interference could result from any of the several well-characterized mechanisms, such as quorum sensing and bacteriocin production (Parret & De Mot 2002; Lerat & Moran 2004). Whatever the mechanism, these results indicate that while previous research has focused almost exclusively on the host–symbiont interaction, interactions among the symbionts themselves are likely to play a critical role in determining the distributions of symbionts in natural populations, and merit further study.

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