Effects of bacterial secondary symbionts on host plant use in pea aphids

A. H. C. McLean1, M. van Asch1, J. Ferrari2 and H. C. J. Godfray1,*

1Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK
2Department of Biology, University of York, PO Box 373, York YO1 5YW, UK

Aphids possess several facultative bacterial symbionts that have important effects on their hosts’ biology. These have been most closely studied in the pea aphid (Acyrthosiphon pisum), a species that feeds on multiple host plants. Whether secondary symbionts influence host plant utilization is unclear. We report the fitness consequences of introducing different strains of the symbiont Hamiltonella defensa into three aphid clones collected on Lathyrus pratensis that naturally lack symbionts, and of removing symbionts from 20 natural aphid–bacterial associations. Infection decreased fitness on Lathyrus but not on Vicia faba, a plant on which most pea aphids readily feed. This may explain the unusually low prevalence of symbionts in aphids collected on Lathyrus. There was no effect of presence of symbiont on performance of the aphids on the host plants of the clones from which the H. defensa strains were isolated. Removing the symbiont from natural aphid–bacterial associations led to an average approximate 20 per cent reduction in fecundity, both on the natural host plant and on V. faba, suggesting general rather than plant-species-specific effects of the symbiont. Throughout, we find significant genetic variation among aphid clones. The results provide no evidence that secondary symbionts have a major direct role in facilitating aphid utilization of particular host plant species.

Keywords: aphid; secondary symbiont; mutualism; Acyrthosiphon pisum; host specialization

1. INTRODUCTION

Although insects have long been known to form symbioses with bacteria, it is only in the last few decades that the extent to which this occurs has become apparent. Many insects have obligate mutualistic relationships with micro-organisms that provide essential substances missing in their diet [1]. Examples of these primary symbionts include Buchnera (which is present in nearly all species of aphid and compensates for the deficiency of amino acids and other compounds in their phloem diet [2]) and Wigglesworthia (which performs an analogous function for tsetse flies (Glossina) feeding on vertebrate blood [3]). In other insect–bacterial associations, the symbionts are not essential for normal host growth and development, which raises the questions of how and why such facultative or secondary symbioses are maintained. In some cases, the bacterium manipulates host reproduction in ways that allow it to spread through the population, such as in the very widespread Wolbachia [4]. In other associations, the symbiont appears to increase host fitness, but only under certain ecological conditions.

Secondary symbionts that confer conditional adaptive advantages to their hosts have been particularly well studied in aphids. In the pea aphid (Acyrthosiphon pisum; Hemiptera: Aphididae), the best-characterized secondary symbionts are three species of γ-Proteobacteria: Regiella insecticola, Hamiltonella defensa and Serratia symbiotica [5]. A large majority of aphid clones contain one and sometimes two of these symbionts [6,7], which are transmitted with high fidelity from mother to offspring and can also be transmitted paternally in the sexual generation [8]. Experiments that have either removed or introduced symbionts have shown that the bacteria can increase their hosts’ ability to defend themselves from parasitoids [9] and entomopathogenic fungi [10], as well as to withstand heat shock [11–13]. Aphid secondary symbionts have also been shown to influence aspects of aphid life history such as the frequency of production of winged morphs [14], though the benefits, if any, of these effects are less clear.

Pea aphids feed on plant species belonging to the family Fabaceae and the pea aphid taxon consists of a series of genetically differentiated host-associated populations connected to differing degrees by gene flow [15–17]. However, a curious feature of pea aphid biology is that while most clones collected in the wild are specialized on a particular host plant species, they nearly all perform well on certain species of vetch (Vicia) [18,19]; gene flow between aphid populations might therefore occur on these species. Pea aphids have been frequently used to study the evolution of specialization and ecological speciation [17,18,20–24], and hence it is natural to ask if secondary symbionts have a role in the adaptation of their hosts to different food plants. Surveys of secondary symbionts clearly show that particular species are strongly associated with aphids feeding on certain food plants—most pea aphid clones on clover (Trifolium), for example, harbour R. insecticola, while those on alfalfa (Medicago) usually carry H. defensa [7,25–28]. While these patterns may reflect a role of secondary symbionts in host plant use, they may also arise because of factors...
coated with host plant use (risk of exposure to natural enemies for example) or simple historical contingency.

Studies designed to distinguish between these explanations have given somewhat contradictory results. Tsuchida et al. [29] used antibiotics to remove R. insecticola from a clover-associated pea aphid clone and found that performance on Trifolium, but not Vicia, was negatively affected. However, in a similar experiment, Leonardo [30] found no fitness effects of removing R. insecticola from two clones of aphid specialized on Trifolium. A third study [31] found that the artificial introduction of R. insecticola into five symbiont-free clones not previously associated with clover could have positive, negative or no effect on performance of Trifolium (there was no overall effect of the introduction but a significant clone by treatment interaction). These results suggest that interactions involving the genotype of either the host or symbiont (or both) can influence host plant use, as has been found in the study of other traits [13,32,33], and also emphasizes the importance of using replicate genotypes in investigations of symbiont biology.

To clarify the role of secondary symbionts in host plant use by pea aphids, we manipulated symbiont composition and then assessed aphid fitness on different species of plant using a much broader range of aphid clones (from different host plant-associated populations and with different symbionts) than hitherto studied. We did this in two ways. Aphids specialized on Lathyrus pratensis have unusually low rates of infection with secondary symbionts; first, we took such clones without secondary symbionts and established novel infections by injecting strains of the secondary symbiont H. defensa that had been harvested from aphids associated with different host plants. The fitness of the same clone of Lathyrus aphid with and without the symbiont was measured on (i) Lathyrus, (ii) the plant species from which the symbiont’s host was collected (where possible) and (iii) the widely acceptable host, Vicia faba. Second, we took 20 clones of aphids drawn from five different host-associated populations, each of which contained the secondary symbiont most commonly associated with their host plant species. By oral administration of antibiotics that are known not to affect the primary symbiont [34], aphid lineages that did not contain secondary symbionts were created. The fitness of aphid clones with and without secondary symbionts was compared on the host plant from which they were collected and on Vicia. This allows us to investigate whether or not any observed fitness effects of secondary symbionts are specific to the host plants with which they are found associated in nature, and thus assess the likelihood that the symbionts are influencing aphid specialization.

2. MATERIAL AND METHODS
(a) Experimental organisms
All pea aphid clones were derived from single individuals originally collected in Berkshire and Oxfordshire (southern England) during June and July 2003, and July and August 2008. It was confirmed that these aphid clones were specialized on the plants from which they were collected by assaying their survival and fecundity on the collection plant species. The aphid clones were confirmed as being genetically distinct from one another through microsatellite typing [18].

The facultative symbiont complement of the different aphid clones was assessed by repeated amplification (or attempted amplification) of the bacterial 16S ribosomal RNA gene using a ‘universal’ bacterial primer pair (10F, 35R) with partial sequencing of the fragment [11,35]. These primers span the 16S–23S rRNA genes and therefore detect a wide range of Eubacteria, with the primary symbiont Buchnera being a notable exception. This was followed by diagnostic PCRs using primers specific to the 16S ribosomal RNA genes of four known pea aphid facultative symbionts (Hamiltonella, Regiella, Serratia and a bacterium currently referred to as ‘X-type’) to check for multiple infections. ‘X-type’ is a less well-characterized γ-Proteobacteria secondary symbiont [36]. Aphid clones were screened using diagnostic PCR for the presence of Richettsia and Spiroplasma infections, both of which have been recorded as facultative associates of pea aphids [37–39]. Appropriate negative and positive controls were used in each case, and details of all primers used are given in the electronic supplementary material, table S1. The secondary symbionts found in the different aphid clones are shown in table 1.

(b) Creating artificial secondary symbiont infections
Novel associations between pea aphids and the facultative symbiont H. defensa were created by injecting naturally symbiont-free aphids (the ‘recipient’ clones; shown to be symbiont-free by the absence of PCR product after attempted amplification using the universal 10F/35R bacterial primer pair and specific primers for Richettsia and Spiroplasma) with haemolymph extracted from four different naturally infected pea aphid clones (the ‘donor’ clones).

### Table 1. The aphid clones used in the symbiont-removal experiments, the host plants on which they are specialized and the secondary symbionts they contained.

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Symbiont</th>
<th>Clones</th>
<th>Clone codes</th>
</tr>
</thead>
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<tr>
<td>Medicago sativa</td>
<td>Hamiltonella defensa</td>
<td>5 clones; one (341) also contained the ‘X-type’ symbiont, one (222) also contained R. insecticola, one (161) also contained Spiroplasma</td>
<td>161, 222, 328, 340, 341</td>
</tr>
<tr>
<td>Trifolium repens</td>
<td>Regiella insecticola</td>
<td>5 clones</td>
<td>102, 126, 313, 317, 319</td>
</tr>
<tr>
<td>Lotus pedunculatus</td>
<td>Hamiltonella defensa</td>
<td>5 clones; three (141, 184 and 208) also contained Rickettsia</td>
<td>132, 141, 184, 208, 224</td>
</tr>
<tr>
<td>Pum sativum</td>
<td>Serratia symbiotica</td>
<td>2 clones</td>
<td>256, 316</td>
</tr>
<tr>
<td>Ononis spinosa</td>
<td>Hamiltonella defensa</td>
<td>3 clones</td>
<td>101, 123, 133</td>
</tr>
</tbody>
</table>
Four different donor clones were used: two from *Lotus pedunculatus*, one from *Medicago sativa* and one from *Ononis spinosa*. All were infected with *H. defensa*, the facultative symbiont most commonly found in aphids on each of these host plants. One clone (208) also contained *Rickettsia* and one (161) *Spiroplasma*. Following injection, recipient aphids were maintained at 14 °C in Petri dishes containing healthy leaves of *V. faba* (cv. The Sutton), which were kept fresh by inserting the petiole into 2 per cent agar gel. *Vicia faba* is a host plant species on which almost all pea aphid clones have been found to perform well [18,20,40]. Each artificially infected clone was derived from a single injected individual and was screened regularly to confirm the continued presence of *H. defensa* (we observed no loss of symbionts after the first few generations; in the case of the two co-infections, both symbionts were established). Experiments were carried out at least six generations after the initial injection.

(c) Curing natural secondary symbiont infections

Natural γ-Proteobacteria secondary symbiont infections were eliminated from 20 aphid clones using oral administration of antibiotics. The clones were collected on *L. pedunculatus*, *M. sativa*, *O. spinosa*, *Pisum sativum* and *Trifolium pratense*, and all possessed the secondary symbiont most commonly associated with that host plant (table 1). Cut leaves of *V. faba* were placed in 1.5 ml Eppendorf tubes containing 100 μg ml⁻¹ Ampicillin, 50 μg ml⁻¹ Cefotaxime and 50 μg ml⁻¹ Gentomicin [34,41], and second instar aphids were allowed to feed on them for 3–4 days at 14 °C. Surviving aphids were then transferred to a *Vicia* leaf with its petiole inserted into agar gel. Once adult, the aphids were separated and the late offspring (over tenth in birth order) retained. After they had begun reproducing, second generation aphids were tested for the presence of secondary endosymbionts using symbiont-specific 16S rRNA primers as described above. Offspring of adults found to lack γ-Proteobacteria were retained to find an infection-free clonal line, which was considered cured if there was a complete lack of amplification at least six generations following antibiotic treatment. The absence of γ-Proteobacteria was reconfirmed after the experiments had been conducted.

Our antibiotic curing protocol did not affect *Spiroplasma* or *Rickettsia*. We found the former in one clone and the latter in three clones (table 1) and confirmed that the infection status was the same in both the original lines and in the lines cured of the γ-proteobacterial secondary symbionts. Differences between the two sets of lines were thus owing to changes in the γ-Proteobacteria symbiont infection.

(d) Effects of artificial infection with Hamiltonella defensa on aphid fitness

Aphids were reared on pre-flowering plants of *V. faba* for two generations prior to the experiments in cultures maintained at 20 °C, 60 per cent relative humidity and a 16 L:8 D light:dark cycle. Experiments were carried out under the same environmental conditions (further details of the plants used in the experiments can be found in the electronic supplementary material, table S2). The fitness of aphids with and without the symbiont *H. defensa* was assessed on *Lathyrus*, *Vicia* and on the plant species from which the donor aphid was collected (except that for logistic reasons this was not possible for *O. spinosa*). Aphids were allowed to reproduce on fresh leaves of *Vicia* for up to 24 h, and 8–10 of the resultant offspring (in a very small number of cases fewer) were placed on similarly sized, fresh and healthy plants of the appropriate species. The total number of offspring produced by the aphids between days 7 and 12, controlling for the number of aphids alive at day 7, was used as our measure of fitness. This composite measure incorporates differences in development time, age at first reproduction and number of offspring produced in early adulthood. Five replicates (stratified across five temporal blocks) were carried out for aphid clones tested on *Lathyrus*, *Lotus* and *Medicago* and six replicates (in three temporal blocks) for tests on *Vicia*.

(e) Effects of secondary symbiont removal upon aphid fitness

Aphid pre-treatment and experiments were conducted under the same conditions as described in §2d above. The fitness of aphids with and without their natural facultative symbiont infections was assessed on the plant species from which they were collected and on *Vicia*. Adult aphids were allowed to reproduce on fresh leaves of either *Vicia* or their natural host for 24 h and the offspring produced (typically 6–10) transferred to plants of the same species. After 7 days, any second-generation offspring and all except three of the adult aphids were removed, and the total number of further offspring produced over the next 8 days was recorded as a measure of fitness (in the case of *Vicia*, the three adult aphids were transferred to a fresh plant as the quality of the seedling plant declines rapidly when fed upon). On average, six replicates were carried out for each aphid clone on each plant species, distributed across nine (*Lotus, Medicago* and *Trifolium*), three (*Ononis*) or four (*Pisum*) temporal blocks.

(f) Statistical analysis

The data were analysed using generalized linear modelling techniques. The data on fecundity are counts and hence were analysed using log-linear techniques with the assumption of quasi-Poisson error variance to allow for overdispersion. All analyses were performed using R (v. 2.6.1; http://www.r-project.org). In six of the 31 replicates used to estimate the fecundity of artificially infected aphids feeding on *Medicago*, all aphids died in the first 7 days and hence the replicates were omitted from the analysis.

3. RESULTS

(a) Symbiont injection

If secondary symbionts improve the ability of their aphid hosts to feed on a particular plant species, then we would expect improved performance of the recipient clone on the plant to which the donor strain’s host is specialized. We were able to test this for *Lotus* and *Medicago*. The uninfected recipient clones performed very poorly on *Lotus*, producing no offspring on this species. This poor performance was not affected by the injection of *H. defensa* derived from *Lotus* aphids; infected aphids also produced no offspring. Uninfected recipient clones were able to survive and reproduce on *Medicago*, though their fecundity on this host plant was considerably less than that on *Lathyrus* (*F*₁,₁₀₀ = 251.4, *p* < 0.001). However, contrary to the hypothesis, lines that had been injected with *H. defensa* derived from aphids collected on *Medicago* produced fewer offspring on *Medicago* than the uninfected controls (*F*₁,₂₆ = 5.53, *p* = 0.029). We also observed significant differences among recipient clones in their
response to the introduction of *H. defensa* when feeding on *Medicago* ($F_{2,20} = 7.01, p = 0.005$).

If carriage of secondary symbionts incurs costs for their hosts, then we might expect reduced fitness on the natural host of the recipients (*Lathyrus*) or on *Vicia*, the host on which most aphid clones perform well. We found that carrying *H. defensa* reduced the fecundity of recipient clones on their natural host (figure 1 and table 2), and also observed that the strength of this effect was significantly influenced by donor strain. Interestingly, the co-infections (one *Spiroplasma*, one *Rickettsia*) appear to have, if anything, a slightly less severe impact on their hosts than the infections with *Hamiltonella* alone. On *Vicia*, there was a large and significant difference in the performance of the aphid genotype (recipient clones), with one performing very poorly irrespective of infection status. For the remaining recipient clones, we found no effect of infection status on fecundity on *Vicia* (figure 1 and table 2).

(b) Symbiont removal

Secondary symbionts were removed from 20 clones of aphids collected on five species of host plant (details in table 1). We initially analysed the complete dataset together, fitting a ‘minimal model’ that controlled for all the factors that were not of immediate interest, before adding terms representing infection status and its interactions. The minimal model included terms for collection plant species, the test plant species on which the aphids’ fitness was assessed (this was either the collection plant species or *Vicia*), aphid clone, experimental block, the aphid clone × test plant interaction and the proportion of alate adults among the progeny produced in the experiment. An analysis of the components of the minimal model is given in the electronic supplementary material, table S3.

Loss of the symbiont on average reduced aphid fecundity. Adding a term for infection status to the minimal model significantly increased the explanatory power of the model (table 2) and for 1 d.f. explained 11.1 per cent of the remaining variation (the residual deviance after fitting the minimal model) in the data. On average, the loss of symbionts leads to a 21 per cent reduction (s.e. 19–24%) in offspring numbers. However, there was significant variation in the response of aphids collected on different host plants, as well as among the different aphid clones themselves (table 3). We explored whether symbiont loss affected aphid performance differently on their collection plant or on *Vicia* by adding the interaction of test plant and infectious status to the statistical model containing all the above terms. A significant difference was found, with fecundity on average being more affected on *Vicia*, but the effect was not large (it explained only 0.6% of the variation in the data for 1 d.f.) and a significant three-way interaction revealed that its strength varied among aphids from different collection plants (table 3).

To understand better how aphids from different host plant-associated populations responded to the loss of their symbionts we conducted separate analyses for the clones collected from each of the five host plants (figure 2; further details in the electronic supplementary material, table S4). Aphids from *Lotus* and *Trifolium* on average experienced reduced fecundity after symbiont removal, and this effect was stronger on *Vicia* than on their collection plant. There were also significant differences in response to symbionts across clones for aphids from *Trifolium*. Clones from *Pisum* and *Ononis* again on average showed reduced fecundity in the absence of the symbiont, though this effect was not consistently stronger when the aphids were tested on *Vicia*. With the *Ononis* clones, there was a significant three-way interaction; this group contained the only clone to be disadvantaged on its collection plant, but not on *Vicia*, by the loss of the symbiont. Finally, the response of *Medicago* clones was variable: different clones had unchanged or lower fecundity when their symbiont was removed (and hence there was no clear overall response), though the response of infected and uninfected aphids within a clone was similar when tested on either *Medicago* or *Vicia*.

4. DISCUSSION

We found no evidence that secondary symbionts have a major effect on host-plant specialization in the pea aphid. Introducing symbionts to aphid lineages that naturally had no bacteria did not improve aphid performance on the plant species with which the symbionts were originally associated. In fact, while aphid performance on *Vicia* was little affected by introduced symbionts, performance on their natural host plant, *Lathyrus*, was reduced. Removing symbionts from a variety of natural aphid–bacteria associations on average reduced fecundity by approximately 20 per cent, but the drop in fitness was greater on *Vicia* than on the original host plant, suggesting

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**Figure 1.** The effect of symbiont presence on aphid fecundity when feeding on (a) *Lathyrus* and (b) *Vicia*. The figure compares three recipient clones (codes 145, 178 and 191) when they carry no symbiont or after the injection of symbiont from four donor clones (codes 101, 132, 208 and 161). In one case, indicated by an asterisk, we failed to establish an infection. Symbiont status: Light grey bars, none; dotted bars, 101; dark grey bars, 132; white bars, 208; striped bars, 161.
that this was a general rather than a plant-species-specific cost of symbiont removal. Throughout, we found that the effects on aphid fitness of removing secondary symbionts tended to vary among aphid genotypes.

Strong correlative associations have been reported between certain symbiont species and aphid populations adapted to feeding on particular plant species; for example, *Trifolium*-feeding clones normally carry *R. insecticola*. This combination has previously been investigated experimentally by Tsuchida et al. [29], Leonardo [30] and Ferrari et al. [31]; our study included five such clones. We found that curing aphids of their natural *R. insecticola* infections had a negative impact on host fitness, in agreement with Tsuchida et al. [29]; however, the results on *Vicia* confirm the conclusions of Leonardo [30] and Ferrari et al. [31] that the presence of *R. insecticola* does not provide specific fitness advantages on *Trifolium*. We obtained similar results for the four other host plants we investigated. Overall, we conclude that no symbiont studied provides any specific advantage to feeding on the host plant with which it is associated, though the presence of often substantial interactions between host genotype and infection status may mean that a particular clone enjoys this benefit, as observed by Tsuchida et al. [29]. We cannot of course rule out the possibility that there is geographical variation in the influence of secondary symbionts on host plant use.

There remains the question of why secondary symbiont distribution is correlated strongly with particular host-plant-associated aphid populations. It seems unlikely that the associations are merely founder effects, an echo of the symbiont flora that was coincidentally associated with the aphids that first colonized a new host plant. Some associations, such as that between *R. insecticola* and *Trifolium*-feeding aphids, are found throughout the world [7,25–28]. It is therefore much more likely that the symbiont–host-plant correlation occurs because different secondary symbionts are selectively advantageous on different host plants for reasons other than direct nutrition-related fitness benefits. It would be difficult but very interesting to see if, for example, aphids on *Trifolium* are more often subject to infection

Table 2. Effects of artificial symbiont infection on the cumulative number of offspring produced by groups of aphids over the first 12 days of adult life, measured on either *Lathyrus* or *Vicia*. Terms were added in the order shown in the table, and the change in degrees of freedom and the deviance explained (the raw figure and as a percentage of the total deviance) are given along with the *F*-statistic and its associated probability (in bold when < 0.05).

<table>
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<tr>
<th>factor(s)</th>
<th>d.f.</th>
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<th>% deviation</th>
<th><em>F</em></th>
<th><em>p</em></th>
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Table 3. Effects of artificial symbiont removal on the cumulative number of offspring produced by groups of three aphids aged between 8 and 15 days. A minimal model described in the text and analysed further in the electronic supplementary material was fitted to the data. Further terms were added in the order shown in the table and the change in degrees of freedom and the deviance explained (the raw figure and as a percentage of the total deviance) are given along with the *F*-statistic and its associated probability (in bold when < 0.05).

<table>
<thead>
<tr>
<th>factor(s)</th>
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<th>deviance</th>
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<td>infection × test plant</td>
<td>1</td>
<td>44.1</td>
<td>0.6</td>
<td>5.00</td>
<td>0.026</td>
</tr>
<tr>
<td>infection × test plant × collection plant</td>
<td>4</td>
<td>129.4</td>
<td>1.9</td>
<td>3.67</td>
<td>0.006</td>
</tr>
<tr>
<td>infection × test plant × clone</td>
<td>15</td>
<td>224.4</td>
<td>3.3</td>
<td>1.70</td>
<td>0.049</td>
</tr>
<tr>
<td>remaining deviance</td>
<td>419</td>
<td>4113.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Secondary symbionts of aphids  A. H. C. McLean et al.  765

Were secondary symbionts to have had a consistent effect on host plant use, it would have complicated the interpretation of the many studies of the evolution of specialization and ecological speciation that have made use of the pea aphid system [17,18,20–24]. Our results suggest that direct effects of symbiont presence on host plant species use are not pervasive, although they do not rule out indirect effects mediated by other aspects of the aphid’s biology that might be correlated with host plant species.

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REFERENCES


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Figure 2. The effects of eliminating the natural secondary symbiont infections from aphid clones collected on *Lotus*, *Medicago*, *Ononis*, *Pisum* and *Trifolium*. The figure shows fecundity of infected (dotted) and uninfected (undotted) aphids when feeding on the plant species on which they were collected (grey bars) or *Vicia* (white bars).


