ZOOLOGICAL SCIENCE 10: 779–785 (1993)

Nitrogen Recycling in the Endosymbiotic System of the Pea Aphid, *Acyrthosiphon pisum*

TETSUHIKO SASAKI and HAJIME ISHIKAWA

Zoological Institute, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

ABSTRACT—In an effort to investigate the metabolic importance of amide amino acids glutamine and asparagine in the endosymbiotic system of aphids, symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*, were maintained on a holidic diet, and the effects of omission of these amides from the diet on the aphid performance, in terms of growth and reproduction, were investigated. While omission of amides depressed the growth and reproduction of symbiotic aphids, the omission of glutamine improved the performance of aposymbiotic aphids. In the honeydew excreted by aposymbiotic aphids on the control diet, glutamine and asparagine were the most abundant amino acid constituents, while the honeydew from symbiotic aphids contained these amides only in trace amounts. In addition, aposymbiotic aphids excreted these amides, especially glutamine, even when kept on diets not containing the amides. The tissues of symbiotic and aposymbiotic aphids contained the same level of glutamine synthetase activity. It was suggested that: (1) amides are important nitrogen sources for symbiotic aphids, but not for aposymbiotic ones; (2) aposymbiotic aphids excrete amides as nitrogenous wastes that are reutilized by symbiotic ones.

INTRODUCTION

Aphids have an association with obligatory endosymbionts of either bacteria or yeast varieties. The bacterial symbionts of aphid are harbored by the mycetocyte, a cell differentiated specifically for this purpose. A number of functions have been hypothesized for symbionts: biosynthesis (i.e., amino acids, sterols and vitamins), energy production and osmoregulation [5]. These hypotheses have revolved around their unique feeding habit (i.e., intracellular within phloem tissue) and the nutritional deficiencies associated with their diet.

Most nitrogen compounds that aphids obtain from their diet are free amino acids with grossly unbalanced composition [2]. The roles of symbionts in the host's amino acid metabolism have been often studied by using holidic diets and aposymbiotic aphids, aphids from which viable symbionts have been depleted by experimental methods such as treatment with antibiotics. Taking full advantage of these experimental techniques, Mittler clearly demonstrated that the symbionts provide the host with several essential amino acids [11].

While nitrogen recycling is also hypothesized as a possible function of the symbionts, we do not yet know what is the waste product of aphid's nitrogen metabolism. The honeydew of aphids, a copious watery secretion rich in carbohydrate, does not contain any of urea, uric acid, allantoin, allantoic acid, creatine, and creatinine, but amino acids and a small amount of ammonia [8]. Amino acids make up the bulk of nitrogenous material in the honeydew, and its amino acid composition is similar to that of the plant juice ingested [1, 10]. This has lead one to conclude that most amino acids found in the honeydew are not aphid metabolic products, but constituents of the diet which were voided without being absorbed or subjected to metabolic manipulation. Thus, we are far from understanding of the nitrogen excretion of aphids.

As described in a previous paper, we analyzed amino acids in the honeydew excreted by symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*, and those in the phloem sap of young broad bean plants, *Vicia faba* on which the aphids were

Accepted June 13, 1993
Received April 30, 1993

feeding [13]. In the phloem sap, the amide amino acids, asparagine and glutamine, were the two most abundant constituents that amounted to approximately half of the total amino acid content. In the honeydew of symbiotic aphids, the relative level of amides was considerably lower than that in the honeydew of aposymbiotic aphids, suggesting that symbiotic aphids utilize amides more efficiently than the aposymbiotic ones. It was also proposed that the amides are nitrogenous waste products of the insects, and that symbiotic aphids are capable of reutilizing them.

In the present study, in an attempt to further clarify the functions of amides in the nutritional physiology and excretory metabolism of the endosymbiotic system of aphids, symbiotic and aposymbiotic aphids were maintained on a chemically defined synthetic diet, and the effects of amide omission on the growth, reproduction and amino acid excretion of the aphids were analyzed.

MATERIALS AND METHODS

Insects

The aphids used in the present study were obtained from a long-established parthenogenetic clone of pea aphids, *Acyrthosiphon pisum* (Harris). The stock culture of aphids was maintained on young broad bean plants, *Vicia faba* (L.) at 15°C with a photoperiod of 17 hr. To minimize alate production in the experiments, a small number of adults (5–10 individuals) were allowed to deposit nymphs for 24 hr on a plant. The parents were removed and the nymphs found at this time were considered to be 1 day old. Aposymbiotic aphids were obtained by rifampicin injection [6]. An adult aphid was anaesthetized in a stream of carbon dioxide and injected with 0.1 μl of rifampicin at 200 μg/ml. The F1 generation produced by the rifampicin-injected aphid was aposymbiotic. It was found that this antibiotic treatment selectively disrupts the bacterial endosymbionts with minimal effects on the host insect [7].

Maintenance of aphids on synthetic diet

The constituents of the control diet were as in diet A1 of Febvay *et al.* [4] except that β-alanine and ornithine were omitted. The total amino acid concentration in this diet was 255 mM, and asparagine and glutamine were 20 mM and 30 mM, respectively. Diets in which asparagine or glutamine, or both amides were omitted from the control diet were designated as Diet(-Asn), Diet(-Gln) or Diet(-Asn, -Gln), respectively. The solution was filtered through 0.22 μm filter and aseptically enclosed in stretched Parafilm membranes as described previously [15]. Aphids born on the plant were transferred to a synthetic diet in groups of 20 or 25 nymphs within 24 hr of birth and maintained at 15°C (17L : 7D). The diet sachets were changed twice a week. Each experiment was performed with five batches, and the data were statistically analyzed using the paired-sample *t*-test.

Amino acid analysis in the honeydew

Drops of honeydew excreted by aphids on synthetic diets were collected by washing them off the lower part of the cage. The samples were then dried *in vacuo* and mixed with coupling solution (ethanol : water : triethylamine : phenylisothiocyanate = 7 : 1 : 1 : 1 v/v). After coupling at room temperature for 25 min, the solution was evaporated to dryness *in vacuo*. The resulting phenylthiocarbamyl amino acids were analyzed by reverse phase HPLC as described previously [14]. The reference amino acid mixture was Type-H of Wako Co., supplemented with asparagine and glutamine.

Determination of glutamine synthetase activity

Glutamine synthetase activity was determined by measuring the formation of γ-glutamylhydroxamate from glutamic acid and hydroxylamine [9]. Whole tissues of aphid were homogenized in 250 mM sucrose and 50 mM Tris-HCl (pH 7.2). The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was used for the assay. The reaction mixture (final volume 0.5 ml) was 100 mM imidazole-HCl buffer (pH 7.2) containing 25 mM 2-mercaptoethanol, 50 mM sodium L-glutamate (pH 7.2), 10 mM NaATP, 20 mM $MgCl_2$ and 125 mM hydroxylamine. After incubation at 30°C for 15 min, 0.75 ml of ferric chloride reagent (370 mM $FeCl_3$, 670 mM HCl and

200 mM trichloroacetic acid) was added, and the precipitated protein was removed by centrifugation. The absorbance of the solution at 535 nm was read in a spectrophotometer. A standard curve was prepared using commercially obtained γ-glutamylhydroxamate. One unit of glutamine synthetase activity was defined as the amount of enzyme that catalyzed the synthesis of 1 μmol of γ-glutamylhydroxamate in 1 hr at 30°C.

The protein content of the sample was determined using the BCA protein assay reagent (Pierce Co.) with bovine serum albumin as standard.

RESULTS

Growth and reproduction of aphids on synthetic diets

Growth and reproduction of the aphids on control diet and amide-omitted diets were summarized in Table 1. When the normal symbiotic aphids were maintained on the control diet, the first 50% of the population underwent final ecdysis to adulthood when they were 10.6±0.6 days old. On a diet from which either asparagine or glutamine was omitted, their development was delayed by about a day. When both amides were omitted simultaneously, the aphids' development was delayed by another day. The weights attained by the aphids raised on Diet(-Asn) and Diet(-Gln) were both about 80% of that of aphids grown on the control diet. Simultaneous omission of the two amides resulted in a further weight reduction and a considerable decrease in the number of nymphs deposited, while individual omissions did not have significant effects on reproduction.

Depletion of symbionts had potent influences on aphid performance. Compared with symbiotic aphids, aposymbiotic aphids needed a longer time for their development, weighed less, and deposited fewer nymphs. While symbiotic aphids performed poorer on any of amide-omitted diets than on the control diet as described above, the performance of aposymbiotic aphids was rather improved by amide omission. The single omission of glutamine improved both growth and reproduction of aposymbiotic aphids. Also on Diet(-Asn, -Gln), they deposited more nymphs than on the control diet. When asparagine was omitted independently, no significant effect was observed.

Amino acid content of honeydew

In Fig. 1, are shown the amino acid contents of the honeydew collected from a population consisting of 4th instar larvae and non-bearing young adults. In the honeydew of symbiotic aphids raised on the control diet, the total amount of amino acids excreted by an aphid per day was 3.6±1.1 nmol (Fig. 1a). Arginine was the predominant constituent that amounted to 54.7 mol% of the total amino acids. Other major constituents were histidine, lysine and phenylalanine. The omission

Table 1. The effects of amide omission on the growth and reproduction of aphids

aphid	diet	developmental time (days)	body weight (mg/aphid)	reproduction (nymphs/aphid)
symbiotic	control	10.6±0.6	2.00±0.08	22.4±0.9
	Diet (-Asn)	11.4±0.7*	1.59±0.07*	19.9±1.0
	Diet(-Gln)	11.8±0.6*	1.60±0.04*	19.9±0.9
	Diet(-Asn, -Gln)	12.8±0.9*	1.27±0.05*	14.5±0.3*
aposymbiotic	control	16.7±0.5	1.16±0.07	1.0±0.2
	Diet(-Asn)	16.2±0.1	1.24±0.02	2.3±0.8
	Diet(-Gln)	15.6±0.4*	1.39±0.04*	4.9±0.3*
	Diet(-Asn, -Gln)	16.1±0.3	1.24±0.02	5.0±0.8*

Experiments were conducted with five groups of 20 symbiotic aphids or 25 aposymbiotic aphids on each diet. The developmental time is the duration in days of the full development of the first 50% of the population, and the weight is that of 7 days old adults. Values are expressed as means±SE.
* Significantly different from equivalent control value ($P<0.05$).

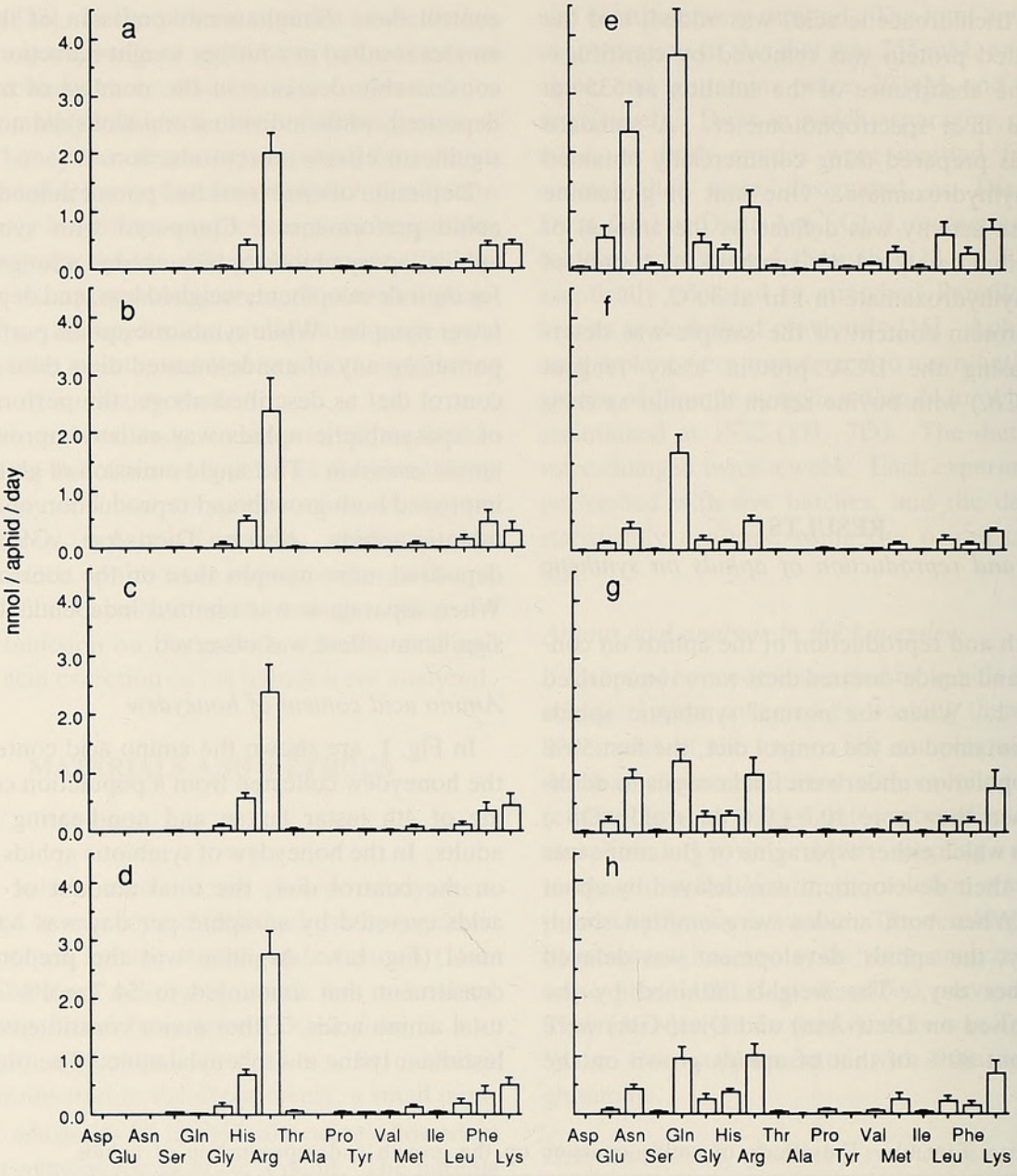

FIG. 1. Amino acid excretion of pea aphids maintained on synthetic diets. Values are expressed in nmol/aphid/day as means ± SE (n = 5). (a) Symbiotic aphids on the control diet; (b) symbiotic aphids on Diet(-Asn); (c) symbiotic aphids on Diet(-Gln); (d) symbiotic aphids on Diet(-Asn, -Gln); (e) aposymbiotic aphids on the control diet; (f) aposymbiotic aphids on Diet(-Asn); (g) aposymbiotic aphids on Diet(-Gln); (h) aposymbiotic aphids on Diet(-Asn, -Gln).

of either one or both amides from the diet had no effect on the amino acid excretion of symbiotic aphids (Fig. 1b-d).

Depletion of symbionts dramatically changed the amino acid constituents of the honeydew. The honeydew of aposymbiotic aphids raised on the control diet (Fig. 1e) contained great amounts of glutamine and asparagine, which were 35.2 mol% and 22.3 mol% of the total amino acids, respectively, while symbiotic aphids scarcely excreted these amides (Fig. 1a). Other amino acids occurring in moderate amounts included arginine, lysine, glutamic acid, leucine, glycine, histidine, methionine and phenylalanine. In total, an aposymbiotic aphid excreted 10.9 ± 4.9 nmol of amino acids in a day.

TABLE 2. Glutamine synthetase activity in aphid tissues

aphid	enzyme activity (units/g of tissue)	protein content (mg/g of tissue)	specific activity (units/mg of protein)
symbiotic	115.0±13.5	69.1±3.0	1.66±0.17
aposymbiotic	99.0± 6.9	58.1±2.6	1.70±0.08

One unit of glutamine synthetase activity was defined as the amount of enzyme that catalyzed the synthesis of 1 μmol of γ-glutamylhydroxamate in 1 hr at 30°C. Values are expressed as means ±SD (n=5).

Not only when aposymbiotic aphids were raised on the control diet, but when raised on diets without amide(s), their honeydew contained appreciable amounts of asparagine and glutamine. On Diet(-Asn), an aposymbiotic aphid excreted asparagine at 0.38 nmol per day (Fig. 1f). On Diet(-Gln), glutamine was still the most abundant amino acid in the honeydew that amounted to 25.3 mol% of the total amino acids (Fig. 1g), suggesting that glutamine is a major nitrogenous waste of aposymbiotic aphids. The excretion rates of asparagine and glutamine on Diet(-Asn, -Gln) were 0.43 nmol/aphid/day and 0.96 nmol/aphid/day, respectively (Fig. 1h).

Glutamine synthetase activity in the aphid tissue

Amino acid analyses of the honeydew collected from aposymbiotic aphids suggested that they actively synthesized amides, especially glutamine. By measuring the formation of γ-glutamylhydroxamate, it was found that tissues of aposymbiotic aphids contain glutamine synthetase at 1.70±0.08 units/mg of protein (Table 2). Although the symbiotic aphids scarcely excreted glutamine, the enzyme activity in their tissues (1.66±0.17 units/mg of protein) was the same as that in aposymbiotic aphids.

DISCUSSION

One major finding in the present study is that aposymbiotic aphids excrete a large amount of glutamine even when kept on a diet from which glutamine is omitted (Fig. 1g). This is the first report that demonstrates that a major amino acid found in the honeydew is a metabolic product of aphids, rather than a constituent of the diet they ingest. It is likely that ammonia, an inevitable waste product of the amino acid metabolism, is assimilated into glutamine in a reaction catalyzed by glutamine synthetase. While in most insects, excessive glutamine, thus produced, is subjected to the formation of uric acid [3], the aposymbiotic aphids may excrete glutamine without further manipulation.

The present result suggested that growth and reproduction of aposymbiotic aphids is better on diets without glutamine, than on a control diet (Table 1). This is the result from omission of the particular amino acid rather than the reduction of total amino acid concentration. For the omission of asparagine did not have significant effects. In addition, when the aphids were kept on a diet in which the amino acid composition is the same as in the control diet but its concentration was reduced by 50 mM, they performed equally as on the control diet (data not shown). It is possible that assimilation of ammonia into glutamine is inhibited, to some extent, by glutamine absorbed from the diet.

Growth and reproduction of symbiotic aphids were retarded by the omission of glutamine, suggesting that glutamine is an important nitrogen source for them. Unlike the honeydew of aposymbiotic aphids, that of the symbiotic aphids contained little glutamine (Fig. 1a), while the glutamine synthetase activity in their tissues was comparable to that in aposymbiotic aphids (Table 2). This suggests that symbiotic aphids can fully utilize glutamine molecules both imbibed from the diet and produced through assimilation of ammonia resulted from the amino acid metabolism. We previously investigated the utilization of amide-nitrogen of glutamine by maintaining aphids on a diet containing [^{15}N]glutamine, and observed that: (1) ^{15}N incorporation into proteins is more than

tenfold higher in symbiotic aphids than in aposymbiotic ones; (2) only symbiotic aphids are able to utilize the amide-nitrogen for the synthesis of so-called essential amino acids as well as non-essential amino acids [14]. In conjunction with these previous results, the present findings suggest that the aphids, with the aid of symbionts, reutilize ammonia through glutamine for the production of essential amino acids and subsequent protein synthesis.

In addition to glutamine, asparagine was excreted as a metabolic product by aposymbiotic aphids (Fig. 1f). Symbiotic aphids excreted little asparagine (Fig. 1a), and the omission of asparagine from the diet depressed the aphid performance, suggesting that asparagine is an important nitrogen source that the aphids are able to utilize efficiently. It is likely that in symbiotic aphids asparagine also takes part in nitrogen recycling.

Mainly because of an increase in amide excretion, aposymbiotic aphids excreted amino acids approximately threefold as much as symbiotic ones. It is likely that aphids owe efficient utilization of amino acid sources to the nitrogen recycling by symbionts. In fact, the retention of amino acids, calculated from the rate of feeding and excretion, is significantly higher in symbiotic aphids than in aposymbiotic ones [14].

Analyzing amino acids excreted by plant-reared aphids, we previously found that amino acid composition in honeydew changes depending on the age of aphids [13]. While the amide content in the honeydew of aposymbiotic aphids is high throughout their lives, that in the honeydew of symbiotic aphids is the lowest when they are actively producing progeny. Nitrogen recycling through amides may function especially for the continuous synthesis of tissue proteins during the aphid's parthenogenetic reproduction.

The mycetocyte symbionts of cockroaches have been shown to utilize the uric acid reserves under the conditions of nitrogen shortage and release nitrogenous compounds, perhaps as amino acids, to the host [12, 16]. The differences in nitrogen recycling between cockroaches and aphids may be related to their feeding behavior. Unlike polyphagous cockroaches, aphids feed exclusively on the phloem sap which is poor in nitrogen content. This may have lead aphids to evolve a more efficient nitrogen recycling system than that in cockroaches. It is equally possible that the difference is simply due to the difference of bacteria that the two insects acquired as endosymbionts.

ACKNOWLEDGMENTS

This research was supported by Grants-in-Aid for General Research (No. 03454020) and for Scientific Priority Areas, "Symbiotic Biosphere: Ecological Interaction Network Promoting Coexistence of Many Species" (No. 04264103) from the Ministry of Education, Science and Culture of Japan. The research was also supported by the grants from Kirin Brewery Co. Ltd.

REFERENCES

1 Auclair JL (1958) Honeydew excretion in the pea aphid, *Acyrthosiphon pisum* (Harr.) (Homoptera: Aphididae). J Insect Physiol 2: 330–337

2 Barlow CA, Randolph PA (1978) Quality and quantity of plant sap available to the pea aphid. Ann Entomol Soc Am 71: 46–48

3 Bursell E (1967) The excretion of nitrogen in insects. Adv Insect Physiol 4: 33–67

4 Febvay G, Delobel B, Rahbe Y. (1988) Influence of the amino acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera: Aphididae). Can J Zool 66: 2449–2453

5 Houk EJ, Griffiths GW (1980) Intracellular symbiotes of the Homoptera. A Rev Ent 25: 161–187

6 Ishikawa H (1978) Intracellular symbionts as a major source of the ribosomal RNAs in the aphid mycetocytes. Biochem Biophys Res Commun 81: 993–999

7 Ishikawa H, Yamaji M (1985) Symbionin, an aphid endosymbiont-specific protein—I. Production of insects deficient in symbiont. Insect Biochem 16: 155–163

8 Lamb KP (1959) Composition of the honeydew of the aphid *Brevicoryne brassicae* (L.) feeding on swedes (*Brassica napobrassica* DC). J Insect Physiol 3: 1–13

9 Meister A (1985) Glutamine synthetase from Mammalian Tissues. Methods Enzymol 113: 185–199

10 Mittler TE (1958) Studies in the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae) II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew. J exp Biol 35: 74–84

11 Mittler TE (1971) Dietary amino acid requirements of the aphid *Myzus persicae* affected by antibiotic uptake. J Nutr 101: 1023–1028

12 Pierre LL (1964) Uricase activity of isolated symbionts and the aposymbiotic fat body of a cockroach. Nature 201: 54–55

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

14 Sasaki T, Hayashi H, Ishikawa H (1991) Growth and reproduction of the symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum* maintained on artificial diets. J Insect Physiol 37: 749–756

15 Srivastava PN, Auclair JL (1971) Influence of sucrose concentration on diet uptake and performance by the pea aphid, *Acyrthosiphon pisum*. Ann Entomol Soc Am 64: 739–743

16 Valovage WD, Brooks MA (1979) Uric acid quantities in the fat body of normal and aposymbiotic German cockroaches, *Blattella germanica*. Ann Entomol Soc Am 72: 687–689