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Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria

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The interactions between herbivorous insects and their symbiotic micro-organisms can be influenced by the plant species on which the insects are reared, but the underlying mechanisms are not understood. Here, we identify plant nutrients, specifically amino acids, as a candidate factor affecting the impact of symbiotic bacteria on the performance of the phloem-feeding aphid *Aphis fabae*. *Aphis fabae* grew more slowly on the labiate plant *Lamium purpureum* than on an alternative host plant *Vicia faba*, and the negative effect of *L. purpureum* on aphid growth was consistently exacerbated by the bacterial secondary symbionts *Regiella insecticola* and *Hamiltonella defensa*, which attained high densities in *L. purpureum*-reared aphids. The amino acid content of the phloem sap of *L. purpureum* was very low; and *A. fabae* on chemically defined diets of low amino acid content also grew slowly and had elevated secondary symbiont densities. It is suggested that the phloem nutrient profile of *L. purpureum* promotes deleterious traits in the secondary symbionts and disturbs insect controls over bacterial abundance.

Keywords: aphid; *Buchnera aphidicola*; plant usage; secondary symbiont; symbiosis

1. INTRODUCTION

Many herbivorous insects are chronically infected by symbiotic micro-organisms, some of which supplement the nutritionally poor plant diet with vitamins and essential amino acids (Buchner 1965; Douglas 1998; Moran & Telang 1998). These symbiotic micro-organisms are generally beneficial for the insect host but, as with some other symbioses (e.g. Redman *et al.* 2001; Backhed *et al.* 2005), their impact on the host is predicted to vary with host and symbiont genotype and with environmental conditions.

The plant usage traits of some insects are influenced by their complement of symbiotic micro-organisms (Chen *et al.* 2000; Hosokawa *et al.* 2007). This study explores the pattern and underlying mechanisms of microbial impacts on plant usage by the phloem-feeding aphid *Aphis fabae*. The microbiota of most aphids is dominated by the γ-proteobacterium *Buchnera aphidicola*, which provides the aphid with essential amino acids and is required by the insect: aphids experimentally deprived of *B. aphidicola* by antibiotic treatment ('aposymbiotic' aphids) grow very slowly and are reproductively sterile (Douglas 1998). Some aphids also bear bacterial 'secondary symbionts', e.g. *Serratia symbiotica*, *Hamiltonella defensa* and *Regiella insecticola* (Moran *et al.* 2005), which can, variously, promote resistance to parasitoids (Oliver *et al.* 2005) and entomopathogenic fungi (Scarborough *et al.* 2005), aphid usage of certain plants (Tsuchida *et al.* 2004) and thermal tolerance (Russell & Moran 2006). An interaction between secondary symbionts and the nitrogen nutrition of aphids is suggested by the demonstration that the density of *S. symbiotica* is elevated in aphids reared on low-nitrogen diets (Wilkinson *et al.* 2007), although other experimental data suggest that secondary symbionts do not contribute to the amino acid nutrition of aphids (Douglas *et al.* 2006*a*).

The basis for this study is the finding that untreated and aposymbiotic *A. fabae* grew at similar, low rates on the labiate plant *Lamium purpureum* (Adams & Douglas 1997), raising the possibility that symbiosis dysfunction induced by *L. purpureum* contributed to the poor aphid performance on this plant. Consistent with this interpretation, bacterial production of essential amino acids, especially threonine, was depressed in *A. fabae* on *L. purpureum*, even though the bacterial density was elevated (Wilkinson *et al.* 2001). These effects of *L. purpureum* cannot be attributed to low growth rates because *A. fabae* reared on other plant species that support poor growth rates did not have elevated bacterial densities or modified amino acid nutrition (Wilkinson *et al.* 2001).

Our approach to investigate how the symbiotic bacteria and plant nutrients affect *A. fabae* performance on *L. purpureum* involved experimental manipulation of the microbial complement of the aphids and analysis of aphid nitrogen nutrition. Following the evidence that aphid genotype can influence secondary symbiont impacts on aphid traits (e.g. Russell & Moran 2006), the experiments used multiple clones of *A. fabae*. The choice of secondary symbiont taxa for these experiments was decided by an investigation of the prevalence of secondary symbiont taxa in natural populations of *A. fabae* colonizing *L. purpureum* and other host plants of *A. fabae*; and these data are presented first.

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2. MATERIAL AND METHODS

(a) *Field aphid populations*

Vicia faba cv. The Sutton, *L. purpureum*, *Chenopodium album* and *Papaver dubium* were raised from seed in a common garden experiment, following the protocol of Darby *et al.* (2003). Twice-weekly between 10 June and 30 September 2003, eight pre-flowering plants of each species were examined for colonizing *A. fabae* and all aphids on the plants were removed. The bacterial complement of all adults which had colonized (as defined by proximity to young larvae, presumably deposited by the adult) was determined by diagnostic PCR.

(b) *Experimental aphids and plants*

Sixteen clones of *Aphis fabae fabae* (clones 1–8 lacking secondary symbionts, clones 9–12 with the secondary symbiont *H. defensa* and clones 13–16 with *R. insecticola*) were reared at 18°C with 18 hours L : 6 hours D on the test plants: pre-flowering *V. faba* cv. The Sutton, on which all *A. f. fabae* perform well (Stroyan 1984), and *L. purpureum*. All clones were reared on *Rumex obtusifolius* for at least 10 generations prior to this study, to ensure that their response to the test plants was not influenced by prior experience of different host plants.

To investigate aphid performance on plants, newborn aphids were caged individually in mesh-covered clip-on cages (2.5 cm internal diameter) on the test plants for 10 days, when they were final instar larvae. Aphid relative growth rate (RGR) was assessed by the formula ln(day-10 weight/day-0 weight)/10, with each of either eight aphids (for experiments with natural symbioses) or nine aphids (for manipulated symbioses) weighed to the nearest microgram on an MT5 microbalance (Mettler). For diet experiments, 10 replicate aphids were reared individually from birth for eight days on chemically defined diets containing 0.5 M sucrose and 25, 50, 100 or 150 mM amino acids (Wilkinson & Douglas 2003), and then weighed individually.

(c) *Manipulations of secondary symbiont complement in aphids*

The secondary symbiont complement of *A. fabae* clones was manipulated in aphids reared on chemically defined diets. *Hamiltonella defensa* was eliminated from clones 10 and 11, and *R. insecticola* from clones 13 and 15 by feeding on diets supplemented with 50 µg ampicillin, cefotaxime and gentamicin ml^{-1} (Douglas *et al.* 2006*a*). Aphids were infected orally with *H. defensa* (clones 5 and 6) and *R. insecticola* (clones 7 and 8) added to the diet (Darby & Douglas 2003), using symbionts obtained from pea aphids *Acyrthosiphon pisum* because *A. fabae* consistently performed poorly when fed on diets containing *A. fabae* extracts. The aphids with manipulated secondary symbiont complements were maintained for at least 10 generations on *R. obtusifolius* prior to analysis, and their status was confirmed by the complementary methods of diagnostic PCR assay and fluorescence *in situ* hybridization. Without exception, congruent results were obtained by the two methods, and the localization of bacteria in aphids infected naturally and experimentally with secondary symbionts was indistinguishable (electronic supplementary material, figure 1).

(d) *Identification and quantification of symbiotic bacteria in aphids*

Buchnera aphidicola, *S. symbiotica*, *H. defensa* and *R. insecticola* were detected by diagnostic 16S rRNA gene endpoint PCR, following the protocol of Douglas *et al.* (2006*a*). The bacteria in clone 13 were quantified by the direct counting method of Wilkinson *et al.* (2001) and in clones 5–8, 11, 12, 15 and 16 by TaqMan quantitative PCR (Q-PCR; Douglas *et al.* 2006*b*) using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, UK). The primers and probes designed for the *dnaK* gene were as follows: *B. aphidicola* forward TGCGGCACTTGCATATGGT, reverse CGAAAGT CCCCCCACCTAAG, probe AGGCCAAGGGAACCGG ACTATAGCT GT; *H. defensa* forward CAAGCGGATT ATTAATGAACCCA, reverse TGGTGCT ATTCCC TTTTCCCT, probe CGCGGCCATTGCCTACGGTTT; and *R. insecticola* forward CCGTAAAGACGTCAATCCC G, reverse GCACCCCTCCTTGAATAGCA, probe C GAAGCGGTTGCGGTCGGTG. For the probe, FAM (6-carboxy fluorescein) was the 5′-terminal reporter dye and TAMRA (tetramethyl carboxyrhodamine) was the 3′-terminal quencher dye. Gene copy number was estimated from the standard curve generated with serial dilutions of linearized cloned *dnaK* gene fragments (the standards) and normalized to aphid weight. The standards and the DNA extracted from each of the eight replicate aphids per treatment were run in duplicate with template-free controls.

(e) *Collection and quantification of free amino acids*

Phloem sap from *L. purpureum* and *V. faba* was collected from the severed stylets of *A. fabae* (one sample per plant) obtained by high-frequency microcautery following the protocol of Pescod *et al.* (2007). Known weights of honeydew were collected over 24 hours from aphids confined to a leaf in clip-on cages lined with pre-weighed aluminium foil, and washed off the foil in a known volume of 80% methanol. Amino acids were separated by reverse-phase HPLC, following derivatization with *o*-phthaldialdehyde (Jones *et al.* 1981), using a Hewlett-Packard HP1100 Series autosampling LC system with C_{18} ZORBAX Eclipse XDB-C8 column (Agilent, UK) and fluorescence detection. AA-S-18 amino acid standards (Sigma, UK) supplemented with asparagine, glutamine and tryptophan were used for calibration.

(f) *Statistical analysis*

The frequency of aphids colonizing different plants and with different bacterial complements was analysed by χ^2-test. All other datasets were normally distributed with homogeneous variances, by the Ryan–Joiner one-sample test and Bartlett's test, respectively. The impact of two fixed factors, rearing plant ('plant') and either presence/absence of secondary symbionts ('symbiosis') or the identity of secondary symbionts ('secondary symbiont'), on the performance and bacterial abundance of aphids was investigated by ANOVA, with clone as a random factor nested within either symbiosis or secondary symbiont. For experiments using aphids with manipulated symbioses, the term 'treatment' was included as a third fixed main factor in the ANOVA. The error term for each source in the ANOVA was chosen from the pattern of components in expectations of mean squares obtained using standard rules (Steele & Torrie 1960). Full outputs from the statistical tests are provided in the electronic supplementary material, table 1.

Table 1. Prevalence of symbiotic bacteria in field populations of the aphid *A. fabae* colonizing four plant species between June and September 2003.

	number of aphids[a]				
plant species	total	with *Ba* only	with *Ba*+*Ss*	with *Ba*+*Hd*	with *Ba*+*Ri*
C. album	75	43	4	7	21
L. purpureum	80	24	7	17	32
P. dubium	71	38	1	9	23
V. faba	92	34	15	13	30

[a] *Ba*, *B. aphidicola*; *Ss*, *S. symbiotica*; *Hd*, *H. defensa*; *Ri*, *R. insecticola*.

3. RESULTS

(a) *Prevalence of symbiotic bacteria in field populations of* A. fabae

The field plot was colonized by 318 wild *A. fabae*. *Buchnera aphidicola* was detected in every aphid tested and secondary symbionts were detected in 179 (56%) aphids. No individual aphid bore more than one secondary symbiont. The most prevalent secondary symbiont was *R. insecticola*, and *S. symbiotica* was present at low frequencies in aphids from most plant species (table 1). The frequency of aphids with different bacterial complements varied significantly with plant species ($\chi^2_9 = 26.66$, $0.01 > p > 0.001$), and this could be attributed principally to the relatively high prevalence of *S. symbiotica* in aphids on *V. faba*.

(b) *Impact of rearing plant and bacterial symbiont complement on aphid performance*

The RGR of the 16 *A. fabae* clones was significantly depressed in aphids on *L. purpureum* relative to *V. faba* ($F_{1,14} = 225.22$, $p < 0.001$), but to a greater extent in clones with secondary symbionts than in secondary symbiont-free clones ($F_{1,14} = 23.83$, $p < 0.001$). Underlying these effects was significant variation among clones ($F_{14,224} = 2.38$, $0.01 > p > 0.001$), which was greater on *L. purpureum* than on *V. faba* ($F_{14,224} = 2.88$, $p < 0.001$; figure 1*a*; with full statistical analysis in the electronic supplementary material, table 1*a*). The performance of aphids bearing *H. defensa* and *R. insecticola* did not differ significantly ($p > 0.05$).

The negative impact of secondary symbionts on aphid performance on *L. purpureum* is open to two alternative explanations: that the secondary symbionts exacerbate the negative effect of *L. purpureum* on aphid performance; and that certain aphid genotypes are particularly prone to infection with secondary symbionts and, independently, are deleteriously affected by *L. purpureum*. To discriminate between these explanations, the performance of aphids with manipulated complements of secondary symbionts was investigated. Aphid performance on *L. purpureum* is predicted to be changed by the manipulations only if the secondary symbionts, and not aphid genotype, are responsible for the exacerbated negative effect of this plant on performance. Elimination of the secondary symbionts by antibiotic treatment improved the mean performance of all four clones tested on *L. purpureum* (figure 1*b*) to a greater extent than for aphids on *V. faba*-reared aphids (plant×treatment: $F_{1,128} = 13.33$, $p < 0.001$). In the reverse experiment, infection of clones

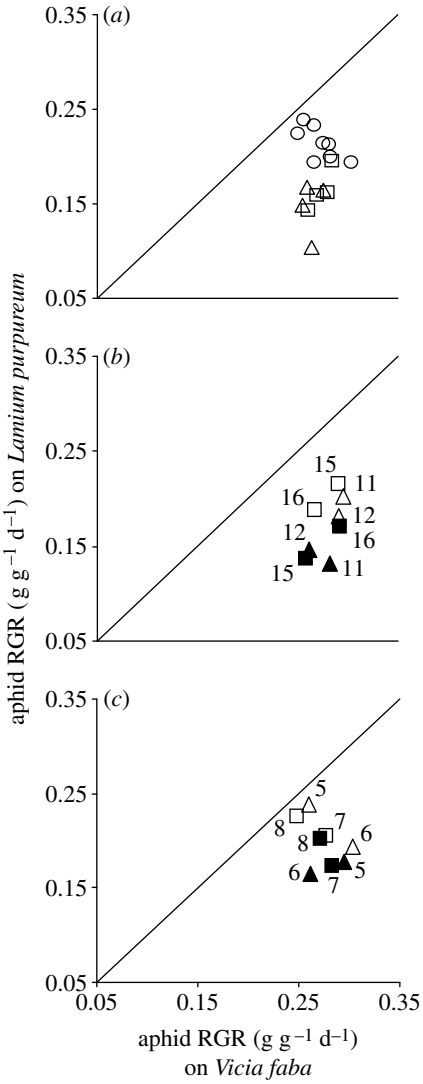

Figure 1. Aphid performance on *V. faba* and *L. purpureum*. (*a*) Clones with 'natural' bacterial complement ($n = 8$; open circles, secondary symbiont-free aphids; open triangles, aphids with *H. defensa*; open squares, aphids with *R. insecticola*); (*b*) clones cured of their natural secondary symbiont infections ($n = 9$; filled triangles, aphids with *H. defensa*; filled squares, aphids with *R. insecticola*; open triangles, aphids cured of *H. defensa*; open squares, aphids cured of *R. insecticola*); (*c*) clones experimentally infected with secondary symbionts ($n = 9$; filled triangles, aphids experimentally infected with *H. defensa*; filled squares, aphids experimentally infected with *R. insecticola*; open triangles, aphids lacking *H. defensa*; open squares, aphids lacking *R. insecticola*). Error bars are not displayed for clarity. Numbers in (*b*) and (*c*) indicate aphid clone numbers.

naturally lacking these bacteria had a more negative effect on the performance of aphids reared on *L. purpureum* than on *V. faba* (figure 1*c*: plant×treatment: $F_{1,128} = 72.41$, $p < 0.001$). Aphid performance also varied between the clones, as influenced by curing (electronic supplementary material, table 1*b*), plant species and infection (electronic supplementary material, table 1*c*).

(c) *Abundance of bacteria in aphids*

We hypothesized that aphid control over the populations of symbiotic bacteria may be disturbed in aphids reared on *L. purpureum*. The copy number of the *B. aphidicola dnaK* gene per unit aphid weight was significantly elevated

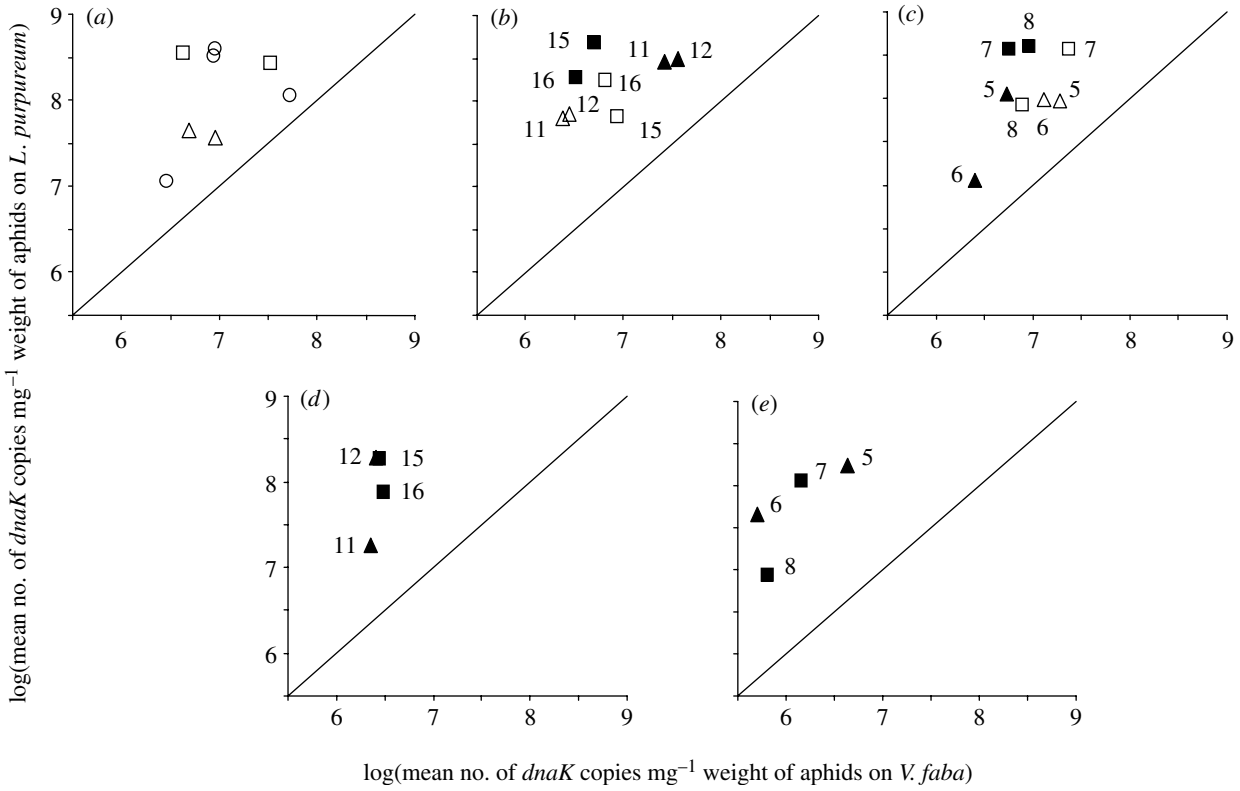

Figure 2. Impact of rearing plant on bacterial populations, quantified as copy number of the gene *dnaK*. (*a*) *Buchnera aphidicola* in clones with 'natural' bacterial complement (open circles, secondary symbiont-free aphids; open triangles, aphids with *H. defensa*; open squares, aphids with *R. insecticola*); (*b*) *B. aphidicola* in clones cured of their natural secondary symbiont infections (filled triangles, aphids with *H. defensa*; filled squares, aphids with *R. insecticola*; open triangles, aphids cured of *H. defensa*; open squares, aphids cured of *R. insecticola*); (*c*) *B. aphidicola* in clones experimentally infected with secondary symbionts (filled triangles, aphids infected with *H. defensa*; filled squares, aphids infected with *R. insecticola*; open triangles, aphids lacking *H. defensa*; open squares, aphids lacking *R. insecticola*); (*d*) secondary symbionts in clones with 'natural' bacterial complement (filled triangles, aphids with *H. defensa*; filled squares, aphids with *R. insecticola*); (*e*) secondary symbionts in clones experimentally infected with secondary symbionts (filled triangles, aphids infected with *H. defensa*; filled squares, aphids infected with *R. insecticola*). For all assays, $n=8$. Error bars are not included for clarity. Numbers in (*b*) to (*e*) indicate aphid clone numbers.

in all aphids on *L. purpureum*; both clones with natural secondary symbiont complements (figure 2*a*: $F_{1,7}=54.59$, $p<0.001$) and experimentally manipulated symbioses (figure 2*b*: $F_{1,112}=168.53$, $p<0.001$; figure 2*c*: $F_{1,112}=103.89$, $p<0.001$). The ANOVAs for all datasets revealed significant interclonal variation in bacterial abundance that, for the manipulated symbioses, was also influenced by treatment (electronic supplementary material, table 1*d–f*).

The copy number of the *dnaK* gene in the secondary symbionts was also significantly elevated in aphids reared on *L. purpureum* relative to those on *V. faba* for both aphid clones bearing their natural complement of bacteria (figure 2*d*: $F_{1,56}=75.32$, $p<0.001$) and aphids experimentally infected with secondary symbionts (figure 2*e*: $F_{1,56}=166.15$, $p<0.001$). A significant clonal effect was obtained for the latter experiment. Further analysis revealed no relationship between the performance of the various clones and the density of secondary symbionts on either plant species (data not shown).

(d) *Determinants of the aphid response to* L. purpureum

The honeydew (egesta) of *A. fabae* feeding from *L. purpureum* contained 5 ± 1.6 nmol amino acids mg^{-1} ($n=9$), 40-fold lower than 200 ± 60 nmol amino acids mg^{-1} honeydew produced by *A. fabae* on *V. faba* ($n=9$). These data demonstrate that the amino acid content of *L. purpureum* phloem sap is very low. *Lamium purpureum* is one of a number of plants ill-suited to direct quantification of phloem sap because sap flow ceases very rapidly on stylet severance. Even so, sufficient sap was obtained from three plants of *L. purpureum* to establish: first, that *L. purpureum* phloem sap is dominated by the sugar sucrose and free amino acids; and, second, that the mean amino acid content was 77 ± 30.1 mM (mean $\pm$ s.e., $n=3$), a value at 'the lower end' of published values (50–800 mM; Douglas 2003) and just 20% of the mean amino acid content of *V. faba* phloem sap, at 389 ± 51 mM ($n=4$).

To investigate the significance of the low amino acid content of *L. purpureum* for the *A. fabae* symbiosis, the impact of dietary amino acid concentration on the aphids was investigated using chemically defined diets with 25–150 mM amino acids (table 2). All aphids of clone 13 (which bears *R. insecticola*) survived the 8-day experimental period and weight gain was strongly dependent on dietary amino acid concentration. As previously observed for aphids on *L. purpureum*, the density of the secondary symbiont was elevated two- to threefold on the 25 mM amino acid diet relative to the

Table 2. Response of symbiosis in *A. fabae* clone 13 to dietary amino acid concentration over 8 days of larval development. (Values of mean ± s.e. (10 replicates) are shown; values in one column with the same superscript letter are not significantly different by Tukey's *post hoc* test ($p > 0.05$).)

dietary amino acids (mM)	aphid weight (mg)	density of bacteria ($10^7 \times$ number of bacteria mg^{-1} fresh weight)	
		B. aphidicola	*R. insecticola*
150	0.56 ± 0.016^{a}	2.28 ± 0.096	0.10 ± 0.007^{a}
100	0.33 ± 0.025^{b}	2.49 ± 0.136	0.13 ± 0.008^{a}
50	0.19 ± 0.015^{c}	2.27 ± 0.146	0.14 ± 0.010^{a}
25	0.13 ± 0.004^{d}	2.53 ± 0.072	0.29 ± 0.023^{b}
ANOVA: $F_{3,36}$	126.9, $p < 0.001$	1.4, $p > 0.05$	36.6, $p < 0.001$

other diets with higher amino acid concentrations. The density of *B. aphidicola* in the aphids did not vary significantly with diet.

4. DISCUSSION

A key result of this study is that, in *A. fabae* reared on *L. purpureum*, secondary symbionts exacerbate the deleterious impact of the plant on aphid performance (figure 1). This effect is accompanied by an elevated density of secondary symbionts in the aphids (figure 2*d*,*e*). Secondary symbiont densities are also high in aphids reared on low nitrogen diets (table 2), suggesting that the low nitrogen content of *L. purpureum* phloem sap (results of this study) might contribute to the high secondary symbiont density in the aphids. The amino acid composition of *L. purpureum* phloem sap and diets differ; and the high secondary symbiont densities on *L. purpureum* with 77 mM amino acids but on diets with 25 mM amino acids raise the possibility that secondary symbiont density is responsive to specific amino acids and not to the total amino acid concentration. Factors contributing to the greater responsiveness of the secondary symbionts than *B. aphidicola* to the diet may include differences between the bacteria in metabolic capabilities or access to aphid nutrients.

Elevated secondary symbiont densities and poor aphid performance have been identified previously for *Ac. pisum* reared on low nitrogen diets (Wilkinson *et al.* 2007) or infected with more than one secondary symbiont (Oliver *et al.* 2006), but poor performance is not always accompanied by high secondary symbiont numbers. For example, the poor performance of *A. fabae* on white clover *Trifolium repens* and *Ac. pisum* on high sucrose diets are not accompanied by elevated secondary symbiont densities (D. Adams and A. E. Douglas 1995, unpublished results; E. Jones and A. E. Douglas 2005, unpublished results). The relationship between secondary symbiont density and aphid performance may be causal, i.e. the high bacterial populations consume nutrients and cause other physiological disturbances that collectively depress aphid growth. Alternatively, the depressed growth and high bacterial populations may not be related causally, except in the sense that they are independent consequences of a dysfunction that remains to be identified.

Significantly, no consistent difference in the impact of the two secondary symbiont taxa, *H. defensa* and *R. insecticola*, on aphid usage of *L. purpureum* was evident, indicating that the effect is not dictated by genes specific to one taxon of secondary symbiont. Other effects of secondary symbionts on ecologically important traits of aphids are more taxon specific. For example, secondary symbiont-mediated resistance to parasitoids in the pea aphid is displayed by *H. defensa* and not by *R. insecticola* (Ferrari *et al.* 2004; Oliver *et al.* 2005). The considerable interclonal variation in the impact of secondary symbionts on aphid traits, as identified here and by various previous studies (e.g. Chen *et al.* 2000; Scarborough *et al.* 2005; Russell & Moran 2006), illustrates how the genotype of aphid and symbiont and their interactions can influence microbial impacts on insect–plant interactions.

The present study illustrates the importance of environmental context (here, rearing plant) on the impact of these micro-organisms on their hosts. Further research is required to establish whether the negative effects of secondary symbionts are an important factor contributing to the variable incidence of these bacteria in field populations of *A. fabae* and other aphids. The secondary symbionts, unlike *B. aphidicola*, are horizontally transmissible (Sandström *et al.* 2001; Darby & Douglas 2003; Moran & Dunbar 2006). The fitness of secondary symbionts may be enhanced by their high population density, even though their aphid host performs poorly, because the high population densities might enhance the opportunity for horizontal transmission away from the relatively unfit aphid host on *L. purpureum*.

In conclusion, this study offers the first identification of a candidate plant factor, low phloem amino acid concentration, contributing to the impact of secondary symbionts on aphid performance on plants. The interaction between the *A. fabae* symbiosis and *L. purpureum* is not a 'laboratory phenomenon'. *Lamium purpureum* in the field is colonized by *A. fabae* from natural populations, including individuals bearing secondary symbionts (table 1), and the aphid numbers increase slowly on *L. purpureum* to form small but persistent colonies (Raymond *et al.* 2000). Host plant-dependent impacts of symbiotic micro-organisms on the fitness of herbivorous insects may be a widespread and currently unrecognized dimension in insect–plant interactions.

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