Population dynamics of defensive symbionts in aphids

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Vertically transmitted micro-organisms can increase in frequency in host populations by providing net benefits to hosts. While laboratory studies have identified diverse beneficial effects conferred by inherited symbionts of insects, they have not explicitly examined the population dynamics of mutualist symbiont infection within populations. In the pea aphid, *Acyrthosiphon pisum*, the inherited facultative symbiont, *Hamiltonella defensa*, provides protection against parasitism by the wasp, *Aphidius ervi*. Despite a high fidelity of vertical transmission and direct benefits of infection accruing to parasitized aphids, *Hamiltonella* remains only at intermediate frequencies in natural populations. Here, we conducted population cage experiments to monitor the dynamics of *Hamiltonella* and of another common *A. pisum* symbiont, *Serratia symbiotica*, in the presence and absence of parasitism. We also conducted fitness assays of *Hamiltonella*-infected aphids to search for costs to infection in the absence of parasitism. In the population cages, we found that the frequency of *A. pisum* infected with *Hamiltonella* increased dramatically after repeated exposure to parasitism by *A. ervi*, indicating that selection pressures from natural enemies can lead to the increase of particular inherited symbionts in insect populations. In our laboratory fitness assays, we did not detect a cost to infection with *Hamiltonella*, but in the population cages not exposed to parasitism, we found a significant decline in the frequency of both *Hamiltonella* and *Serratia*. The declining frequencies of *Hamiltonella*-infected aphids in population cages in the absence of parasitism indicate a probable cost to infection and may explain why *Hamiltonella* remains at intermediate frequencies in natural populations.

Keywords: endosymbiont; defensive mutualism; parasitoid; proteobacteria; *Regiella*

1. INTRODUCTION

Symbiotic associations between invertebrate animals and inherited microbes are common in nature (e.g. Buchner 1965; Werren & Windsor 2000; Zchori-Fein & Perlman 2004). These microbes are transmitted vertically from parent to offspring and can increase in frequency in host populations by manipulating host reproduction or providing net benefits to hosts (Bull 1983; Werren & O'Neill 1997). Experimental laboratory assays have identified diverse beneficial roles conferred by inherited symbionts of invertebrates (e.g. Montllor *et al.* 2002; Oliver *et al.* 2003; Tsuchida *et al.* 2004). These studies identify potential mechanisms by which symbionts may invade and persist in host populations, but do not explicitly test the population dynamics of the mutualist symbiont infections within host populations.

A striking example of the diverse roles performed by inherited symbionts can be found in the pea aphid, *Acyrthosiphon pisum*, one of few invertebrates to have many components of its complex microbiota studied. Virtually all aphids, including *A. pisum*, harbour the obligate, 'primary' bacterial symbiont, *Buchnera*, which is housed in specialized host cells called bacteriocytes, and provides amino acids and other nutrients scarce in their plant sap diet (reviewed in Douglas 1998). In addition to *Buchnera*, aphids may also be infected with one or more facultatively associated, inherited bacteria, called secondary symbionts (SS). Recent laboratory studies have implicated *A. pisum* SS in mediating several phenotypes important to aphid survival and reproduction, including resistance to parasitic wasps (Oliver *et al.* 2003, 2005), resistance to fungal pathogens (Scarborough *et al.* 2005), performance on particular host plants (Tsuchida *et al.* 2004) and thermal tolerance (Chen *et al.* 2000; Montllor *et al.* 2002; Russell & Moran 2006).

Two *A. pisum* SS have been shown to confer some level of resistance to parasitism by the wasp *Aphidius ervi* in laboratory assays. Of the two, *Hamiltonella defensa* genotypes appear to generally confer greater resistance than *Serratia symbiotica* and also confer direct fitness benefits to the infected, parasitized aphid (Oliver *et al.* 2003, 2005, 2006). The laboratory results suggest a mechanism for the spread of *Hamiltonella* within populations exposed to parasitism. To date, no fitness costs of *Hamiltonella* infection have been detected in laboratory assays (Russell & Moran 2006). Thus, we might predict that, given the documented high fidelity of vertical transmission of SS to progeny, SS, and *Hamiltonella* in particular, would maintain high frequencies or become fixed in natural populations. However, worldwide, surveys indicate that *A. pisum* SS are found at intermediate frequencies in nature (Sandström *et al.* 2001; Tsuchida *et al.* 2002; Simon *et al.* 2003; Oliver *et al.* 2006). Here, we examined the fitness costs and benefits to SS infection in two types of experiments. We examined the fitness effects of *Hamiltonella* in aphids not exposed to parasitism in a laboratory assay. We also conducted population cage experiments with and without parasitism to monitor SS dynamics in aphid populations in the presence and absence of parasitism.

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2. MATERIAL AND METHODS

(a) *Study organisms*

The three most common *A. pisum* SS are members of the Enterobacteriaceae (Gammaproteobacteria) and have been described with nomenclature replacing several provisional labels (Moran et al. 2005). *Serratia symbiotica* was previously called PASS or R-type, *H. defensa* was called PABS or T-type and *Regiella insecticola* was called PAUS or U-type (Chen & Purcell 1997; Darby et al. 2001; Sandström et al. 2001; Tsuchida et al. 2002).

The pea aphid is a pest of herbaceous legumes, including fava bean, *Vicia faba*. Most populations of this aphid are cyclical parthenogens; they reproduce asexually most of the year and have a single sexual generation in autumn. Clonal lines can therefore be maintained in the laboratory indefinitely by rearing under long daylight conditions. Clonal *A. pisum* lines were maintained on *V. faba* at $20 \pm 1°C$ under long daylight conditions on a 16 L : 8 D cycle.

Aphidius ervi (Haliday; Hymenoptera: Braconidae) is a solitary endoparasitoid and the dominant parasitoid of *A. pisum* in North America (Angalet & Fuester 1977). The adult female wasp lays an egg inside the aphid host, and the resulting larva feeds and develops inside the living aphid over a period of 5–8 days, eventually killing the host. The wasps were maintained in the laboratory on an uninfected aphid line (5A) at $20 \pm 1°C$ on a 16 L : 8 D cycle. Honey and water were provided to adult wasps.

(b) *Fitness parameters of* Hamiltonella-*infected versus* Hamiltonella-*uninfected lines*

To determine whether *Hamiltonella* genotypes confer a fitness cost to aphids in the absence of parasitism, we measured basic fitness parameters of clonal lines of aphids with and without *Hamiltonella* under standard laboratory conditions. We performed assays to measure cumulative fecundity, generation time, defined here as time-to-first reproduction (TFR), and fresh weight at adulthood. In aphid clonal line 5A (one of the pink colour morphs found in *A. pisum* and the line used in the population cage experiments), we compared the uninfected aphid line (5A) and two lines of the same clone infected with different *Hamiltonella* strains. The strains were UT1→5A, the strain used in the population cage that confers high levels of resistance to parasitism in the 5A background, and 82B→5A, a strain that confers moderate levels of resistance (Oliver et al. 2005). We used two strains of *Hamiltonella* that differed in levels of protection conferred to allow us to examine whether costs incurred are proportional to protective benefits received. We also performed these measurements in a second aphid background, using uninfected clonal line A2E (a green colour morph) and *Hamiltonella*-infected line 82B→A2E. This experimental line of *Hamiltonella*-infected *A. pisum* was established for a previous study (Oliver et al. 2005) and was also found to confer moderate levels of protection (Oliver et al. 2005). To measure cumulative fecundity, we placed five fourth instar aphids of a particular line on a single *V. faba* plant, with a vented, plastic inverted cup as a lid (Oliver et al. 2003). Each of the five aphid lines was replicated 15 times. All lines were incubated at $20 \pm 1°C$ and 16 L : 8 D. The number of adult aphids and the number of offspring they produced were counted every 3 days after the onset of reproduction. Offspring were removed after they were counted so that they could not mature to adulthood and reproduce. The assay ran for 21 days, but in all lines most offspring were produced by day 15. Cumulative fecundity distributions through day 15 were not normally distributed and log (ln) transformations were performed prior to conducting an ANOVA. We also examined cumulative fecundities for surviving aphids. These were estimated by simply summing the fecundity at each time period divided by the number of surviving aphids.

To examine generation time (TFR) and fresh weights at adulthood, newborn nymphs were removed and placed on *V. faba* plants within 3 h of birth in cohorts of approximately 10 aphids and maintained at $20 \pm 1°C$ and 16 L : 8 D. We began monitoring cages 7 days later at approximately 4–5 hour intervals (except during the 8D period) to detect the onset of reproduction in individual aphids. Reproducing aphids were removed from the cages and immediately weighed to assess fresh weight at adulthood.

(c) *Population cages*

In the population cages, we used three *A. pisum* lines that differ in SS composition but share the same aphid and *Buchnera* genetic background. Experimental lines were created by microinjection of SS from infected lines into a single uninfected line (5A; Chen & Purcell 1997; Oliver et al. 2003). Line 5A is uninfected with SS and is highly susceptible to parasitism by *A. ervi*, line 2BB→5A is infected with *S. symbiotica* and receives moderate protection from parasitism (Oliver et al. 2003), and line UT1→5A is infected with *Hamiltonella* and receives high levels of protection from parasitism (Oliver et al. 2005). The 5A clone has been in culture since 1999 (Sandström et al. 2001) and has been divided into several laboratory-reared lines. Some of the long-term cultures have fixed a mutation in the *Buchnera* genome that affects heat shock response (Russell & Moran 2006; Dunbar et al. 2007). All of the lines used in the current experiment had the ancestral *Buchnera* genotype. The experiment was carried out for a minimum of 18 months after the most recent artificial infection (UT1→5A). Thus, previous findings of reduced fitness of *A. pisum* within the first eight months following establishment of an artificial infection (Koga et al. 2003) are unlikely to be a concern here.

We established six population cages, each with an equal number and proportion of *A. pisum* representing three infection states: uninfected with SS (line 5A), infected with *S. symbiotica* (2BB→5A) and infected with *Hamiltonella* (UT1→5A). The cages (50 cm × 50 cm × 50 cm) were kept in chambers at $20 \pm 1°C$ on a 16 L : 8 D cycle. While every attempt was made to ensure all cages were exposed to similar conditions (e.g. lighting), cages were routinely rotated during cage maintenance to randomize any positional effects. Cages were initially seeded with 60 second to third instar aphids of each infection status (180 total aphids). On each of three pots containing *V. faba* (each pot contained three plants, approx. 35 cm in height), 20 aphids of each infection type were placed. In each cage, aphids were allowed to reach adulthood and reproduce until the bulk of offspring were second and third instar nymphs. Half of the cages were kept as controls while the other half were exposed to parasitism by *A. ervi*. For the first bout of parasitism, 30 mated *A. ervi* females were introduced for 24 h in each of the three treatment cages. Subsequent bouts of parasitism resulted from wasps emerging from previous bouts of parasitism. Wasps were allowed to emerge, mate and forage for aphids for approximately 24 h. Cages were checked once daily and adult wasps were removed to prevent decimation of aphid populations. In both cage types, pots containing dead or dying plants were removed and new plants (with no aphids)

Table 1. Comparison of fecundity, generation time and fresh weights (at adulthood) among five *A. pisum* clonal lines. We examined two aphid genotypes, 5A (pink) and A2E (green), which are either uninfected with SS or infected with *H. defensa* strains UT1 (confers high levels of protection) or 82B (moderate levels of protection). Different letters in columns signify a statistically significant difference at the 0.05 level.

A. pisum lines	assay			
	mean cumulative fecundity per cage to d15 (five aphids/cage), (ln back-transformed)	mean cumulative fecundity $\pm$ s.e. per 'surviving' aphid—see §2	generation time $\pm$ 3 h median, mean	fresh weights (g) $\pm$ s.e. at adulthood
5A	393[a] ($N=13$)	99.9 $\pm$ 2.9[a]	214, 211[b] ($N=37$)	3.95 $\pm$ 0.08[a] ($N=33$)
UT1→5A	405[a] ($N=15$)	101.2 $\pm$ 2.8[a]	209, 205[c] ($N=32$)	3.83 $\pm$ 0.11[a] ($N=20$)
82B→5A	428[a] ($N=14$)	101.6 $\pm$ 2.9[a]	209, 206[c] ($N=66$)	3.72 $\pm$ 0.08[a] ($N=36$)
A2E	328[b] ($N=10$)	85.7 $\pm$ 4.7[b]	223, 220[a] ($N=45$)	3.33 $\pm$ 0.08[b] ($N=39$)
82B→A2E	356[ab] ($N=10$)	93.8 $\pm$ 4.0[ab]	214, 214[b] ($N=41$)	3.34 $\pm$ 0.08[b] ($N=39$)

were inserted. Dead plant material bearing wasp pupae was not removed from cages until after wasp eclosion. We exposed the population cages to six rounds of parasitism (approx. 12 weeks). Following parasitism rounds one, two, four and six, we removed a sample of 60 aphids from each cage, conducted DNA extractions and performed diagnostic PCR to determine aphid infection status and estimate symbiont frequencies in each cage. The extraction protocol, diagnostic PCR primers and reactions conditions can be found in Oliver *et al.* (2003, 2006).

We started the experiment with three control (no wasp) cages, but we eventually found wasps in one. We removed wasps and mummies diligently from the contaminated cage, but were unable to eliminate wasps, and this cage was therefore not included in the control cage analyses. As we were left with only two control cages, we decided to conduct a second block of the experiment, in which only the control was replicated. In addition to serving as a baseline to compare treatment cages, the control population cages were well suited to identify costs associated with infection (see more detail in §4). We used the same experimental design for the second block of population cages except that aphids were removed every three weeks to assess SS frequencies.

We estimated selection for particular infection types from the linear regression of the logit (infection frequency) against time (all trials ran for 12 weeks). A similar method has been used for estimating changes in frequencies of mitochondrial haplotype in *Drosophila* (Ballard & James 2004). All analyses were conducted in JMP v. 4.0.3 (SAS Institute, Inc.).

3. RESULTS
(a) *Fitness parameters of* Hamiltonella-*infected versus* Hamiltonella-*uninfected lines*

Our measurements of cumulative fecundity, generation time and fresh weight at adulthood indicated no significant costs to SS infection, but rather some modest benefits (table 1). The only significant differences in cumulative fecundity (0–15 days) were between the two uninfected clones 5A (pink) and A2E (green; *t*-test, $p=0.012$). While infection status did not significantly influence cumulative fecundity, there was a trend towards greater fecundity of both pink and green clones when they were infected with *Hamiltonella* (table 1). There were also clear differences in fresh weight between the green (A2E) and pink (5A) clones (*t*-test, $p<0.0001$). However, fresh weights were neither significantly different between uninfected (A2E)

and *Hamiltonella*-infected (82B→A2E) aphids of the green clone (*t*-test, $p=0.96$) nor among infected and uninfected aphids of the pink clone (ANOVA, $p=0.12$). Both clone and infection status influenced generation time. Generation time (TFR) was significantly faster for *Hamiltonella*-infected aphids in both green and pink clones. In the pink clone, both UT1→5A and 82B→5A matured faster than the uninfected line (5A; Wilcoxon rank-sum test, $\chi^2=0.03$ and 0.003, respectively). For the green clone, the *Hamiltonella*-infected line (82B→A2E) matured more quickly than the uninfected line (A2E; Wilcoxon rank-sum test, $\chi^2<0.0001$). Generation time was also faster in the pink clone (5A) than the green clone (A2E; Wilcoxon rank-sum test, $\chi^2<0.0001$).

(b) *Population cages*

The infection frequencies of *A. pisum* facultative symbionts were significantly influenced by parasitism pressure from the wasp, *A. ervi*. Final infection frequencies differed significantly from starting frequencies (33.3% of each type) in both the cages with wasps (likelihood ratio $\chi^2=343$, $p<0.0001$) and in control cages lacking wasps (likelihood ratio $\chi^2=177$, $p<0.0001$). In cages exposed to wasps, the proportion of aphids infected with *Hamiltonella* increased to near fixation at the end of the experiment (mean = 97.2%; figure 1). An ANOVA of the linear regression of the logit of *Hamiltonella* frequency against time is significantly different than zero (a slope of zero would indicate no change in infection frequency; table 2). At the end of the experiment, uninfected aphids were found at very low frequencies (mean = 2.8%) and aphids infected with *S. symbiotica* were completely eliminated (figure 1, table 2).

Control cages containing no wasps were conducted in two blocks. An ANOVA of the slopes of each SS between the two blocks indicated no significant differences between blocks, so the data were pooled. In cages not exposed to wasps, we found a significant increase in the number of uninfected aphids by the end of the experiment (mean = 71%), while the frequency of aphids infected with *S. symbiotica* and *Hamiltonella* decreased significantly (figure 1, table 2). Final infection frequencies of *Hamiltonella* (13.3%) and *S. symbiotica* (15.3%) were similar in cages not exposed to parasitism ($F_{1,8}=0.054$, $p=0.82$). As mentioned in §2, one of our three control cages was accidentally contaminated with wasps. While this cage was dropped from the analysis, it is interesting to
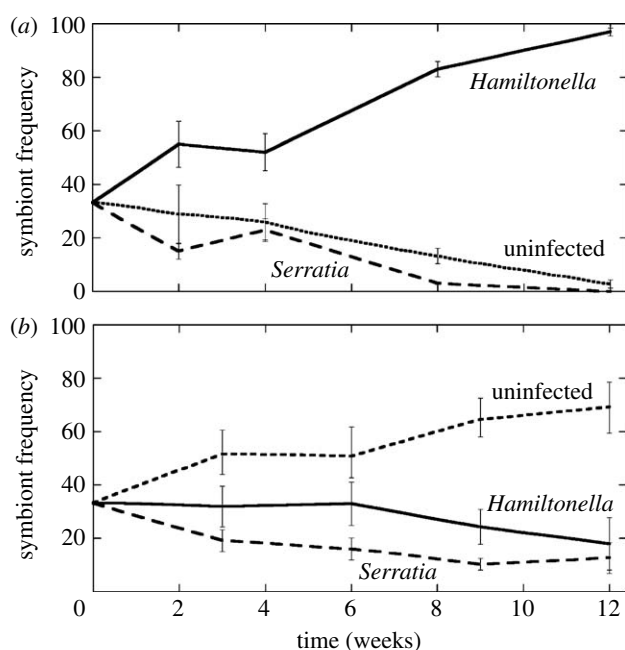
Figure 1. Infection frequencies of *A. pisum* secondary symbionts over time. Aphids may be infected with either *Hamiltonella* (solid line), *Serratia* (long-dashed line) or uninfected with secondary symbionts (short-dashed line). (*a*) Infection frequencies (with s.e. at sampling points) in the presence of parasitic wasps and (*b*) infection frequencies in the absence of wasps. For clarity, infection frequencies in the absence of wasps are shown only for one of two experimental blocks. There were no significant differences in the slopes of the infection frequencies between blocks.

note that *Hamiltonella*-infected aphids increased in this cage, to a final infection frequency of 57%. This value is intermediate relative to control and treatment cages, possibly reflecting the intermediate level of parasitism characterizing this cage. We also found that the frequency of aphids infected with *S. symbiotica* declined to very low frequencies (1.7%) in this contaminated cage. Finally, we did detect one possible incidence of horizontal transmission of SS. In the second block of the control cages, we detected, using diagnostic PCR, a double infection with *Serratia* and *Hamiltonella* in one aphid at the second sampling period (six weeks). Because SS are found in aphid haemolymph and honeydew (Fukatsu *et al.* 2000; Darby & Douglas 2003), it is possible that external contamination of the aphid could explain this result. However, in the subsequent (nine-week) sampling event of the same control cage there were two doubly infected aphids. No doubly infected aphids were found in the final sampling (12 weeks), nor in any other cage.

4. DISCUSSION

Our results indicate that selection pressures from natural enemies can lead to the increase of particular inherited symbionts in insect populations. We found that the frequency of *A. pisum* infected with the facultative symbiont, *Hamiltonella*, increased dramatically in population cages after repeated exposure to parasitism by the wasp, *A. ervi* (figure 1, table 2). Previous experimental studies indicated that *A. pisum* infected with *Hamiltonella* receive protection from attack by *A. ervi* (Oliver *et al.* 2003, 2005). Another study found direct benefits to

infection with *Hamiltonella* in the presence of parasitism; parasitized, *Hamiltonella*-infected aphids produce far more offspring than parasitized uninfected control aphids (Oliver *et al.* 2006). This increase of *Hamiltonella*-infected aphids in population cages exposed to parasitism indicates that parasitism pressure probably influences *A. pisum* symbiont frequencies in nature. This is the first report that natural enemies can influence symbiont frequencies in populations of animal hosts. A field study by Clay *et al.* (2005) found that the proportion of *Lolium arundinaceum* (tall fescue grass) infected with the inherited endophytic fugal symbiont, *Neotyphodium coenophialum* increased rapidly in the presence of herbivores. The fungal endophytes produce alkaloids that defend the grasses against herbivory (reviewed in Clay 1996).

After six rounds of parasitism by *A. ervi*, we did not detect any aphids infected with *S. symbiotica* (figure 1). This result is perhaps not surprising, because this isolate of *S. symbiotica* was found to confer only moderate resistance to wasps, and parasitism interfered with reproduction such that infected, parasitized aphids received no direct fitness benefit to infection (Oliver *et al.* 2003, 2006). Other reports indicate that *S. symbiotica* provides thermal protection in *A. pisum* (Chen *et al.* 2000; Montllor *et al.* 2002; Russell & Moran 2006), and this effect may explain the persistence of this inherited symbiont in aphid populations in nature.

Our results are the first to suggest probable fitness costs of SS infection of aphids in the absence of parasitism, providing insight into the unanswered question of why uninfected lineages occur at high frequencies in field populations worldwide (Sandström *et al.* 2001; Tsuchida *et al.* 2002; Simon *et al.* 2003; Oliver *et al.* 2006). Given the evidence presented here that *Hamiltonella*-infected aphids increase rapidly in frequency in the presence of parasitism, one would predict that *Hamiltonella* would be fixed or much more prevalent in natural populations where *A. ervi* is a common and important natural enemy (Angalet & Fuester 1977). Do fitness costs to infection keep the symbiont at intermediate frequencies in field populations? Previously, Gwynn *et al.* (2005) reported a trade-off between *A. pisum* fecundity and resistance to *A. ervi*, but these authors did not link either parasitoid resistance or reduced fecundity to infection with SS. Fitness costs to *Hamiltonella* infection are not evident in the direct estimates of fitness parameters reported in this study (table 1). A previous study, using similar measures, also found no fitness costs to infection with either *Hamiltonella* or *Serratia* (Russell & Moran 2006). However, the significant decline of both SS infections in the population cages not exposed to parasitism (figure 1) indicates the probable presence of fitness costs not detected in the laboratory assays. An alternative, but unlikely, explanation is that the observed reductions in the frequency of SS-infected lines were due to the loss of SS in infected lines rather than due to costs associated with infection. However, in parthenogenetic lines maintained under 20°C on *V. faba* in the laboratory (same conditions as population cages), infections are extremely stable: no SS-free lines have been generated from numerous SS-bearing lines after more than 175 generations in the laboratory.

What explains the difference between results from laboratory and population cage experiments? Unlike our population cage experiment, fitness assays performed by

Table 2. (*a*) Estimates of selection (s) from linear regression of logit (symbiont frequency) against time in population cages exposed to parasitic wasps. All cages held at $20 \pm 1°C$ on a 16 L : 8 D light/dark cycle. (*b*) Estimates of selection (s) from linear regression of logit (symbiont frequency) against time in cages not exposed to wasps.

symbiont type	replicate	block	cage slope	mean slope	*t*-test
(*a*)					
H. defensa	1		0.338		
	2	1	0.192[a]	0.278 ± 0.022	$p = 0.003$
	3		0.303		
S. symbiotica	1		−0.330[a]		
	2	1	−0.222[a]	-0.294 ± 0.018	$p = 0.001$
	3		−0.330[a]		
uninfected	1		−0.220		
	2	1	−0.100[a]	-0.169 ± 0.018	$p = 0.009$
	3		−0.188		
(*b*)					
H. defensa	1	1	0.004		
	2	1	−0.125		
	1	2	−0.142	-0.119 ± 0.018	$p = 0.011$
	2	2	−0.220		
	3	2	−0.111		
S. symbiotica	1	1	−0.191		
	2	1	−0.026		
	1	2	−0.220	-0.133 ± 0.021	$p = 0.012$
	2	2	−0.188		
	3	2	−0.041		
uninfected	1	1	0.102		
	2	1	0.147		
	1	2	0.226	0.168 ± 0.016	$p < 0.001$
	2	2	0.258		
	3	2	0.109		

[a] Denotes that the particular cage fixed at 100 or 0% at end of experiment (12 weeks). Therefore, slopes were estimated through parasitism bout four (third SS frequency estimate at week 8). Logit $= \ln(y/1-y)$; $y =$ proportion infected.

rearing aphids on separate cages would have neither detected effects due to competition nor any density-dependent effects due to the decline of plant quality. Also, slight differences in fitness are more likely to be revealed by competition experiments than by comparing fitness measures from lines reared separately. Rearing lines separately results in statistical noise, because cage or plant differences are affecting estimates of fitness components. This variation is eliminated in competition experiments, in which the different lines are subjected to the same local conditions within each replicate. However, the single line assays indicated slight fitness *benefits* from SS infection, an effect that was reversed in the population cage trials. The environmental conditions of the population cages are presumably closer to that encountered by field populations.

These results underscore the importance of population-level assays to validate the conclusions reached from individual or small-group fitness experiments. Xi *et al.* (2005) also found a substantial difference between single or small-group assays and population cages. Laboratory assays of the fitness of a cytoplasmic incompatibility *Wolbachia* infection in mosquitoes showed little cost, but population cage studies suggested a cost of approximately 15%.

The incidence of SS infection in natural populations is predicted to be determined by the rate of acquisition of symbionts by uninfected lineages, the rate of loss of symbionts by infected lineages, the relative fitness of infected versus uninfected lineages, and possible reproductive manipulation during the sexual stage in aphid

populations. Evidence gathered to date suggests that loss is rare. As noted above, SS infections are very stable in the laboratory. In nature, heat or other sources of stress might result in some loss of symbionts, but it seems unlikely that such losses would be sufficient to explain the high frequencies of SS-free lines observed in some populations. Horizontal transfers of SS within and among aphid species are indicated by phylogenetic analyses (Sandström *et al.* 2001; Russell *et al.* 2003). The mechanisms and frequencies of such transfers remain largely unknown (Darby & Douglas 2003). One potential route for the lateral transfer of SS is via contaminated ovipositors of parasitoids, but attempts to substantiate this route of transfer in laboratory trials have been unsuccessful (K. M. Oliver 2004, unpublished data). We did detect one probable instance of horizontal transmission of *A. pisum* SS in our population cages. However, this occurred in a control cage (no wasps) indicating that lateral transfer of SS may occur through the host plant or via aphids ingesting *Hamiltonella*-infected substances, such as honeydew, on the plant surface. *Hamiltonella* has been detected in *A. pisum* honeydew, and in laboratory assays *A. pisum* can become infected with *Hamiltonella* after feeding on artificial diets containing these bacteria (Darby & Douglas 2003). Moran & Dunbar (2006) recently demonstrated that paternal SS infections are readily transferred to uninfected sexual females and that some of these are stably inherited in the descendant lines of parthenogenetic females. On the other hand, maternal infections are occasionally lost during the sexual egg stage

(Moran & Dunbar 2006). Thus, whether the sexual phase of the life cycle results in a net increase or decrease in infection frequency in nature is unknown. Very high frequencies have been observed in the fundatrix generation in New York (N. A. Moran 2002, unpublished data), suggesting that losses during the sexual and overwintering stages are not a major source of uninfected lineages.

In natural populations that experience fluctuations in selection pressures, symbiont frequencies will reflect the incidence of different environments. Infections with little effect on fitness will be governed mainly by drift. If a symbiont is brought to high frequency due to selection by natural enemy pressure or environmental stress, and the effect of the symbiont on fitness is essentially neutral, then the frequency may remain high for some time after this selective force is removed. We expect that most *A. pisum* populations do encounter frequent selection by parasitism by *A. ervi* and, thus, that *Hamiltonella* frequency would be near fixation if this symbiont were neutral with respect to fitness in the absence of parasitoids. Furthermore, strains of both *Hamiltonella* and *S. symbiotica* have been shown to improve tolerance to heat stress, suggesting that episodes of high temperature would bring them to higher frequency relative to uninfected lineages. Given the expected frequency of selection events favouring SS-infected lineages, the observed frequencies of *A. pisum* lacking SS appear to be too high to be explained by spontaneous losses, which appear to be extremely rare. Therefore, the continued presence of SS-uninfected lineages in spite of clearly demonstrated benefits to *SS* infection strongly argues for a cost to SS infection. The population cage experiment described here provides the first clear evidence of such a cost, and may explain why *Hamiltonella*, in particular, remains at intermediate frequencies in nature.

REFERENCES

Angalet, G. W. & Fuester, R. 1977 *Aphidius* parasites of pea aphid in the eastern half of United-States. *Ann. Entomol. Soc. Am.* **70**, 87–96.

Ballard, J. W. O. & James, A. C. 2004 Differential fitness of mitochondrial DNA in perturbation cage studies correlates with global abundance and population history in *Drosophila simulans*. *Proc. R. Soc. B* **271**, 1197–1201. (doi:10.1098/rspb.2004.2709)

Buchner, P. 1965 *Endosymbiosis of animals with plant microorganisms*. New York, NY: Interscience Publishers.

Bull, J. J. 1983 *Evolution of sex determining mechanisms/James J. Bull*. Menlo Park, CA: Benjamin/Cummings Pub. Co.

Chen, D. Q. & Purcell, A. H. 1997 Occurrence and transmission of facultative endosymbionts in aphids. *Curr. Microbiol.* **34**, 220–225. (doi:10.1007/s002849900172)

Chen, D. Q., Montllor, C. B. & Purcell, A. H. 2000 Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. *Entomol. Exp. Appl.* **95**, 315–323. (doi:10.1023/A:1004083324807)

Clay, K. 1996 Interactions among fungal endophytes, grasses and herbivores. *Res. Popul. Ecol.* **38**, 191–201. (doi:10.1007/BF02515727)

Clay, K., Holah, J. & Rudgers, J. A. 2005 Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proc. Natl Acad. Sci. USA* **102**, 12 465–12 470. (doi:10.1073/pnas.0503059102)

Darby, A. C. & Douglas, A. E. 2003 Elucidation of the transmission patterns of an insect-borne bacterium. *Appl. Environ. Microbiol.* **69**, 4403–4407. (doi:10.1128/AEM.69.8.4403-4407.2003)

Darby, A. C., Birkle, L. M., Turner, S. L. & Douglas, A. E. 2001 An aphid-borne bacterium allied to the secondary symbionts of whitefly. *FEMS Microbiol. Ecol.* **36**, 43–50. (doi:10.1111/j.1574-6941.2001.tb00824.x)

Douglas, A. E. 1998 Nutritional interactions in insect–microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annu. Rev. Entomol.* **43**, 17–37. (doi:10.1146/annurev.ento.43.1.17)

Dunbar, H. E., Wilson, A. C. C., Ferguson, N. R. & Moran, N. A. 2007 Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biol.* **5**, 1006–1015. (doi:10.1371/journal.pbio.0050096)

Fukatsu, T., Nikoh, N., Kawai, R. & Koga, R. 2000 The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **66**, 2748–2758. (doi:10.1128/AEM.66.7.2748-2758.2000)

Gwynn, D. M., Callaghan, A., Gorham, J., Walters, K. F. A. & Fellowes, M. D. E. 2005 Resistance is costly: trade-offs between immunity, fecundity and survival in the pea aphid. *Proc. R. Soc. B* **272**, 1803–1808. (doi:10.1098/rspb.2005.3089)

Koga, R., Tsuchida, T. & Fukatsu, T. 2003 Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont Buchnera in an aphid. *Proc. R. Soc. B* **270**, 2543–2550. (doi:10.1098/rspb.2003.2537)

Montllor, C. B., Maxmen, A. & Purcell, A. H. 2002 Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecol. Entomol.* **27**, 189–195. (doi:10.1046/j.1365-2311.2002.00393.x)

Moran, N. A. & Dunbar, H. E. 2006 Sexual acquisition of beneficial symbionts in aphids. *Proc. Natl Acad. Sci. USA* **103**, 12 803–12 806. (doi:10.1073/pnas.0605772103)

Moran, N. A., Russell, J. A., Koga, R. & Fukatsu, T. 2005 Evolutionary relationships of three new species of *Enterobacteriaceae* living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.* **71**, 3302–3310. (doi:10.1128/AEM.71.6.3302-3310.2005)

Oliver, K. M., Russell, J. A., Moran, N. A. & Hunter, M. S. 2003 Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl Acad. Sci. USA* **100**, 1803–1807. (doi:10.1073/pnas.0335320100)

Oliver, K. M., Moran, N. A. & Hunter, M. S. 2005 Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc. Natl Acad. Sci. USA* **102**, 12 795–12 800. (doi:10.1073/pnas.0506131102)

Oliver, K. M., Moran, N. A. & Hunter, M. S. 2006 Costs and benefits of a superinfection of facultative symbionts in aphids. *Proc. R. Soc. B* **273**, 1273–1280. (doi:10.1098/rspb.2005.3436)

Russell, J. A., Latorre, A., Sabater-Munoz, B., Moya, A. & Moran, N. A. 2003 Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Molecular Ecology* **12**, 1061–1075. (doi:10.1046/j.1365-294X.2003.01780.x)

Russell, J. A. & Moran, N. A. 2006 Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc. R. Soc. B* **273**, 603–610.

Sandström, J. P., Russell, J. A., White, J. P. & Moran, N. A. 2001 Independent origins and horizontal transfer of bacterial symbionts of aphids. *Mol. Ecol.* **10**, 217–228. (doi:10.1046/j.1365-294X.2001.01189.x)

Scarborough, C. L., Ferrari, J. & Godfray, H. C. J. 2005 Aphid protected from pathogen by endosymbiont. *Science* **310**, 1781. (doi:10.1126/science.1120180)

Simon, J. C., Carre, S., Boutin, M., Prunier-Leterme, N., Sabater-Munoz, B., Latorre, A. & Bournoville, R. 2003 Host-based divergence in populations of the pea aphid: insights from nuclear markers and the prevalence of facultative symbionts. *Proc. R. Soc. B* **270**, 1703–1712. (doi:10.1098/rspb.2003.2430)

Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T. & Fukatsu, T. 2002 Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. *Mol. Ecol.* **11**, 2123–2135. (doi:10.1046/j.1365-294X.2002.01606.x)

Tsuchida, T., Koga, R. & Fukatsu, T. 2004 Host plant specialization governed by facultative symbiont. *Science* **303**, 1989. (doi:10.1126/science.1094611)

Werren, J. H. & O'Neill, S. L. 1997 *Influential passengers: inherited microorganisms and arthropod reproduction*. Oxford, UK; New York, NY: Oxford University Press.

Werren, J. H. & Windsor, D. M. 2000 Wolbachia infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. B* **267**, 1277–1285. (doi:10.1098/rspb.2000.1139)

Xi, Z. Y., Khoo, C. C. H. & Dobson, S. L. 2005 Wolbachia establishment and invasion in an *Aedes aegypti* laboratory population. *Science* **310**, 326–328. (doi:10.1126/science.1117607)

Zchori-Fein, E. & Perlman, S. J. 2004 Distribution of the bacterial symbiont Cardinium in arthropods. *Mol. Ecol.* **13**, 2009–2016. (doi:10.1111/j.1365-294X.2004.02203.x)