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The Role of Extrafloral Nectar Amino Acids for the Preferences of Facultative and Obligate Ant Mutualists

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Abstract Plants in some 300 genera produce extrafloral nectar (EFN) to attract ants as a means of indirect defence. Among Mesoamerican *Acacia* species, obligate myrmecophytes produce EFN constitutively to nourish symbiotic ant mutualists, while non-myrmecophytes induce EFN secretion in response to herbivore damage to attract non-symbiotic ants. Since symbiotic *Acacia* ants entirely depend on the host-derived food rewards while non-symbiotic ants need to be attracted to EFN, this system allows comparative analyses of the function of EFN components in ant nutrition and attraction. We investigated sugar and amino acid (AA) composition in EFN of two myrmecophytes (*Acacia cornigera* and *Acacia hindsii*) and two related non-myrmecophyte species (*Acacia farnesiana* and *Prosopis juliflora*). AA composition allowed a grouping of myrmecophytes vs. non-myrmecophytes. Behavioural assays with obligate *Acacia* inhabitants (*Pseudomyrmex ferrugineus*) and non-symbiotic ants showed that AA composition affected ant preferences at high but not at low AA/sugar ratios. Most interestingly, behavioural responses differed between the two types of ants tested: Symbiotic ants showed a clear preference for higher AA concentrations and preferred nectar mimics with those four AAs that most significantly characterised the specific nectar of their *Acacia* host plant. In contrast, non-symbiotic ants distinguished among nectars containing different sugars and between solutions with and without AAs but neither among nectars with different AA/sugar ratios nor among mimics containing different numbers of AAs. Our results confirm that both AAs and sugars contribute to the taste and attractiveness of nectars and demonstrate that the responses of ants to specific nectar components depend on their life style. AAs are a chemical EFN component that likely can shape the structure of ant–plant mutualisms.

Keywords *Acacia* · Ant–plant interaction · *Pseudomyrmex* · Mutualism · Nectar

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Introduction

Nectar is an aqueous solution of substances that mainly comprise primary metabolites such as sugars and amino acids and generally serves the attraction of mutualistic animals to plants (Baker and Baker 1975; Baker et al. 1978). Resulting benefits for plants include pollination in the case of floral nectar and protection from herbivores through the attraction of carnivores in the case of extrafloral nectar (EFN; Koptur 1992; Heil 2007, 2008). EFN is usually secreted outside the flowers, and—in contrast to floral nectar—it is not involved in pollination (Bentley 1977; Koptur 1992).

EFN has been described for plants in more than 300 genera (Bentley 1977; Koptur 1992; see also URL: www.biosci.unl.edu/emeriti/keeler/extrafloral/worldlistfamilies.htm). Plant species secreting EFN to attract defending ants (De la Fuente and Marquis 1999; Heil and McKey 2003) are, therefore, commonly called 'myrmecophilic' (i.e. 'ant-loving'). Ants benefit from attending plants, since they use EFN as a nutritive resource and since they are guided to

herbivores in the case of herbivore-induced EFN flow. Compounds that are mainly regarded responsible for the attraction of ants are sugars (Baker and Baker 1973; Blüthgen and Fiedler 2004; Heil et al. 2005) and amino acids (Lanza 1988, 1991; Lanza et al. 1993; Blüthgen and Fiedler 2004). Ants generally appear to prefer sugar solutions that contain amino acids over pure sugar solutions (Lanza 1991), but even the detailed identity of amino acids could elicit varying ant responses to artificial EFNs (Blüthgen and Fiedler 2004).

Other plants, in contrast, secrete EFN in a slightly different functional context. Obligate myrmecophytes are inhabited by specialised ants (Heil and McKey 2003). In these cases, there is no need for the plant hosts to attract ants from the vicinity, while the nutritive value of EFN might be more important due to the dependency of the inhabiting ants on the host-derived food rewards. A comparative approach using a set of related ant–plants that are characterised by these different levels of specificity allows, thus, a deeper understanding of the function that certain EFN components might play in ant attraction and nutrition.

For the present study, we used Mesoamerican *Acacia* species. In this species group, the obligate myrmecophytes secrete EFN constitutively (Heil et al. 2004) to nourish symbiotic ant colonies. These ants, which belong to the *P. ferrugineus* group of the genus *Pseudomyrmex*, obligatorily inhabit particular host plants and are nutritionally adapted to (and entirely dependent on) the host-derived food sources (Heil et al. 2004, 2005; Clement et al. 2008; Kautz et al. 2009). In contrast, non-myrmecophyte species of *Acacia* and of the related genera secrete EFN only in response to herbivore attack (Heil et al. 2004) to attract non-symbiotic ants, i.e. generalist species from the vicinity (Heil and McKey 2003). Thus, although EFN of both myrmecophyte and non-myrmecophyte *Acacia* species fulfils nutritive functions, an attractive function appears important only for the non-myrmecophytes, while EFN of myrmecophytes has likely a higher nutritional importance for the symbiotic ants.

The aim of our study was to evaluate how soluble amino acids affect ant preferences. We predicted that both sugars and amino acids should differ among EFN of myrmecophyte and non-myrmecophyte plant species. Both compound classes were analysed by high-performance liquid chromatography (HPLC), and then, behavioural assays were applied to study the attractiveness of nectar mimics differing in the quantity and concentration of those single compounds that most strongly contributed to the chemical differences among EFNs. Thereby, we aimed to determine whether specific amino acids, their concentration, or their mere number have any specific function in shaping different types of ant-plant mutualisms.

Methods and Materials

Plant Material and Study Site

We investigated the chemical composition of EFN of two myrmecophytes [*Acacia hindsii* Benth. and *Acacia cornigera* (L.) Willendow], of one non-myrmecophyte *Acacia* [*Acacia farnesiana* (L.) Willendow] and of one non-myrmecophytic, sympatric species of another genus, yet the same subfamily, the Mimosoideae (*Prosopis juliflora* Swartz). EFN was collected from plants growing naturally in the coastal area of the state of Oaxaca (México), 5 km northwest of Puerto Escondido (Pacific coast; ~15°55′ N and 97°09′ W; elevation, 15 m), in March and April 2007 and 2008. Species were determined following Janzen (1974) and Seigler and Ebinger (1995) and by comparison with specimens held at the Herbario MEXU at UNAM (Mexico City).

Nectar Collection and Quantification

Branches of myrmecophytes were deprived of ants and other insects the day before nectar collection: thorns were cut off, ants were mechanically removed, and the branch was then placed in a mesh bag after isolating it from the rest of the plant by applying a ring of sticky resin (Tangletrap, The Tanglefoot Corp., Grand Rapids, MI, USA). Branches of non-myrmecophyte species were induced by applying 1 mmol aqueous jasmonic acid solution (Heil et al. 2004) and then placed in mesh bags. After 1 day, nectar production rates were quantified as amounts of soluble solids per 24 h and per gram leaf dry mass by quantifying the nectar volume with microcapillaries (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) and the nectar concentration with a refractometer (Atago Co. Ltd.) as described previously (Heil et al. 2000, 2001). The leaves bearing the EFN were then collected and dried (50°C for 48 h). EFN was collected from five individuals per species, which, in earlier studies, (Heil et al. 2004, 2005) turned out a sufficiently high sample size to represent the chemical variability of EFN secretion in the populations under investigation.

Determination of Carbohydrates and Amino acids

EFN was stored at −20° until analysis. For carbohydrate analysis, 30 µL of nectar were diluted in 600 µL de-ionised water. After centrifugation and membrane filtration (Vivaspin 500, Vivascience Sartorius Group, Stonehous, UK), sugars were immediately separated by HPLC on an anion exchange column and quantified by pulsed amperometric detection (Dionex Series 4500 Chromatography System, Dionex, Idstein, Germany). For the analysis of amino acids,

30 µL of nectar were diluted in 200 µL de-ionised water. After centrifugation and membrane filtration, 100 µL of the supernatant were diluted with 20 µL sulfosalicylic acid (12.5%). After incubation at 4°C for 30 min and a second centrifugation, 50 µL of sample buffer were added to 100 µL of the supernatant. Samples were then analysed using an Amino Acid Analyzer LC 5001 (Biochrom 20 Plus, Cambridge, England). To control for differences in overall nectar concentration, the content of each amino acid was related to the sugar content of the respective sample and is expressed in millimole amino acid per millimole sugar. Differences in amino acid concentrations among the four species were evaluated with a Kruskal–Wallis analysis of variance (ANOVA) (N=5 individual per species). Different individuals were used as replicates to avoid pseudoreplication. Considering that data were not normally distributed, amino acid composition was evaluated with a non-metric multidimensional scaling (NMDS), in order to identify putative associations among the species (NMDS allows to reduce a multidimensional data set to two dimensions and thus appeared an appropriate approach for this question). Ordination was carried out using the following parameters: Bray–Curtis as distance measure, stability criterion of 0.005, 200 iterations, ten runs with real data and ten runs with randomised data. The software used for this analysis was PC-ORD v. 4.2 (McCune and Mefford 1999). Values of NMDS axes were compared among species using a univariate ANOVA.

Behavioural Assays

To study the behavioural responses of ants (symbiotic vs. non-symbiotic ants) to EFN with differing composition, 'cafeteria'-style experiments were carried out under field conditions. Such 'cafeteria' experiments allow to simultaneously offer different types of food sources to animals that freely can choose among them.

The NMDS of EFN amino acids revealed strongest differences between EFNs of *A. hindsii* and *Prosopis* (Fig. 3). We, therefore, focused on these two plant species for the behavioural assays and evaluated the attraction of obligate *Acacia* symbionts (*Pseudomyrmex ferrugineus* Smith F.) and of non-symbiotic ants to EFNs of these two plant species and to different artificial nectars that mimicked the major differences between the two plant species (see Table 1).

Experiment 1: High and Low AAs A first field eperiment was conducted in March 2007. EFN of *A. hindsii* and *Prosopis* was first collected from several individual plants (N=3–5) in the field and then pooled to achieve higher nectar volumes. Then, EFN collected of *A. hindsii* was adjusted with distilled water to a concentration of 4% (w/v), which was the highest concentration found in nectar of *Prosopis* in the field. Six nectar mimics were applied at the same concentration (4%): solution (sol.) 1 contained fructose (F)+glucose (G)+sucrose (S) at a ratio of 3:3:1 to mimick sugar ratio as found in the EFN of *Prosopis*, sol. 2 contained F+G at a ratio of 1:1, mimicking the sugars found in EFN of *A. hindsii*. Three nectar mimics were prepared with different AA compositions: Sol. 3 was a sugar solution (F/G=1:1) containing methionine, isoleucine, leucine, valine, threonine, phenylalanine, proline and serine (i.e., those AA that were highly correlated with Axis 1, see below, and that most strongly contributed to the chemical difference between EFN of *A. hindsii* and of *Prosopis*). Sol. 4 was a sugar solution (F/G=1:1) with those four AA that were highly dominant in EFN of *A. hindsii* (see below, Table 2), and sol. 5 was a sugar solution (F/G=1:1) containing phenylalanine and proline, which both appear particularly important AA in the physiology of insects (Chapman 1983; Dafni and Kevan 1994; Micheu et al. 2000). Pure water was offered as a control (sol. 6; Table 1).

These six artificial solutions and fresh EFNs of *A. hindsii* (sol. 7) and *Prosopis* (sol. 8) were offered to ants in their natural habitat. Two different AA/sugar ratios were used to evaluate whether ants are able to distinguish among different artificial solutions when these contain different AA/sugar ratios: (1) a ratio of each amino acid to fructose and glucose of 1:50 ('high-AA EFNs', N=10 cafeterias) and (2) a ratio of each amino acid to fructose and glucose of 1:1,000 ('low-AA-EFNs', N=17 cafeterias). The ratio 1:50 represents the values that we found in EFN of *Acacia* species (see Table 2).

Independent experiments were conducted for symbiotic and non-symbiotic ants. For *P. ferrugineus*, a 10-µl drop of each of the eight solutions was offered on a horizontal branch of an *A. hindsii* host plant (one cafeteria per plant). For generalist ants, the eight solutions were offered on branches of *Prosopis* that were cut off the plants and placed then on the soil to facilitate the access of generalist ants. In both cases, the individual droplets were offered 10–15 cm apart from each other, and the spatial order varied among the cafeterias. Solutions that had evaporated or that had been entirely consumed were replaced with a new drop of 10 µL. All ants feeding on the droplets were counted five times during the morning (between 10:00 A.M. and 13:00 P.M.). Each single count lasted 3 min, with an interval of 30–40 min between the individual censuses. Because ant abundance may differ among individual plants, numbers of ants that had been attracted to the individual cafeterias were summed up for every cafeteria to calculate the relative proportion of ants that had been attracted to each individual solution. This percentage of ants was subjected to univariate ANOVA (independent variable, solution

Table 1 Composition of amino acid solutions used for the "cafeteria experiments"

Substances	Sol. 1 F+G+S	Sol. 2 F+G	Sol. 3 F+G +8AA	Sol. 4 F+G +4AA	Sol. 5 F+G +2 AA	Sol. 6 Water	Sol. 7 *A. hindsii*	Sol. 8 *Prosopis*
Fructose	x	x	x	x	x			
Glucose	x	x	x	x	x			
Sucrose	x		x					
Isoleucine			x					
Leucine			x	x				
Methionine			x					
Phenylalanine			x	x	x			
Proline			x	x	x			
Serine			x					
Threonine			x					
Valine			x	x				
Pure water						x		
EFN *A. hindsii*							x	
EFN *Prosopis*								x

8AA, 4AA and 2AA mean the addition of the respective amino acids as shown in the table to the F (fructose)+G (glucose) sugar solution

type) after arcsine transformation (Sokal and Rohlf 1995). A least significant difference (LSD) test was posteriorly applied.

Experiment 2: Number of AAs A second 'cafeteria experiment' was carried out in January 2009 to examine whether the ratio of AAs to sugars or the number of AAs is most important to determine ant preferences. Given that ants were only able to distinguish among solutions at higher AA concentrations (Fig. 4), solutions (4%) at ratios 1:10 and 1:50 of AAs to total sugars were prepared with different numbers of total AAs (2AA, 4AA and 8AA). The following six solutions were prepared: 1:10-2AA, 1:10-4AA, 1:10-8AA, 1:50-2AA, 1:50-4AA and 1:50-8AA. Solutions were

Table 2 Concentration of single amino acids (AAs) (μmol L^{-1}), total AAs (mmol L^{-1}) and total sugars (mmol L^{-1}) in EFN of *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Proposis juliflora*

	A. cornigera	*A. hindsii*	*A. farnesiana*	*Prosopis*
ALA (**)	1,846±336	924±102	364±93	178±51
ARG (**)	0±0	10±10	24±14	280±152
ASN (*)	3,375±187	581±237	7,120±2,187	1275±605
ASP (**)	176±15	335±79	496±126	963±355
GLN (**)	1,186±170	831±449	1,473±408	206±121
GLU (***)	1,922±138	2,441±848	302±46	294±27
GLY (*)	86±10	209±46	256±32	196±65
HIS (**)	2,770±359	1,595±158	278±93	469±62
ILE (***)	857±139	1,808±207	285±125	7±5
LEU (***)	1,405±196	3,462±285	56±22	22±7
LYS (ns)	40±17	46±12	38±10	74±21
MET (***)	400±94	1,148±93	44±21	0±0
PHE (***)	13,127±2,672	12,738±2,085	2,809±527	2,066±150
PRO (***)	1,238±205	912±364	195±96	0±0
THR (*)	450±32	805±83	498±123	124±23
TRP (**)	1,489±399	339±86	452±92	938±158
TYR (*)	4,606±477	1,533±155	1,484±300	4,816±469
SER (**)	941±141	1,001±262	1,368±196	381±94
VAL (***)	1,712±196	4,281±468	620±191	165±36
Total AAs	37±0.6	34±0.6	18±0.3	12±0.2
Total Sugars	827±118	336±34	356±44	562±62

Statistical differences among the four species were evaluated for each AA with a Kruskal–Wallis ANOVA, and significance levels are indicated. For amino acid names, see Table 3. Total AAs refers to the sum of the 19 AAs for each species. Total sugars refer to the sum of fructose and glucose for *A. cornigera* and *A. hindsii* and of fructose, glucose and sucrose for *A. farnesiana* and *Prosopis* (see Fig. 1).

ns $P>0.05$,*$P<0.05$, **$P<0.01$, ***$P<0.001$

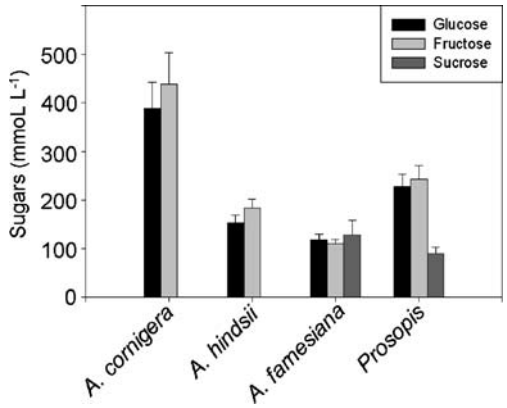

Fig. 1 Sugar quantities in EFN. Concentrations are depicted in mmol sugars per L EFN as means±SE. Sample size N=5 individuals per species

offered in independent experiments to symbiotic (N=10) and non-symbiotic ants (N=10). 'Cafeteria experiments' were conducted as described above. Differences in the percentage of ants attracted to each solution were analysed with univariate ANOVA, after arcsin transformation. LSD was applied then as post hoc test.

Experiment 3: AA/sugar ratios The third experiment was conducted January 2009 to determine which minimal ratio of AAs to total sugars allows ants to differentiate among mimics that contain and that do not contain AAs. Six different 4AA solutions (4%) for symbiotic ants (N=10) and six different 8AA solutions (4%) for non-symbiotic ants (N=10) were prepared at different ratios of AAs to total sugars (1:10, 1:50, 1:100, 1:500 and 1:1,000) and tested in 'cafeteria experiments'. Differences in ant preferences (percentage of ants) among solutions were analysed with univariate ANOVA, after arcsin transformation. A LSD test was posteriorly applied. 'Cafeteria experiments' were conducted as described above.

Results

Sugars and Amino Acids

Sucrose, fructose and glucose were the only sugars detected in EFN of *Acacia* and of the closely related *Prosopis*. EFNs of the two non-myrmecophyte species contained all three sugars, while EFNs of the myrmecophytes only contained the monosaccharides, fructose and glucose (Fig. 1). EFN secretion (in microgram soluble solids per gram leaf dry mass per 24 h) by the myrmecophyte, *A. cornigera*, was significantly higher than for the non-myrmecophyte species ($F_{3,21}$=6.08; P<0.005; univariate ANOVA; Fig. 2). No significant differences were observed in EFN secretion between *A. cornigera* and *A. hindsii* (P>0.05, Tukey test) and between *A. hindsii* and the non-myrmecophyte species (P>0.05, Tukey test).

Amino acid concentrations varied strongly among the four species, and 'species' was a significant source of variation in the concentrations of 17 of the 19 amino acids detected (Table 2). The qualitative compositions differed much less, as only two of the four species contained less than 19 amino acids (*A. cornigera*, arginine missing; *Prosopis*, methionine and proline missing), while in EFN of *A. hindsii* and *A. farnesiana*, all the 19 amino acids were present.

Non-metric Multidimensional Scaling

Both axes contributed significantly to the variation among the species (axis 1, $F_{3,16}$=63.0, P<0.001, univariate ANOVA; axis 2, $F_{3,16}$=22.4, P<0.001, univariate ANOVA), allowing a grouping of myrmecophyte vs. non-myrmecophyte species. *A. hindsii* and *Prosopis* were most distant from each other (Fig. 3). For axis 1, there were no significant differences among myrmecophyte species and among non-myrmecophytes, but the myrmecophtes differed significantly from the non-myrmecophytes. For axis 2, *Prosopis* was significantly different from all other three species.

Methionine, isoleucine, leucine, valine, threonine, phenylalanine, proline and serine were the components with the highest contribution to both axes (amino acids with higher correlation coefficients, see Table 3), suggesting that these eight amino acids did increase the C value and thus contributed most strongly to the differentiation among the species. All these eight amino acids where present at much higher concentrations in *A. hindsii* EFN than in EFN of *Prosopis* (Table 2).

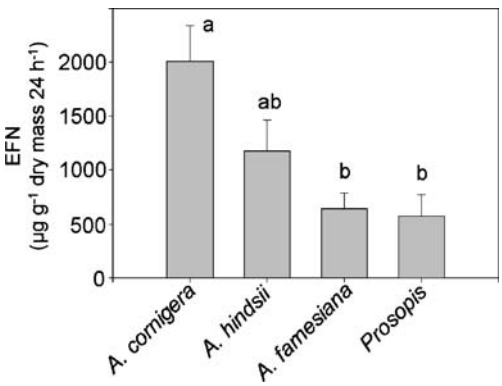

Fig. 2 EFN secretion rates. Amounts of total soluble solids (µg secreted per g leaf dry mass and per 24 h) are depicted for *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Prosopis* as means±SE. Sample size N=5 individuals. *Different letters* indicate significant differences (P<0.05 according to post hoc Tukey test) among the species

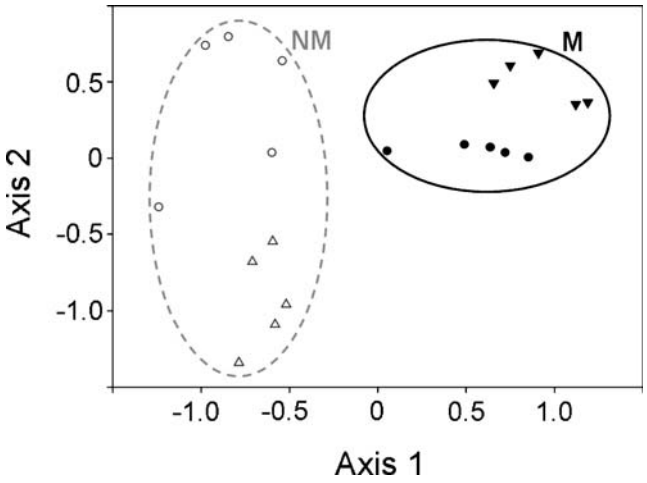

Fig. 3 Non-metric multidimensional scaling (NMDS) ordination diagram of amino acid composition in EFNs. *Black circles*, *A. cornigera*; *black triangles*, *A. hindsii*; *white circles*, *A. farnesiana*; *white triangles*, *Prosopis*; *M* myrmecophytes; *NM* non-myrmecophytic species

Amino Acids and Ant Attraction

In the experiment using low AA EFNs (ratio of each AA to fructose=1:1,000 in the artificial mimics), mutualistic ants preferred EFN of *A. hindsii* over EFN of *Prosopis* (Fig. 4a), whereas non-mutualistic ants showed the opposite preference (Fig. 4b). In general, 'solution type' significantly affected the percentage of ants attracted to the different solutions. This remained true both for symbiotic ants ($F_{7,128}$=8.31; P<0.001; univariate ANOVA) and for non-symbiotic ants ($F_{7,128}$=7.49; P<0.001; univariate ANOVA). Nevertheless, neither symbiotic nor non-symbiotic ants discriminated among the various AA-containing artificial solutions (Fig. 4a, b). For high AA EFNs, the percentages of ants attracted to the different solution types also were significantly different both for symbiotic ants ($F_{7,72}$=10.89; P<0.001; univariate ANOVA) and non-symbiotic ants ($F_{7,72}$=10.83; P<0.001; univariate ANOVA; Fig. 4c, d). Moreover, ants under these conditions distinguished among the artificial solutions, since symbiotic ants significantly preferred the artificial solution with four amino acids (leucine, phenylalanine, proline and valine), while no significant differences were observed among the other artificial solutions. Again, symbiotic ants preferred EFN of *A. hindsii* over the EFN of *Prosopis* (Fig. 4c). On the other hand, non-symbiotic ants significantly preferred the sugar solutions with sucrose over the solution without sucrose, and the sugar–amino acid solutions over sugar-only solutions, although they did not discriminate among the different solutions with amino acids. Consistently with the first experiment, *Prosopis* EFN attracted more non-symbiotic ants than nectar of *A. hindsii* (Fig. 4d).

In the second experiment testing different AA/sugar ratios, significant differences among AA solutions were only observed for symbiotic ants ($F_{5,54}$=6.66; P<0.001; univariate ANOVA). These ants significantly preferred the solution 1:10 over all other solutions, and in fact, ant preference decreased continuously with decreasing AA concentration (Fig. 5a). In contrast, non-symbiotic did not differentiate significantly among solutions with different AA/sugar ratios ($F_{5,54}$=0.27; P>0.05; univariate ANOVA; Fig. 5b). Similar results were obtained in the third experiment, where symbiotic ants distinguished among the different solutions ($F_{5,54}$=0.47; P>0.05; univariate ANOVA, see Fig. 5a) and significantly preferred the solution with 4AAs over the other solutions at both 1:10 and 1:50 ratios ($F_{5,54}$=4.67; P<0.001; univariate ANOVA, see Fig. 5c). Again, non-symbiotic ants did not differentiate significantly among solutions ($F_{5,54}$=0.27; P>0.05; univariate ANOVA; Fig. 5d)

Discussion

EFN is secreted by many plants to attract ants and thus serves as a means of indirect defence (Heil 2008). Sugars and amino acids are generally known as the EFN compounds that are responsible for this attractive function (Koptur 1979; Lanza and Krauss 1984; Lanza 1991; Lanza

Table 3 Correlations between specific amino acids and the two NMDS axes in EFN of three *Acacia* species and *Prosopis*

	NMS I	NMS II
Amino Acids		
ALA (alanine)	0.64	0.31
ARG (arginine)	−0.32	−0.38
ASN (asparagine)	−0.36	0.45
ASP (aspartic acid)	−0.39	−0.33
GLN (glutamine)	0.08	0.66
GLU (glutamic acid)	0.70	0.40
GLY (glycine)	−0.32	0.23
HIS (histidine)	0.81	0.24
ILE (isoleucine)	0.88	0.66
LEU (leucine)	0.91	0.49
LYS (lysine)	−0.14	−0.18
MET (methionine)	0.87	0.51
PHE (phenylalanine)	0.89	0.35
PRO (proline)	0.67	0.37
THR (threonine)	0.61	0.82
TRP (tryptophan)	0.18	−0.26
TYR (tyrosine)	0.02	−0.65
SER (serine)	0.37	0.74
VAL (valine)	0.87	0.56

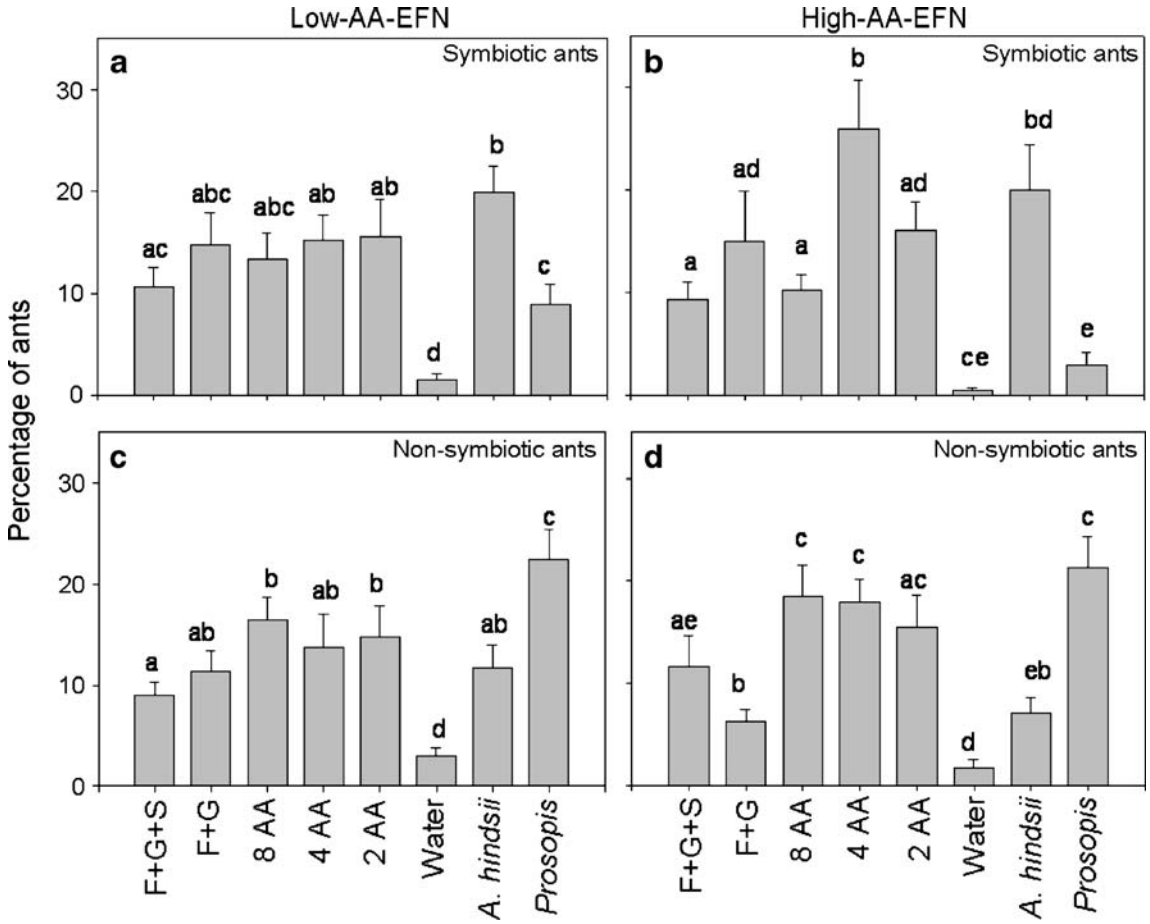

Fig. 4 Ant attraction to EFN mimics. Preferences of symbiotic and non-symbiotic ants to natural EFNs of *A. hindsii* and *Prosopis* and various EFN mimics with and without amino acids (AA). Solution compositions are indicated in Table 1. Low-AA-EFN (**a**, **b**) contained an AA/sugar ratio of 1:1,000 (sample size=17 cafeterias), whereas high AA EFNs (**c**, **d**) contained a ratio of 1:50 (sample size=10 cafeterias). Ant preferences are expressed as means+SE of the percentage of all feeding ants that were attracted to each solution. *Different letters* indicate significant difference in ant attracted among solutions ($P<0.05$ according to post hoc LSD test)

et al. 1993; Blüthgen et al. 2004), but ant preferences to sugars and amino acids may vary among ant species according to their nutritive needs and particularly among functionally different types of mutualisms.

NMDS analysis demonstrated a separation of myrmecophyte species vs. non-myrmecophytes according to the amino acid composition of their EFN: the myrmecophyte, *A. hindsii*, and the non-myrmecophyte, *P. juliflora*, turned out to be the most distant among the four investigated species. Interestingly, these chemical distances mirror the phylogenetic relations: a phylogenetic reconstruction based on chloroplast DNA markers (Heil et al. 2004) also revealed *A. hindsii* and *P. juliflora* to be most distantly related among the species tested in this study. Phenylalanine and proline appeared in much higher concentrations in EFN of the myrmecophytes, *A. cornigera* and *A. hindsii*, than in EFN of the two non-myrmecophytes, which is in line with the very low concentrations of these two amino acids found in EFN of the non-myrmecophyte, *Macaranga tanarius* (Heil et al. 2000) and in other EFNs (Baker et al. 1978; Inouye and Inouye 1980). These two amino acids were among those that most intensively contributed to the differentiation that NMDS revealed among the EFNs studied in this paper.

We found that free amino acids at high concentrations in EFN significantly affected preferences by different ants. Several studies have reported interspecific variability in ant preferences to amino acids (Lanza 1988; Lanza et al. 1993; Blüthgen and Fiedler 2004). Our results generally confirm these studies, since symbiotic and non-symbiotic ants differed in their preferences for artificial amino acid solutions. Nevertheless, differences in ant behaviour were only evident when the relative concentration of single amino acids to sugars were high (1:50), i.e., at concentrations as found in *Acacia* EFN. In contrast, neither symbiotic nor non-symbiotic ants discriminated among artificial mixtures at low amino acid concentrations (1:1,000). This result confirms the study by Lanza (1991), who showed that preferences of fire ants were most obvious when nectar mimics contained high levels of amino acids.

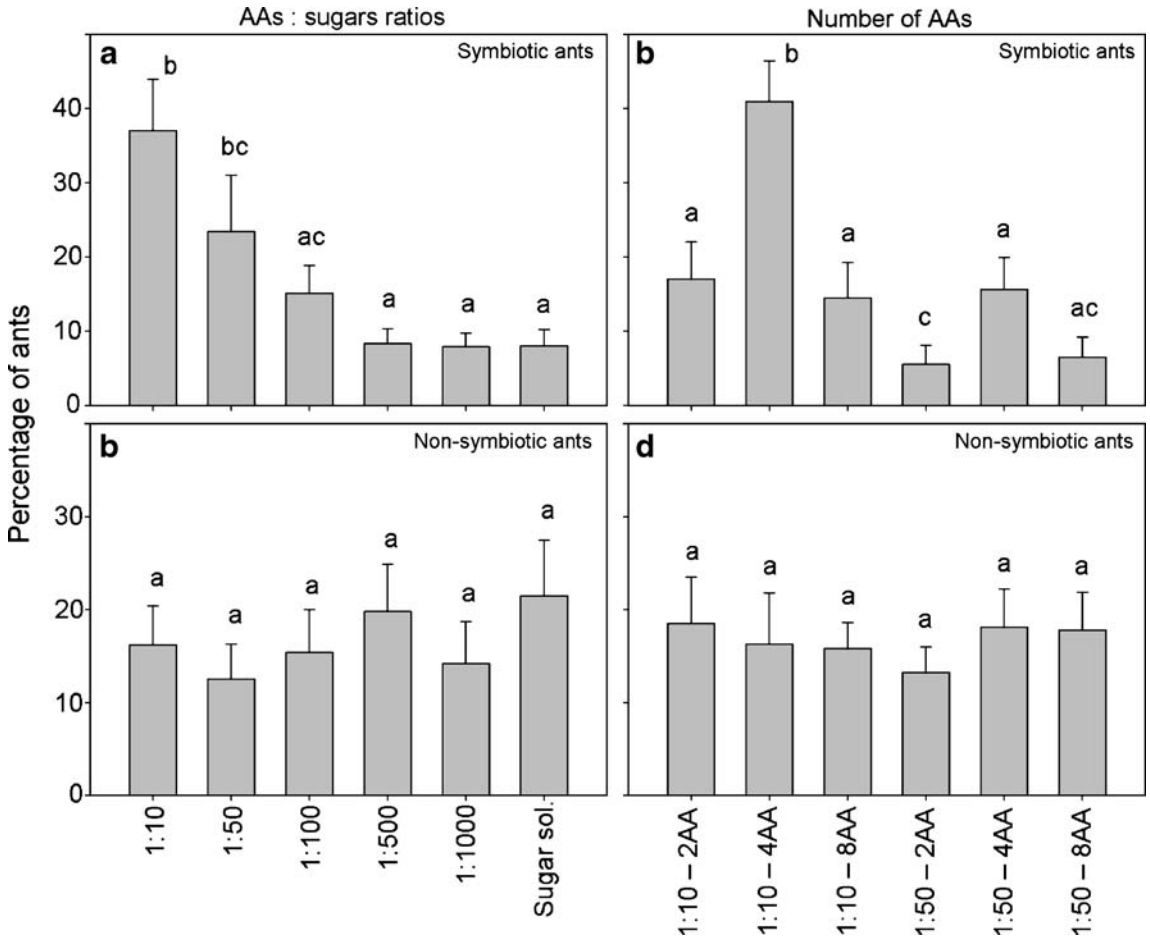

Fig. 5 Preferences of symbiotic and non-symbiotic ants to 4AA and 8AA solutions, respectively, with different AA/sugar ratios (**a**, **b**) (sample size=10 cafeterias) and to solutions with different number of AAs at two different AAs/sugars ratios (**c**, **d**) (sample size=10 cafeterias). Ant preferences are expressed as means+SE of the percentage of all feeding ants that were attracted to each solution. *Different letters* indicate significant difference in ant attracted among solutions ($P<0.05$ according to post hoc LSD test)

Therefore, our results support the general assumption that high concentrations of amino acids in nectar contribute notably to its taste (Gardener and Gillman 2002).

However, ant life history strongly affected whether and how ants responded to certain nectar components and AAs that affected the chemical grouping of myrmecophyte EFNs vs. non-myrmecophyte EFNs determined in part the observed behaviour of symbiotic and non-symbiotic ants. As expected, the symbiotic ants specifically preferred the solution containing those four amino acids that are highly concentrated in the EFN of their host plant (*A. hindsii*). Furthermore, symbiotic ants were able to distinguish this specific solution (1:10-4AA) from other solutions, suggesting that not only AA concentration but also their number and detailed identity determines preferences by symbiotic ants. In contrast, although non-symbiotic ants preferred the solution with eight amino acids in the first experiment, they did not distinguish among nectar mimics that differed only in the number or exact concentration of AAs, while the identity of sugars had a strong and significant effect. Apparently, for generalist ants, just the presence of amino acids in the nectar but not their detailed identity is important, while symbiotic ants are much more selective. Considering that non-symbiotic ants do not establish an obligate mutualism with plants, they must forage on different plant species, unlike symbiotic ants, which are constitutively nourished by one specific host. This different style of life between symbiotic and non-symbiotic ants affects their preferences and selectiveness with respect to detailed chemical composition of their food sources.

These results also suggest that those 4AAs that contributed most to separate myrmecophyte from non-myrmecophyte EFN and that significantly affected the behaviour of symbiotic ants should have a particularly important function for the nutrition of these ants. In fact, high concentrations of phenylalanine and proline have also been reported for different floral nectars (Carter et al. 2006; Petanidou et al. 2006) and thus might be typical for more important types of

nectar-mediated interactions. Phenylalanine is one of the ten essential amino acids for honeybees (Chapman 1983; Dafni and Kevan 1994), while proline is preferentially utilised by insect pollinators during the initial phases of insect flight (Micheu et al. 2000). For ants, comparable information is lacking, and further physiological studies are needed to determine the significance of specific essential amino acids for their metabolism.

Our study suggests that the detailed amino acid composition of EFN is important for its ecological function. Strikingly, behavioural responses differed between symbiotic ants and non-symbiotic ants, with symbiotic ants generally being more selective. Our results confirm that both amino acids and sugars contribute to the taste and attractiveness of nectars and demonstrate clearly that the responses of ants to specific nectar components depend on their life style. Therefore, amino acids are a chemical component of nectar that likely can shape the structure of ant–plant mutualisms.

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