Physiological Entomology (2001) **26**, 86–93

On the functional significance of symbiotic microorganisms in the Homoptera: a comparative study of *Acyrthosiphon pisum* and *Nilaparvata lugens*

T. L. WILKINSON and H. ISHIKAWA
Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo ku, Tokyo 113, Japan

Abstract. All phloem-feeding Homoptera possess symbiotic microorganisms. Although the phylogenetic position and anatomical location of the microorganisms differ, the underlying theme of the symbiosis is the same; the microorganisms improve the nutritional quality of the diet through the provision of essential amino acids. The symbiosis has been well documented in aphids, but little information is available from other homopteran groups. The impact of the loss of bacterial symbionts in the pea aphid *Acyrthosiphon pisum* Harris and eukaryotic yeast-like symbionts in the Asian rice brown planthopper *Nilaparvata lugens* Stål was examined in parallel. The weight and relative growth rate of aphids and planthoppers was significantly reduced by symbiont loss, and characteristic features of aposymbiotic pea aphids, so-called 'metabolic signatures', were, for the first time, observed in aposymbiotic *N. lugens*. For example, the amount of protein per unit fresh weight was reduced by 26 and 10%, and the free amino acid levels increased 1.8- and 1.4-fold, in aposymbiotic *A. pisum* and *N. lugens*, respectively. In addition, the concentration of the amino acid glutamine was elevated in the tissues of aposymbiotic insects. The data are discussed in the context of our current understanding of the nutritional role of the symbiosis and the mechanisms of nitrogen metabolism in the two insect species. It is concluded that the metabolic adjustments of the insects to symbiont loss are broadly equivalent.

Key words. Amino acids, aphid, aposymbiotic, bacteria, insect, planthopper, symbiosis, yeast-like symbionts.

Introduction

All phloem-feeding members of the Homoptera possess symbiotic microorganisms. Although the diversity, location and phylogenetic position of the symbionts varies (see Buchner, 1965; Douglas, 1989), the underlying theme of the symbiosis is the provision of essential amino acids. Phloem sap has an unbalanced amino acid composition dominated by non-essential amino acids, and there is now substantial evidence that the microorganisms in phloem-feeding insects synthesize essential amino acids that are made available to the insect host (see Douglas, 1989, 1998; Baumann *et al*., 1995, for full reviews). Interestingly, those Homoptera, such as typhlocybine leafhoppers, which have switched to feeding on intact plant cells, a diet with a more balanced nutritional content, have secondarily lost the symbiosis. The implication is clear. To quote Douglas (1998): 'the microbial symbiosis is causally linked with phloem feeding'.

The symbiosis in aphids (Sternorrhyncha: Aphidoidea) has been extensively studied. In these insects the symbiotic partners are bacteria located in specialized cells, called mycetocytes or bacteriocytes, loosely aggregated through the abdominal haemolymph (see Douglas, 1998, for full review). The bacteria have been assigned to the genus *Buchnera* (Munson *et al*., 1991). The impact of symbiont loss has also been well documented (e.g. Wilkinson, 1998). For example, bacteria-free or aposymbiotic pea aphids *Acyrthosiphon pisum*, produced by mild antibiotic therapy, have a reduced growth rate, attain a lower adult size, and their reproductive output is curtailed dramatically (Sasaki *et al*., 1991; Douglas, 1992). In addition, they exhibit so-called metabolic 'signatures' which can be attributed directly to the loss of their symbiotic partners.

Correspondence: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K. Tel.: +44 (0)121 4145481; fax: +44 (0)121 4145925; e-mail: T.L.Wilkinson@bham.ac.uk

In particular, the concentration of free amino acids in the tissues of aposymbiotic aphids is elevated, but the levels of protein are reduced, suggesting that protein synthesis is limited by a lack of essential amino acids (Prosser & Douglas, 1991; Liadouze *et al*., 1995; Wilkinson & Douglas, 1996). The amino acid glutamine is also significantly elevated in the tissues of aposymbiotic aphids, even when the insects are reared on diets which contain no glutamine. Loss of the symbiosis induces an elevated ammonia load in aposymbiotic aphids, and the accumulation of glutamine has been linked to the detoxification of ammonia through its incorporation into glutamate (Wilkinson & Douglas, 1995a). The robust nature of these metabolic signatures in aphids has been confirmed by analysis of similar responses in the black bean aphid *Aphis fabae* (Adams *et al*., 1996; Adams, 1996)

Although there is little detailed information from other homopteran groups concerning the functional significance of the symbiotic microorganisms and the response of the insect host to symbiont loss, it is clear that the *Buchnera* in pea aphids and other members of the Aphididae are not exceptional in their ability to improve the nutritional quality of phloem sap for their insect hosts. The study reported here addresses this issue in two phylogenetically distant insects, the pea aphid *Acyrthosiphon pisum* Harris (Sternorrhyncha: Aphididae) and the Asian rice brown planthopper *Nilaparvata lugens* Stål (Auchenorryncha: Delphacidae), and represents, to our knowledge, the first direct comparison of the requirement for symbiotic microorganisms in insects from different suborders within the Homoptera. Both *A. pisum* and *N. lugens* feed on phloem sap containing low concentrations of essential amino acids (Hayashi & Chino, 1990; Douglas, 1993; Sandström & Pettersson, 1994) and possess symbiotic microorganisms that supplement the diet through the provision of essential amino acids (Douglas, 1998; Koyama, 1985). However, the identity of the microorganisms differs considerably. *Acyrthosiphon pisum* contains well-documented populations of bacteria (*Buchnera*; see above), whereas the symbionts in *N. lugens* are eukaryotic and have historically been termed 'yeast-like symbionts' (e.g. Noda, 1974). Recent molecular studies have refined this classification and have placed the symbionts of *N. lugens* (and symbionts from other members of the Delphacidae) in the class Pyrenomycetes in the subphylum Ascomycotina (Noda *et al*., 1995; this study will refer to these microorganisms as yeast-like symbionts). In addition, the yeast-like symbionts in the small brown planthopper *Laodelphax striatellus* have been implicated in the production of 24-methylenecholesterol (Noda *et al*., 1979), whereas there is definitive evidence only for essential amino acid provisioning by the *Buchnera* in aphids.

The diversity of the microorganisms in the two species dictated the experimental design adopted in this study because the symbionts in *N. lugens* are not susceptible to antibiotic therapy. Disruption of the eukaryotic symbionts can be achieved using elevated temperature, and several studies have documented the deleterious impact of heat treatment on these microorganisms (Noda & Saito, 1979; Chen *et al*., 1981; Lee & Hou, 1987; Sasaki *et al*., 1996). Antibiotic and heat treatments were adopted in this study to examine in parallel the impact of symbiont loss in *A. pisum* and *N. lugens*, respectively.

Materials and Methods

Insect material

The insects used in this study were derived from long-term cultures maintained at the University of Tokyo. *Acyrthosiphon pisum* clone TLW97/TU9 was maintained as asexual viviparae on *Vicia faba* seedlings at 20°C, in LD 18 : 6 h regime, and *N. lugens* was maintained as a sexually reproducing colony on *Oryza sativa* seedlings at 25°C, in LD 18 : 6 h conditions.

Production of aposymbiotic insects

The bacterial symbionts in *A. pisum* were disrupted with an oral dose of the antibiotic rifampicin, administered via chemically defined diets (at 50 µg/mL diet) over the first 2 days of nymphal development. The diet was formulation A of Prosser & Douglas (1992), and full details of the antibiotic technique are given in Rahbé *et al*. (1994). The yeast-like symbionts in *N. lugens* were disrupted by rearing newly hatched nymphs at 35°C for 3 days (Noda & Saito, 1979; Chen *et al*., 1981; Sasaki *et al*., 1996). These methods have previously been demonstrated as effective in targeting the symbiotic microorganisms with little or no deleterious effects on the insects themselves (see Wilkinson, 1998, for a review of antibiotic-treatment in aphids, and Chen *et al*., 1981 and Sasaki *et al*., 1996, for studies on the impact of heat-treatment in eliminating the yeast-like symbionts from *N. lugens*). In both cases, symbiotic control insects were maintained under parallel conditions as aposymbiotic insects, but without the disrupting factor, i.e. symbiotic aphids were reared on chemically defined diets lacking antibiotic for the first 2 days of development, and symbiotic planthoppers were reared under standard culture conditions.

To obtain insects of uniform age, two approaches were adopted, reflecting the different biology of the two species. For aphids, adult apterae were isolated on fresh plants and any offspring produced after 16 h were collected and transferred to chemically defined diets (see above). For planthoppers, adult insects were allowed to oviposit on clean rice seedlings for 3 days. Preliminary experiments indicated that maximum egg hatch occurred approximately 10 days after oviposition; any insects that hatched prior to the main collection day were removed to ensure uniformity of age within the experimental animals. Insects hatching within a 16-h 'window' on the tenth day after oviposition were transferred to clean seedlings at either 25°C (symbiotic insects) or 35°C (aposymbiotic insects) for 3 days. Following the pre-treatments to eliminate the symbiotic microorganisms, the insects were returned to standard rearing conditions, either fresh plants for *A. pisum* or fresh plants at 25°C for *N. lugens*, and allowed to continue development.

Insect performance

The weight of first-instar nymphs of *A. pisum* and *N. lugens* within 16 h of birth was determined from a sample of insects removed from the experimental cultures. This value is referred to as initial weight. Insect performance was examined by weighing individual insects on day 8 of nymphal development. Preliminary experiments indicated that all the insects were preadult at this time; symbiotic and aposymbiotic aphids were late fourth instar and late third/early fourth instar, respectively, whereas symbiotic and aposymbiotic planthoppers were third instar and late second/early third instar, respectively. Only insects destined to be flightless (i.e. apterous aphids and brachypterous planthoppers) were used. It was impossible to differentiate between male and female *N. lugens* at this age. Two parameters were scored: insect weight on day 8 and relative growth rate (RGR) over 8 days of nymphal development, calculated using the formula RGR = (ln(final weight) - ln(birth weight))/time. The growth rate analysis included the period during symbiont elimination (2 days on chemically defined diets for *A. pisum* and 3 days at 35°C for *N. lugens*); this approach was adopted to minimize compounding effects of developmental and chronological age, and therefore direct comparisons between growth rates of the insect species are probably misleading (see statistical analysis, below). All insect weights were obtained to the nearest microgram using a Libror AEM 5200 microbalance (Shimadzu, Japan).

Protein and free amino acid content of the insects

The total protein content of individual insects was determined using a protein microassay kit (BioRad Chemical Co., California, U.S.A.) with bovine serum albumin as standard. Insects were weighed on day 8 of nymphal development and homogenized in ice-cold 100 µL 50 mM Tris-HCl pH 7.5. The homogenate was centrifuged to remove debris and the supernatant used in the assay. Total protein is expressed in micrograms per insect and per unit insect fresh weight.

The free amino acids in 8-day-old insects were analysed by reverse-phase high performance liquid chromatography (HPLC) following methanol extraction. Insects of known weight were homogenized in 100 µL ice-cold 80% methanol and the supernatant, following centrifugation at 13 000 *g* for 10 min, was retained. After evaporating to dryness *in vacuo* the amino acids in insect samples and standard samples (see below) were coupled with phenylisothiocyanate under nitrogen for 30 min. The resulting phenylthiocarbamyl amino acids were evaporated to dryness *in vacuo* and stored at – 20°C. For analysis, all samples were reconstituted in a known volume of mobile phase buffer. The sample was filtered using 0.22 µm centrifuge filters and 20 µL of each sample was injected onto a heated ODS-column (Shiwa Chemical Industries, Japan). The amino acids were separated using a buffer system comprising 0.05% triethylamine in 0.14 M sodium acetate (pH 6.35)/acetonitrile (47 : 3 v/v) and 60% acetonitrile (Sasaki *et al*., 1990). Sample peak areas were compared with those of standard samples (Type H of Wako Co., Japan) supplemented with asparagine, glutamine and tryptophan, obtained using a Shimadzu UV detector at 254 nm. All protein amino acids could be assayed by this method, and the concentration of free amino acids in insect tissues is expressed as nanomoles per insect, nanomoles per unit insect fresh weight, and molar percentage of the total concentration.

Statistical analysis

All data sets were analysed using parametric statistical tests. Analysis of variance (ANOVA) was used to investigate the response of the insects to symbiont loss. The data were transformed if necessary to conform to the assumptions of ANOVA, as indicated in the text. The employment of ANOVA in the statistical analysis of the data allowed full interpretation of the relative response of each insect species to symbiont loss, irrespective of the methodology used to produce aposymbiotic insects. The composition of free amino acids in the insects was analysed by repeated measures ANOVA, since the variables are not independent, using ln(nmol amino acid per insect), followed by profile analysis to identify single amino acids which contributed to significant differences between groups (see Douglas, 1996, for full details).

Results

Both symbiotic and aposymbiotic insects fed and continued development following disruption of their symbiotic micro-organisms, as could be observed from the production of honeydew and exuviae. The performance of the insects over the first 8 days of nymphal development is shown in Table 1. Symbiotic *N. lugens* were much smaller than *A. pisum*, and their relative growth rates were significantly lower (see 'species' term in ANOVA). As expected from previous studies (e.g. Lee & Hou, 1987; Douglas, 1992), the performance of aposymbiotic insects was significantly reduced (see 'symbiosis' term in ANOVA) but, as the significant interaction term demonstrates, the impact of symbiont loss was not equivalent between the two species. Aposymbiosis had a much greater impact on the performance of *A. pisum* than that of *N. lugens*. For example, the fresh weight of *A. pisum* and *N. lugens* was reduced by 63% and 42%, respectively, following symbiont loss, and the equivalent values for relative growth rate were 31% for *A. pisum* and 18% for *N. lugens*.

The total protein content of 8-day-old insects is shown in Table 2. The amount of protein was higher in *A. pisum* than *N. lugens*, reflecting the smaller body size of planthoppers, but the amount of protein per unit fresh weight was nearly 1.5-fold higher in the planthoppers. The concentration of protein in aposymbiotic *A. pisum* and *N. lugens* was reduced by 26% and 10%, respectively, and the statistical analysis suggests that the impact of symbiont loss was equivalent in the two insect species (the interaction term in the ANOVA was not significant).

All protein amino acids were detected from the tissues of *A. pisum* and *N. lugens*. In addition, an unknown peak (eluting

Table 1. Performance (mean ± SE, $n = 25$) of symbiotic and aposymbiotic insects over first 8 days of nymphal development. The weights of insects within 16 h of birth for calculation of RGR were 0.131 mg for *A. pisum* and 0.029 mg for *N. lugens*.

Insect	Symbiosis	Fresh weight* (mg)	Mean relative growth rate (mg/mg/day)
A. pisum	Symbiotic	3.38 ± 0.120	0.404 ± 0.0047
	Aposymbiotic	1.24 ± 0.047	0.279 ± 0.0045
N. lugens	symbiotic	0.66 ± 0.021	0.391 ± 0.0042
	Aposymbiotic	0.38 ± 0.017	0.319 ± 0.0057

ANOVA (* data log-transformed prior to analysis):
Species: $F_{1,96} = 1349.5$, $P < 0.001$; 7.6, $0.01 > P > 0.001$.
Symbiosis: $F_{1,96} = 422.4$, $P < 0.001$; 422.7, $P < 0.001$.
interaction $F_{1,96} = 31.1$, $P < 0.001$; 31.1, $P < 0.001$.

Table 2. Total protein content (Mean ± SE, $n = 10$) of 8-day-old symbiotic and aposymbiotic insects.

Insect	Symbiosis	Protein per insect (μg)	Protein per mg insect fresh weight (μg)
A. pisum	Symbiotic	197.2 ± 13.22	60.1 ± 1.17
	Aposymbiotic	58.1 ± 2.04	44.5 ± 0.93
N. lugens	Symbiotic	56.4 ± 1.97	89.0 ± 2.32
	Aposymbiotic	29.8 ± 2.02	80.2 ± 2.43

μg protein per mg fresh weight, ANOVA:
Species: $F_{1,36} = 308.9$, $P < 0.001$.
Symbiosis: $F_{1,36} = 44.1$, $P < 0.001$.
Interaction : $F_{1,36} = 3.4$, $P > 0.050$.

with a retention time between arginine and threonine) was detected in the extracts from *N. lugens*. The peak contributed 6.0% and 5.9% of the total peak area from symbiotic and aposymbiotic insects, respectively ($t_{28} = 0.21$, $P > 0.05$), and was excluded from further analysis. The concentration of free amino acids (see Table 3) was equivalent in both insect species but, as shown in previous studies, the total free amino acid content was elevated in the tissues of aposymbiotic *A. pisum*. A similar effect was observed in aposymbiotic *N. lugens*, with the total amino acid concentration increasing 1.4-fold (see 'symbiosis' term in ANOVA). The impact of aposymbiosis was not equivalent between the two species, as indicated by the significant interaction term, and the discrepancy in total amino acid concentration was highest between symbiotic and aposymbiotic pea aphids.

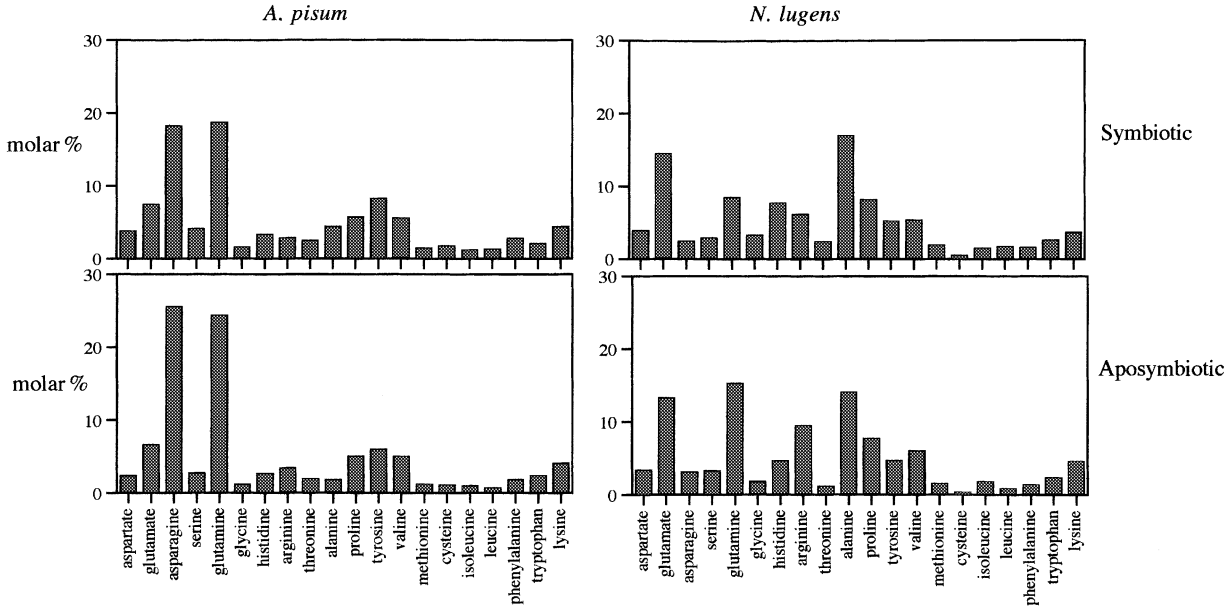
The composition of the free amino acids in *A. pisum* and *N. lugens* is shown in Fig. 1 and Table 3. The free amino acid composition was dominated by non-essential amino acids in both insects. In *A. pisum*, asparagine and glutamine together accounted for 37 and 50 molar % of the amino acids from symbiotic and aposymbiotic insects, respectively, whereas the amino acids in *N. lugens* were dominated by glutamate, alanine and glutamine, at 40 and 43 molar % in symbiotic and aposymbiotic insects, respectively. The contribution of essential amino acids to the total amino acid concentration (see Table 3) was highest in *N. lugens*, at approximately 32 molar % in both symbiotic and aposymbiotic insects, but there was a reduction of 3 molar %, from 26 molar %, in the essential amino acid content of aposymbiotic *A. pisum*. The absolute amount of glutamine in the tissues of *A. pisum* and *N.lugens* is shown in Table 3. There was a 2.4-fold and 2.6-fold increase in the amount of glutamine in the tissues of *A. pisum* and *N. lugens*, respectively, following symbiont disruption, and the non-significant interaction term in the ANOVA indicates that this effect was equivalent in both insect species.

Repeated measures multivariate analysis of variance revealed the composition of amino acids in the insects to be significantly different (see legend to Fig. 1), and profile analysis (detailed results not shown) highlighted the amino acids with the greatest contribution to the overall differences. In brief, the comparison between symbiotic *A. pisum* and *N. lugens* confirmed the gross differences detectable by visual inspection of the data, particularly the dominance of asparagine and glutamine in pea aphids relative to all the other amino acids, and the dominance of alanine and glutamate in planthoppers. The analysis comparing aposymbiotic insects also revealed alanine, asparagine and cysteine to be the main discriminating amino acids between *A. pisum* and *N. lugens*.

Table 3. Amino acid and glutamine content (Mean ± SE, $n = 15$) in the tissues of symbiotic and aposymbiotic pea aphids *Acyrthosiphon pisum* and brown rice planthoppers *Nilaparvata lugens.*

Insect	Symbiosis	Total amino acid concentration (nmol per mg fresh weight)	Essential amino acid content (molar %)	Glutamine concentration† (nmol per mg fresh weight, molar % in parentheses)
A. pisum	Symbiotic	40.8 ± 1.98	26.1	7.53 ± 0.621 (18.6)
	Aposymbiotic	74.6 ± 2.15	22.8	17.92 ± 0.542 (24.3)
N. lugens	Symbiotic	47.3 ± 2.01	32.3	3.87 ± 0.423 (8.4)
	Aposymbiotic	64.6 ± 3.41	32.6	10.14 ± 1.400 (15.3)

ANOVA († data log-transformed prior to analysis):
Species: $F_{1,56} = 0.49$, $P > 0.05$; 40.9, $P < 0.001$.
Symbiosis: $F_{1,56} = 107.9$, $P < 0.001$; 66.8, $P < 0.001$.
Interaction: $F_{1,56} = 11.3$, $0.01 > P > 0.001$; 0.1, $P > 0.050$.



Main effects	Wilks λ	Roys Φ	$F_{19,38}$	P
Species	0.144	0.985	136.3	< 0.001
Symbiosis	0.122	0.878	14.4	< 0.001
Interaction	0.286	0.714	5.0	< 0.001

Fig 1. Free amino acid composition of symbiotic and aposymbiotic *A. pisum* and *N. lugens*. The value for each amino acid is the molar % of the total concentration shown in Table 3. Repeated measures MANOVA ($s = 1$, $m = 8.5$, $n = 18$).

Because the free amino acid composition is likely to be influenced by differences in the amino acid composition of the phloem sap on which the insects feed, the comparisons between insect species are not discussed further.

The impact of aposymbiosis within insect species was not equivalent. The major difference between the profiles of symbiotic and aposymbiotic pea aphids was an increase in alanine in symbiotic aphids relative to 10 other amino acids; elevated glutamine in aposymbiotic pea aphids was not detected by profile analysis. Comparison of the profiles from symbiotic and aposymbiotic *N. lugens* highlighted elevated glutamine and arginine in aposymbiotic planthoppers as contributing most to the overall differences, and also indicated that the essential amino acid leucine was significantly depressed in the tissues of aposymbiotic insects. The mean absolute concentration of leucine in symbiotic and aposym-

biotic *N. lugens* was 0.80 ± 0.078 and 0.51 ± 0.112 nmol per mg fresh weight, respectively (mean $\pm$ SE, $n = 15$; $t_{28} = 2.12$, $0.05 > P > 0.01$). The mean concentrations of two other essential amino acids, histidine and threonine, appeared to be reduced in aposymbiotic *N. lugens* compared with symbiotic insects, but the differences were not statistically significant (data not shown).

Discussion

The key finding from the experiments described in this paper is that the functional significance of symbiotic microorganisms in distantly related members of the Homoptera is broadly equivalent. The data highlight metabolic adjustments to aposymbiosis in *N. lugens* which have been reported previously only in aphids (e.g. Prosser & Douglas, 1991; Liadouze *et al*., 1995; Wilkinson & Douglas, 1995a; Adams *et al*., 1996; Wilkinson, 1998). The implication is that for those insects that rely on the metabolic capabilities of symbiotic microorganisms for essential amino acid provisioning, the physiological response to the loss of the endogenous supply of essential amino acids is the same, irrespective of the phylogenetic position or anatomical location of the symbiotic partners. The purpose of this discussion is two-fold: first, to address the discrepancies in the response of the insects to symbiont loss, and second, to consider the significance of the metabolic signatures to our understanding of the role of the symbiosis in insect physiology.

The impact of aposymbiosis in N. lugens *and* A. pisum

Although it is clear that *A. pisum* and *N. lugens* have an absolute requirement for their symbiotic partners (e.g. Douglas, 1992; Chen *et al*., 1981; Sasaki *et al*., 1996; this paper), the results from the statistical analysis indicate that the impact of symbiont loss is often not equivalent between the two insect species. The relevant term in the analysis of variance is the interaction between species and symbiosis, which for many of the parameters is significant, and it is clear from the data that the impact of the experimental treatment to eliminate the symbionts is greater in *A. pisum* than in *N. lugens.* We consider two possible explanations. First, the greater impact of symbiont loss in *A. pisum* may reflect differences in the developmental age of the insects at the time of study. The development time to adulthood is considerably longer in *N. lugens* than *A. pisum*, and involves five larval instars, as opposed to four larval instars in *A. pisum*, and it is quite plausible that the impact of symbiont loss would appreciate in *N. lugens* as the insects age. However, the experimental period was not extended beyond 8 days because the interpretation of experiments using older aposymbiotic insects becomes problematic as secondary consequences of symbiont loss, i.e. those which cannot be directly related to loss of the endogenous supply of essential amino acids, become apparent (see Wilkinson & Douglas, 1995b; Wilkinson, 1998).

The second possibility relates to the effectiveness of antibiotic- and heat-treatment in eliminating the microorganisms from the insects and methodological problems in their use, particularly elevated temperature. The use of antibiotics to disrupt the symbiotic bacteria in aphids has been addressed thoroughly (Wilkinson, 1998). There is no evidence of bacteria remaining viable or recovering to re-populate the mycetocytes following antibiotic treatment, and the antibiotic specifically targets the bacterial symbionts with no deleterious effects on aphid tissues. However, the effectiveness of heat treatment in completely eliminating the 'yeast-like' symbionts from *N. lugens* is not so definite, and no studies have addressed the effect of heat-treatment on the insect *per se*. For example, histological examinations of both *N. lugens* (Chen *et al*., 1981; Sasaki *et al*., 1996) and *Laodelphax striatellus* (Noda & Saito, 1979) have revealed a low number of symbionts remaining in the fat body following heat treatment (approximately 5% of the number in control *L. striatellus*; Noda & Saito, 1979), but although the cell wall of these symbionts often remains intact, the cytoplasm appears vacuolated and contains residual bodies when viewed under the electron microscope (Noda & Saito, 1979; Chen *et al*., 1981). The possibility that these symbionts are metabolically active cannot be excluded. In addition, it is impossible to discriminate between the impact of elevated rearing temperature on the insect tissue and the symbionts, and in particular the impact of elevated temperature on insect performance irrespective of the symbiotic microorganisms. Despite these methodological problems, the body of evidence presented here shows clearly that the metabolic response of the two insect species to symbiont loss is broadly equivalent.

The significance of the metabolic signatures

The increase in total amino acid titres and the reduction in the concentration of protein in aposymbiotic *N. lugens* parallels the response of aposymbiotic pea aphids to symbiont loss. These metabolic signatures in aposymbiotic pea aphids have been linked to a reduction in protein synthesis, which may be limited by the lack of one or two essential amino acids, leading to the accumulation of non-essential amino acids which contribute to the increase in the total free amino acid pool (Prosser & Douglas, 1991; Wilkinson & Douglas, 1995a). The essential amino acids that have been proposed as limiting to aposymbiotic pea aphids generally belong to the aromatic amino acid family, most notably phenylalanine (Wilkinson & Douglas, 1996). The data in this study suggest that the essential amino acid leucine is a possible candidate for limiting protein synthesis in aposymbiotic *N. lugens*, whereas the analysis of the amino acid composition of the aphid clone used in this study does not highlight any one essential amino acid as being significantly depressed in aposymbiotic aphids; this may reflect variation in amino acid requirements between different aphid clones, as indicated by the variable dietary requirement for phenylalanine in pea aphid clones reported by Srivastava *et al*. (1985), and more generally suggests that extrapolation from experimental studies involving single clonal lineages of aphids may be misleading. However, this does not detract from the

observations in this study from two disparate insect species which, when taken together, highlight the requirement for a functional symbiosis to maintain protein synthesis in phloem-feeding insects.

Elevated glutamine levels in the tissues of aposymbiotic pea aphids has been fully documented (see Introduction), but this is the first demonstration of an equivalent response in aposymbiotic *N. lugens*. There is strong evidence that the glutamine in aphids is derived from the detoxification of waste ammonia, but the metabolic origin of the ammonia remains obscure (Wilkinson & Douglas, 1995a) and the significance of the symbiosis in nitrogen recycling (i.e. the utilization of host waste in the production of essential nutrients) is far from certain (see Douglas, 1998; for discussion). Nitrogen metabolism in *N. lugens* has also been examined in this context, although uric acid has been proposed as the vehicle for nitrogen recycling (Sasaki *et al*., 1996; Hongo & Ishikawa, 1997). For example, *N. lugens* appears to store uric acid in the fat body (i.e. the same anatomical location as the symbionts), but the levels of uric acid decrease during periods of nitrogen deprivation. Uricase, the enzyme responsible for uric acid catabolism, can be detected in whole insects and isolated symbionts, but not in aposymbiotic insects, although aposymbiotic *N. lugens* contain higher concentrations of uric acid. Taken together, the data suggest that uric acid is broken down by the symbionts and utilized (i.e. recycled) to produce essential amino acids (cf. nitrogen recycling in cockroaches; Mullins & Cochran, 1975). However, it is difficult to link elevated glutamine levels in aposymbiotic *N. lugens* with loss of the yeast-like symbionts. In particular, it is unlikely that disruption of nitrogen recycling would lead to elevated glutamine levels, as the endpoint of nitrogen metabolism in the insect is uric acid, which accumulates in aposymbiotic insects.

The question remains why both aposymbiotic aphids and planthoppers have an increased ammonia load requiring detoxification through glutamine. The comparative approach adopted in this study, utilizing two insect species with different mechanisms of nitrogen metabolism, suggests that it is unlikely that ammonia accumulates as a direct consequence of symbiont loss. An alternative explanation relates to the loss of the endogenous supply of essential amino acids and amino acid turnover. Specifically, amino acid metabolism in aposymbiotic insects may undergo futile cycles of inter-conversions, with the net production of ammonia, as the demand for specific amino acids overtakes dietary supply. In other words, the requirement to detoxify ammonia through incorporation into glutamine arises as a consequence of incomplete amino acid metabolism, rather than the loss of a specific sink for ammonia; this prediction can be tested through detailed analysis of amino acid transaminases in aposymbiotic insects.

Acknowledgements

We thank T. Sasaki and Y. Hongoh for advice on the rearing and handling of *Nilaparvata lugens* and A. E. Douglas for critical comments and discussion. Financial support was provided by the Bio-orientated Technology Research Advancement Institution, Ministry of Agriculture, Forestry and Fisheries, Japan.

References

Adams, D. (1996) *Host plant effects on an aphid-bacterial symbiosis*. D.Phil Thesis. University of York, UK.

Adams, D., Wilkinson, T.L. & Douglas, A.E. (1996) The aphid-bacterial symbiosis – a comparison between pea aphids and black bean aphids. *Entomologia Experimentalis et Applicata*, **80**, 275–278.

Baumann, P., Lai, C.-Y., Roubakhsh, D., Moran, N.A. & Clark, M.A. (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera* – intracellular symbionts of aphids. *Annual Review of Microbiology*, **49**, 55–94.

Buchner, P. (1965) *Endosymbiosis of Animals with Plant Microorganisms*. Wiley & Sons, New York.

Chen, C.C., Cheng, L.-L. & Hou, R.F. (1981) Studies on the intracellular yeast-like symbionts in the brown planthopper, *Nilaparvata lugens* Stal. II. Effects of antibiotics and elevated temperature on the symbiotes and their host. *Zeitschrift für angewandte Entomologie*, **92**, 440–449.

Douglas, A.E. (1989) Mycetocyte symbiosis in insects. *Biological Reviews*, **69**, 409–434.

Douglas, A.E. (1992) Requirement of pea aphids (*Acyrthosiphon pisum*) for their symbiotic bacteria. *Entomologia Experimentalis et Applicata*, **65**, 195–198.

Douglas, A.E. (1993) The nutritional quality of phloem sap utilized by natural aphid populations. *Ecological Entomology*, **18**, 31–38.

Douglas, A.E. (1996) Reproductive failure and the amino acid pools in pea aphids (*Acyrthosiphon pisum*) lacking symbiotic bacteria. *Journal of Insect Physiology*, **42**, 247–55.

Douglas, A.E. (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology*, **43**, 17–37.

Hayashi, H. & Chino, M. (1990) Chemical composition of phloem sap from the uppermost internode of the rice plant. *Plant Cell and Physiology*, **31**, 247–251.

Hongoh, Y. & Ishikawa, H. (1997) Uric acid as a nitrogen resource for the brown planthopper, *Nilaparvata lugens*: studies with synthetic diets and aposymbiotic insects. *Zoological Science*, **14**, 581–586.

Koyama, K. (1985) Nutritional physiology of the brown rice planthopper, *Nilaparvata lugens* Stal. (Hemiptera: Delphacidae). II. Essential amino acids for nymphal development. *Applied Entomology and Zoology*, **20**, 424–430.

Lee, Y.H. & Hou, R.F. (1987) Physiological roles of a yeast-like symbiote in reproduction and embryonic development of the brown planthopper *Nilaparvata lugens* Stal. *Journal of Insect Physiology*, **33**, 851–860.

Liadouze, I., Febvay, G., Guillaud, J. & Bonnot, G. (1995) Effect of diet on the free amino acid pools of symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. *Journal of Insect Physiology*, **41**, 33–40.

Mullins, D.E. & Cochran, D.G. (1975) Nitrogen metabolism in the American cockroach: II. an examination of negative nitrogen balance with respect to mobilization of uric acid stores. *Comparative Biochemistry and Physiology A*, **50**, 489–510.

Munson, M.A., Baumann, P. & Kinsey, M.G. (1991) *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the

mycetocyte associated primary endosymbionts of aphids. *International Journal of Systematic Bacteriology*, **41**, 566–8.

Noda, H. (1974) Preliminary histological observation and population dynamics of intracellular yeast-like symbiotes in the smaller brown planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae). *Applied Entomology and Zoology*, **9**, 275–277.

Noda, H., Nakashima, N. & Koizumi, M. (1995) Phylogenetic position of yeast-like symbiotes of rice planthoppers based on partial 18S rDNA sequences. *Insect Biochemistry and Molecular Biology*, **25**, 639–646.

Noda, H. & Saito, T. (1979) Effects of high temperature on the development of *Laodelphax striatellus* (Homoptera: Delphacidae) and on its intracellular yeast-like symbiotes. *Applied Entomology and Zoology*, **14**, 64–75.

Noda, H., Wada, K. & Saito, T. (1979) Sterols in *Laodelphax striatellus* with special reference to the intracellular yeast-like symbiotes as a sterol source. *Journal of Insect Physiology*, **25**, 443–447.

Prosser, W.A. & Douglas, A.E. (1991) The aposymbiotic aphid: an analysis of chlortetracyclinetreated pea aphid *Acyrthosiphon pisum*. *Journal of Insect Physiology*, **37**, 713–19.

Prosser, W.A. & Douglas, A.E. (1992) A test of the hypothesis that nitrogen is upgraded and recycled in an aphid (*Acyrthosiphon pisum*) symbiosis. *Journal of Insect Physiology*, **38**, 93–99.

Rahbé, Y., Delobel, B., Febvay, G. & Chanterel, G. (1994) Aphid-specific triglycerides in symbiotic and aposymbiotic *Acyrthosiphon pisum*. *Insect Biochemistry and Molecular Biology*, **24**, 95–101.

Sandström, J. & Pettersson, J. (1994) Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. *Journal of Insect Physiology*, **40**, 947–995.

Sasaki, T., Aoki, T., Hayashi, H. & Ishikawa, H. (1990) Amino acid composition of the honeydew of symbiotic and aposymbiotic pea aphids *Acyrthosiphon pisum*. *Journal of Insect Physiology*, **36**, 35–40.

Sasaki, T., Hayashi, H. & Ishikawa, H. (1991) Growth and reproduction of the symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. *Journal of Insect Physiology*, **41**, 41–46.

Sasaki, T., Kawamura, M. & Ishikawa, H. (1996) Nitrogen recycling in the brown planthopper, *Nilaparvata lugens*: involvement of yeast-like endosymbionts in uric acid metabolism. *Journal of Insect Physiology*, **42**, 125–129.

Srivastava, P.N., Gao, Y., Levesque, J. & Auclair, J.L. (1985) Differences in amino acid requirements between two biotypes of the pea aphid, *Acyrthosiphon pisum*. *Canadian Journal of Zoology*, **63**, 603–606.

Wilkinson, T.L. (1998) Elimination of intracellular symbionts from insects: a study of antibiotic-treatment in the pea aphid *Acyrthosiphon pisum*. *Comparative Biochemistry and Physiology A*, **119**, 871–881.

Wilkinson, T.L. & Douglas, A.E. (1995a) Why aphids lacking symbiotic bacteria have elevated levels of the amino acid glutamine. *Journal of Insect Physiology*, **41**, 921–927.

Wilkinson, T.L. & Douglas, A.E. (1995b) Aphid feeding, as influenced by disruption of the symbiotic bacteria: an analysis of the pea aphid (*Acyrthosiphon pisum*). *Journal of Insect Physiology*, **41**, 635–640.

Wilkinson, T.L. & Douglas, A.E. (1996) The impact of aposymbiosis on amino acid metabolism of pea aphids. *Entomologia Experimentalis et Applicata*, **80**, 279–82.

Accepted 12 October 2000