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Urea in Weaver Ant Feces: Quantification and Investigation of the Uptake and Translocation of Urea in *Coffea arabica*

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Abstract Weaver ants are tropical insects that nest in tree canopies, and for centuries these ants have been used for pest control in tropical orchards. Trees hosting weaver ants might benefit not only from the pest protective properties of these insects but also an additional supply of nutrients from ant feces deposited on the leaves. In a recent study, we demonstrated that *Coffea arabica* plants hosting *Oecophylla smaragdina* weaver ants under laboratory conditions experienced enhanced nitrogen availability compared with plants grown without ants. Therefore, the aim of the present study was to further investigate the interactions of weaver ants with the host plants with respect to plant nutrition. Here, we report the identification and quantification of urea, a highly effective foliar nutrient present in the fecal depositions of *O. smaragdina*. Feces samples obtained from six *O. smaragdina* colonies were analyzed, and urea concentrations ranging from 1.98 to 31.05 µg/mg ant feces were detected. Subsequently, we investigated the uptake and translocation of $^{15}N_2$-urea in amounts corresponding to the estimated urea contribution via feces depositions on single host plant leaves under laboratory conditions. The results clearly demonstrated that fecal urea was not only assimilated but also translocated within the plant. This evidence strongly supports the hypothesis that the fecal urea of weaver ants is a source of nitrogen for the host trees. Thus, weaver ant feces likely contribute to an improved nutritional status of ant-hosting trees in tropical orchards, thereby adding value to the use of weaver ants for the biocontrol of insect pests.

Keywords *Coffea arabica* · *Oecophylla smaragdina* · Weaver ants · Foliar nutrients · Urea quantification · Nitrogen nutrients

Introduction

Weaver ants (Formicidae, Hymenoptera) are tropical ants comprising only two species: the African *Oecophylla longinoda* (Latreille) and the Asian *Oecophylla smaragdina* (Fabricius). Both species live in colonies containing thousands of workers in the canopies of trees, where these insects create nests in the foliage using the silk from their larvae (Hölldobler and Wilson 1977b). The high numbers of ants require substantial amounts of food. Protein is primarily obtained through preying on other insects, but birds and small mammals are also considered in the diet of the weaver ants (Wojtusiak and others 1995). Sugar is retrieved from plant nectar or by milking honeydew from mutualistic Hemipterans (Davidson 1997). The weaver ant colony comprises a queen in addition to eggs, larvae, and minor and major workers. The minor workers spend most of their lives tending to the queen and larvae, whereas the major workers are responsible for capturing prey and defending the colony (Hölldobler and Wilson 1977a).

For centuries, weaver ants have been used for pest control in tropical orchards, with 304 A.D. as the earliest record of this application (Huang and Yang 1987). These ants are effective against a variety of pest insects, including

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the fruit flies of mango and citrus (for example, *Ceratitis* and *Bactrocera* species), which are major pests of these crops (Van Mele and others 2007; Van Mele 2008). Previous field studies have reported beneficial effects of these ants which cannot be associated with a mere decrease in the number of pest insects. Instead, these effects might reflect the increased nutrient supply resulting from the fecal depositions of these ants. Vietnamese citrus farmers have used *O. smaragdina* for centuries primarily because they believe that these ants produce substances that increase the quality, sweetness, and juiciness of their crops, and some scientific evidence of these claims has been reported (Barzman and others 1996). Similarly, increased nut shine and quality were observed in cashew trees hosting *O. smaragdina* (Peng and others 1999a, b). In Australian mango production, trees hosting *O. smaragdina* produced 20 % more first-quality fruits compared with trees in which pest insects were controlled using pesticides (Peng and Christian 2005).

The transfer of nutrients from ants to plants has previously been demonstrated for several ant species but not for weaver ants. The soil surrounding the mounds of soil-living ant species is often richer in organic matter and phosphorous, nitrogen, and potassium (Folgarait 1998). Wagner and Nicklen (2010) fed ^{15}N-labeled glycine to ants living in the soil beneath *Acacia constricta* (Benth) trees, demonstrating the specific transfer of nitrogen-containing nutrients from soil-living ants to the host trees. After 24 days, significantly higher levels of ^{15}N were observed in *A. constricta* trees under which ant colonies were present in the soil compared with trees located further away. Analogous results were obtained for myrmecophilic plants, which maintain a mutualistic relationship with ants and typically possess specialized structures in which the ants can nest. Fischer and others (2003) fed ^{15}N-labeled glycine to ants of the species *Pheidole bicornis* (Forel), which typically nest in two myrmecophilic *Piper* species. After 6 days, 25 % of the supplied nitrogen was transferred to the plants, thereby demonstrating that ant-derived nutrients can be provided to myrmecophilic plants. Stable isotope analyses have also been applied in studies of nutrient fluxes from ants to myrmecophilic plants. Indeed, Sagers and others (2000) reported that an average of 93 % of the nitrogen of *Cecropia* trees was supplied from *Azteca* ants nesting in the hollow stems of this tree. Similarly, Treseder and others (1995) established that 29 % of the nitrogen in the tropical epiphyte *Dischidia major* [(Vahl) Merr.] was derived from the debris of *Philidris* ants nesting in the hollow structures of this plant.

Weaver ants produce copious amounts of feces, which are deposited on the leaves of host trees in the form of visible droplets (spots). Higher amounts of fecal spots are observed in areas with high ant activity (Offenberg 2007). Thus, trees hosting large ant communities might benefit from the nutrients supplied from the ant feces and absorbed through the leaves of the host trees. In a greenhouse study, utilizing an untargeted metabolomics approach, we observed that *Coffea arabica* L. plants hosting *O. smaragdina* ants displayed metabolic changes similar to plants supplied with high levels of nitrogen (Vidkjær and others 2015), demonstrating that ant-hosting plants are subjected to higher levels of nitrogen compared with control plants. We hypothesized that the observed effect resulted from the increased supply of urea, a known foliar nutrient (Readman and others 2002), detected in the fecal depositions of *O. smaragdina*. The aims of the present study were as follows: (1) to quantify the urea contents in the fecal depositions from the weaver ant *O. smaragdina*; (2) to estimate the urea quantities supplied to a leaf of an ant-hosting plant; and (3) to investigate whether urea is absorbed and translocated from older (source) leaves to younger (sink) leaves in *C. arabica* plants. To our knowledge, this study is the first to address specific nutritious compounds in ant feces and quantitatively examine the supply of a specific compound from ants to plants. New methods to improve and increase the nitrogen supply to crop plants are constantly in demand (Lum and Hirsch 2002; Jannin and others 2013); therefore, the results of the present study are important for further developments in modern tropical agriculture.

Materials and Methods

Reagents and Chemicals

$^{15}N_2$-urea (98 atom %), urea, methoxyamine hydrochloride, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1 % trimethylchlorosilane (TMCS), fatty acid methyl esters (FAMEs), and pyridine were purchased from Sigma-Aldrich (Schnelldorf, Germany). Acetanilide was purchased from Merck (Darmstadt, Germany), and HPLC-grade methanol was obtained from Rathburn (Walkerburn, Scotland). The water used in the present study was purified using a Dionex (Hvidovre, Denmark) MilliQ purifier.

Ant Colonies

Six laboratory *O. smaragdina* colonies, collected in the wild outside Darwin in Northern Australia, were included in the experiment. Two colonies (1 and 2) were maintained at the Department of Agroecology, Aarhus University, Flakkebjerg. The remaining four colonies (3–6) were maintained at the Department of Bioscience, Aarhus University, Silkeborg. All colonies were maintained in *C. arabica* L. plants (24–50 cm in height) under identical

conditions in greenhouses at 25 °C and 70 % humidity on a 12/12-h light cycle. The plants hosting the ant colonies were placed in the middle of a tray filled with water to confine the ants to the host plant. The numbers of ants in colonies 1–3 were similar and considerably higher than those in colonies 4–6, which were also comparable in size. The numbers of ants were estimated according to nest size and worker activity.

Collection of Ant Feces onto Glass Slides

The feces from the six different *O. smaragdina* colonies were collected onto glass slides (25 × 75 × 1 mm). The feces from colonies 1 and 2 were collected, while the ants were intermittently maintained on two different food regimens (A and B) for 84 days (Fig. 1). In diet A, the colonies were fed ad libitum with a 30 % w/w sucrose solution, and in Diet B the ants were fed ad libitum with the same sucrose solution in addition to American cockroaches (*Periplaneta americana* L.). Each diet period lasted 14 days. In each period, on day 4, ten glass slides were mounted in the host plant of each colony, and on day 10 these glass slides were replaced with fresh slides to obtain two 5-day samplings within each diet period (Fig. 1). The diets of colonies 3–6 were not changed for the duration of the feces collection; these ants were fed ad libitum sucrose dissolved in water (20 % w/w) in addition to one imago (adult insect produced after metamorphosis) *P. americana* and one imago *Musca domestica* L. (house fly) per week (Diet C). The feces from colonies 3–6 were collected onto a total of ten glass slides per colony mounted in the host plants for 7 days. Feces were collected from multiple colonies maintained on different diets to obtain reliable estimates of the quantities of urea in *O. smaragdina* feces. All glass slides containing the ant feces samples were stored at −20 °C until extraction.

Extraction of Ant Feces

The feces, clearly visible as spots on the surfaces of the glass slides, were extracted by washing the glass slides with a solution of 20 % methanol in water. For colonies 1 and 2, all fecal specimens collected within the 14-day diet period were pooled, resulting in one sample per diet period per colony. Thus, three samples per colony for each of the two diets (A and B) were available for analysis. For colonies 3–6, each sample comprised all of the feces specimens collected within the 7-day sampling period. During extraction, the number of spots in each pooled sample (25–165) was noted. The resulting extracts were evaporated to dryness using a vacuum centrifuge, and the amount of ant feces in each sample was determined by weighing.

Collection of Feces (Rectal Fluid) Directly from the Ants

Additional feces samples were directly obtained from the major worker ants of colonies 1 and 2, while these ants were fed the sucrose/cockroach diet (Diet B). The feces samples were collected by gently squeezing the gasters of the ants using forceps until a droplet of rectal fluid emerged. This droplet was collected in an Eppendorf tube, and each sample comprised the rectal fluid from five major workers. Three samples from each ant colony were prepared using a total of fifteen ants each from colonies 1 and

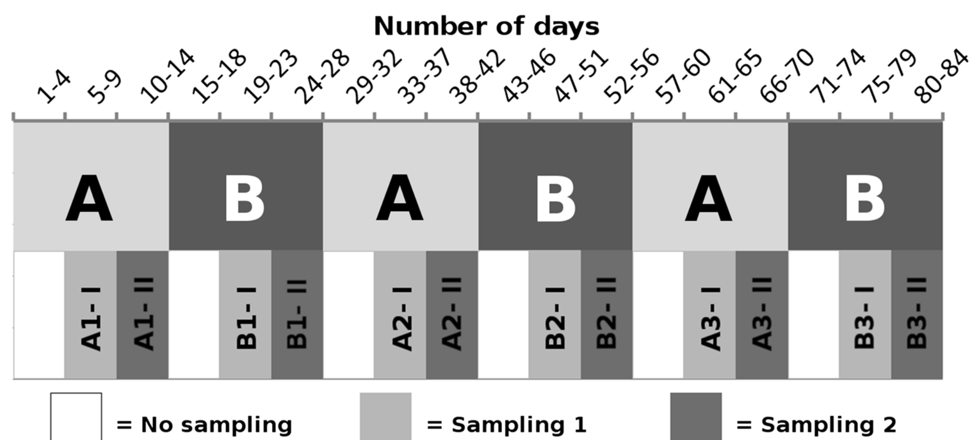

Fig. 1 Diet regimens and fecal samplings from colonies 1 and 2. For 84 days, the two colonies were intermittently kept on diet A (sucrose) and diet B (sucrose + cockroaches). Each diet period lasted 14 days and fecal samplings were initiated on day 4 by mounting glass slides in the host plants of the ant colonies (sampling 1). After 5 days, these glass slides were replaced with new slides and these were left in the plants for another 5 days (sampling 2). The feces collected in samplings 1 and 2 of each diet period were pooled (pooling of, for example, A1-I with A1-II and B1-I with B1-II) and subsequently constituted one sample for each of the diet periods

2. Upon collection, the samples were centrifuged, dried using a vacuum centrifuge, and stored at −20 °C.

GC-TOFMS Quantification of Urea in Ant Feces

Sample Preparation

The dried pooled feces samples originating from the glass slides were re-dissolved in 1 ml of 20 % methanol in water. From each re-dissolved feces sample, three aliquots containing approximately 0.2 mg of ant feces were then transferred to Eppendorf tubes and 1 µg of $^{15}N_2$-urea (100 µl of 10 µg/ml $^{15}N_2$-urea in water) was added to each as an internal standard. For quantification purposes, calibration standards were prepared with unlabeled urea in the following amounts: 0.1, 0.5, 1, 1.5, 2, 3, 4, and 5 µg in addition to 1 µg of $^{15}N_2$-urea as an internal standard. The calibration standards containing 0.1 and 0.5 µg urea were prepared from a solution of 10 µg/ml of urea in water (10 and 50 µl, respectively), whereas the 1–5 µg calibration standards were prepared from a solution of 100 µg/ml urea in water (10–50 µl). All standards and feces samples were then dried using a vacuum centrifuge and subsequently derivatized by first adding 10 µl of methoxyamine in dry pyridine (40 mg/ml) to each of the samples. This mixture was shaken at 1000 rpm for 1.5 h at 30 °C, followed by the addition of 91 µl of MSTFA (+1 % TMCS). Consistent with our standard procedure, the MSTFA was spiked with a mixture of straight-chain FAMEs, used as retention index (RI) markers, at a concentration of 10 µl/ml (mixture of C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30 FAMEs in chloroform at the following concentrations: C8-C16: 0.8 µg/ml and C18-C30: 0.4 µg/ml). The samples were subsequently shaken at 1000 rpm for 30 min at 37 °C, transferred to GC vials, and analyzed within 8 h of derivatization.

GC-TOFMS Analysis

The samples were analyzed in randomized order using a GC-TOFMS system comprising an Agilent 7890A (Santa Clara, CA, USA) gas chromatograph equipped with a Leco Pegasus HT 4010 time-of-flight mass spectrometer (St. Joseph, MI, USA) and Leco ChromaTOF software (version 4.50.8.0) (St. Joseph, MI, USA). One microliter of each sample was injected into the split/splitless inlet, equipped with a straight tube liner with glass wool, using a split ratio of 1:5. The inlet temperature was maintained constant at 250 °C. Chromatography was performed on a 30-m-long 0.25 mm i.d. rtx-5Sil MS column with 0.25 µm 5 % diphenyl/95 % dimethyl polysiloxane film (Restek, Bellefonte, PA, USA) at a constant helium flow of 1 ml/min. The GC temperature was initially maintained at 50 °C for 1 min, subsequently increased at a rate of 20 °C/min until reaching 330 °C, and maintained constant at this temperature for 5 min. The transfer line temperature was 280 °C. Mass spectra were acquired under electron impact (EI) ionization conditions using 70 eV in the mass range of m/z 80–550 at 20 spectra/s using a source temperature of 250 °C and a detector voltage of 1685 V. The recording was initiated after a solvent delay of 280 s and terminated after 20 min. Prior to the series of injections, the instrument was tuned (auto tuning) using perfluorotributylamine (PFTBA).

Urea Standard Curve

The standard curve was prepared in Excel (Microsoft, Seattle, WA, USA), plotting the ratio of the ^{14}N-urea and ^{15}N-urea peak areas against the urea concentration. The standard curve was applied to a linear function, and the R^2 value was 0.98.

Validation of the Analytical Method

A dry pooled feces sample, collected from colony 1 maintained on Diet B (same sample as for the total nitrogen and carbon analysis—see below), was re-dissolved in 20 % methanol in water and twenty nine aliquots, each containing 0.195 mg of ant feces, were transferred to Eppendorf tubes. Three samples were analyzed as described above to establish the concentration of urea in the samples (average urea concentration of 4.92 µg/mg feces, or 1.01 µg/fecal spot). Eighteen samples were split into three groups and spiked with three levels of urea (0.2, 0.4, or 4 µg) to establish the recovery percentages. The recovery percentages were evaluated according to EURACHEM guidelines (EURACHEM 1998). The limit of detection (LOD) and limit of quantification (LOQ) were obtained after determining the standard deviation (stdev) of urea in the spiked feces samples of the replicate recovery experiments at the lowest concentration. According to the EURACHEM guidelines, LOD was determined as 3 stdev, and LOQ was set to 10 stdev. To evaluate the linearity of the urea response in ant feces, the remaining eight samples originating from the pooled colony 1 sample were used to prepare an additional standard curve by adding 1 µg of $^{15}N_2$-urea and 0.1, 0.5, 1, 1.5, 2, 3, 4, or 5 µg of unlabeled urea to these samples. This standard curve was used solely to evaluate the linearity of the urea response, not for quantification purposes. The precision was evaluated based on the relative standard deviations (RSD %) of the three replicates prepared from each of the ant feces samples analyzed.

Total Nitrogen and Carbon Analysis

A pooled sample comprising 165 fecal spots (33.9 mg of dry feces extract) collected onto glass slides during a collection period of five consecutive days from colony 1 maintained on Diet B was prepared as described above (see "Extraction of Ant Feces" section). For the total nitrogen and carbon analysis, two replicate samples (5 mg each) of this dried fecal extract were used for the C/N analysis (Dumas method). A CNS Vario EL-III automatic nitrogen and carbon analyzer (ANCA) was used (Elementar, Hanau, Germany).

Estimation of the Fecal Urea Contributed from *O. smaragdina* Ants to *C. arabica* Host Plants

The amounts of urea used to treat the plants in the present study were estimated using the data obtained from the leaves collected for a previous metabolomics study (Vidkjær and others 2015). In the metabolomics study, the number of fecal spots on the young developing leaves collected from ant-hosting *C. arabica* plants with two-week intervals over a period of 7 months (14 samplings) was within the range of 0–40 fecal spots per leaf. The urea content in the ant feces samples of the present experiment was within the range of 0.24–1.66 µg/fecal spot (Table 1). Thus, each of the leaves collected for the previous experiment had been exposed to 0–9.6 µg urea using the lowest urea content and 0–66.4 µg urea using the highest urea content in the ant fecal spots (Table 1). Based on these values, the highest estimated urea contribution from the ant feces was 66.4 µg per leaf. We used 1/5 of this value (13.28 µg) as the lowest estimated urea contribution per leaf. These amounts of urea were subsequently used to treat the two groups of *C. arabica* plants (high and low urea concentrations), as described below.

Application of $^{15}N_2$-Urea to *C. arabica* Leaves

Young *C. arabica* plants of the same age and approximately 24 cm in height were obtained from a commercial supplier. *C. arabica* was used in the present study to facilitate a comparison with the data obtained from a previous metabolomics study in which *C. arabica* had been used as host plants for *O. smaragdina* ants (Vidkjær and others 2015). In the previous experiment, *C. arabica* was used because plants that were not treated with pesticides could be commercially obtained and serve as suitable host plants for the weaver ants. For the duration of the experiment, the plants received no fertilizer treatment and were watered through drip irrigation using only tap water.

In the present study, three groups (high urea, low urea, and control), each containing five *C. arabica* plants, were included in the $^{15}N_2$-urea leaf absorption experiment. Each of the plant pots contained multiple *C. arabica* plants, and two plants of approximately the same height and with the same number of leaves were selected from each pot for the experiment. On each of the selected plants, the upper leaf surfaces of the two penultimate leaves (Fig. 2) were treated with either $^{15}N_2$-urea (98 atom %) or deionized water (control). The high-urea plant group was treated with 40 µl of 1.66 µg/µl $^{15}N_2$-urea per leaf (132.8 µg $^{15}N_2$-urea per leaf pair), and the low-urea plant group was treated with 40 µl of 0.332 µg/µl $^{15}N_2$-urea per leaf (26.56 µg $^{15}N_2$-urea per leaf pair). The control group was treated with 40 µl of deionized water per leaf. To mimic the fecal depositions of the ants, the solutions were applied in 2-µl spots using a micropipette, and great care was taken not to damage the leaf surface during application. The amounts of urea applied to each of the pairs of source leaves (Fig. 2) corresponded to 12.59 and 62.97 µg ^{15}N for the low- and high-urea treatments, respectively. One plant per pot was harvested after 4 days (A, Fig. 2), and the other plant was harvested after 11 days (B, Fig. 2). From each of the harvested plants, the pair of treated source leaves (1, Fig. 2) and developing pair of sink leaves located directly above the source leaves (2, Fig. 2) were sampled. One pair of source or sink leaves was treated as one sample and the total ^{15}N content was analyzed. Approximately 4 h prior to collection, the source leaves were washed with deionized water to remove any unabsorbed urea. After collection, the leaf pairs were weighed (fresh weight), frozen in liquid nitrogen, freeze-dried, weighed (dry weight), and finely ground using a Genogrinder.

Analysis of ^{15}N in *C. arabica* Leaves

The total nitrogen content and isotopic ratio of $^{15}N/^{14}N$ were measured in solid samples using Dumas combustion (1020 °C) on an elemental analyzer (CE 1110, Thermo Electron, Milan, Italy) coupled in continuous flow mode to a Finnigan MAT Delta PLUS isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Briefly, approximately 3 mg of the homogenized and dried samples were weighed in tin combustion cups for elemental analysis. Acetanilide was used for elemental analyzer mass calibration. As a working standard for isotope ratio analysis, we used pure N_2 gas calibrated against atmospheric air. The analysis performance (Qa/Qc) was assessed through the inclusion of reference samples of biological origin (Peach leaves (NIST 1547), National Institute of Standards and Technology, Gaithersburg, MD, USA). The amount of ^{15}N taken up in the *C. arabica* plants was calculated according to Powlson and Barraclough (1993) using the following equation:

Table 1 Quantification of urea in *O. smaragdina* feces collected onto glass slides

Colony	Sampling period[a]	Urea (μg/mg feces)[b,c]	Urea (μg/mg feces) per sampling day	Urea (μg)/fecal spot[b]
Diet A: 30 % w/w sucrose				
1	A1-I + II	3.77	0.75	0.75
1	A2-I + II	4.01	0.80	0.95
1	A3-I + II	1.98	0.40	0.24
2	A1-I + II[d]	–	–	–
2	A2-I + II[d]	–	–	–
2	A3-I + II	7.34	1.47	1.03
Diet B: American cockroaches + 30 % w/w sucrose				
1	B1-I + II	4.69	0.94	0.96
1	B2-I + II	3.94	0.79	0.93
1	B3-I + II	7.37	1.47	1.34
2	B1-I + II[d]	–	–	–
2	B2-I + II[d]	–	–	–
2	B3-I + II	8.65	1.73	0.98
Diet C: American cockroaches + house fly + 20 % w/w sucrose				
3	NA	21.67	3.10	1.13
4	NA	31.05	4.44	1.66
5	NA	18.39	2.63	0.53
6	NA	27.53	3.93	0.84

Colonies 1 and 2 were intermittently maintained on two different diets consisting of either sucrose alone (Diet A) or sucrose in addition to cockroaches (Diet B) for a total of six diet periods. The details of these diet schemes are described in Fig. 1 and in the "Materials and Methods" section. The diet of colonies 3–6 was not changed and these colonies were maintained exclusively on sucrose, cockroaches, and house flies (Diet C). The numbers represent average values of three samples taken from the same pooled fecal sample

[a] Refers to the sampling periods listed in Fig. 1

[b] The relative standard deviation (RSD %) of the three replicates was less than 10 %

[c] LOD: 0.55 μg/mg feces; LOQ: 1.83 μg/mg feces

[d] Not enough feces to perform urea quantification

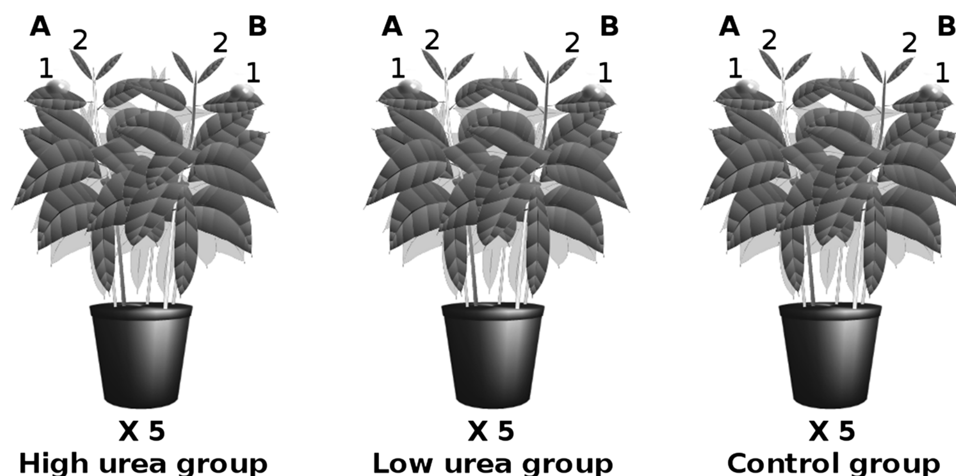

Fig. 2 Three groups of *C. arabica* plants included in the $^{15}N_2$-urea leaf absorption experiment. On each of the two selected plants in each pot, the upper leaf surfaces of the two penultimate leaves (*1*) were treated with either $^{15}N_2$-urea (98 atom %) or deionized water (control). The leaf pairs of the high-urea plant group were treated with 132.8 μg $^{15}N_2$-urea and the low-urea plant group was treated with 26.56 μg $^{15}N_2$-urea per leaf pair. One plant per pot was harvested after 4 days (*A*) and the other plant was harvested after 11 days (*B*). From each of the harvested plants, the pair of treated source leaves (*1*) and developing pair of leaves (sink) located above the source leaves (*2*) was sampled and the total content of ^{15}N in these was analyzed

$$^{15}N_{\text{Taken up}} = N_{\text{Total}} \times \frac{\left(^{15}N_{\text{Sample}} - ^{15}N_{\text{Background}}\right)}{^{15}N_{\text{Excess fertilizer}}}$$

where N_{Total} is the total nitrogen content in the sample, $^{15}N_{Sample}$ is the total ^{15}N content of the sample, $^{15}N_{Background}$ is the mean ($n = 5$) of the ^{15}N in the control plants, and $^{15}N_{Excess\ fertilizer}$ is %Enriched urea (98 %) minus the natural abundance of ^{15}N (0.3663 %). The results were converted into µg.

Statistical Analysis

Statistical analyses were performed using Statgraphics plus 5.1. Variance homogeneity was assessed using Levene's test (all data exhibited variance homogeneity) and subjected to analysis of variance (ANOVA) followed by Tukey's post hoc test. Prior to statistical testing of the urea concentrations in ant feces, the three replicates were averaged.

Results

Performance of the Analytical Method

The recovery percentages were within the acceptable 80–120 % according to the EURACHEM guidelines for the samples spiked at 4 µg levels. The samples spiked with 0.4 µg were nearly within these limits, with recovery percentages of 110–139 %, whereas the samples spiked with the lowest urea level (0.2 µg) showed recovery percentages of 188–232 %. The recovery estimates were not used to correct the data from the experiment. The LOD and LOQ were 0.55 and 1.83 µg urea/mg ant feces, respectively. All samples had urea concentrations above both the LOD and LOQ, except for the rectal fluid samples collected from colony 2, which were below the LOQ but above the LOD. The rectal fluid samples from colony 2 were nonetheless included because quantitation with higher uncertainties than the *p* value defined by EURACHEM is possible and recommended when further statistical analysis is performed (Analytical Methods Committee 1987). The precision of the quantification was high, with relative standard deviations of 1.6–7.2 %. In addition, the linearity was considered high, as the R^2 value of the standard curve prepared in ant feces was 0.98.

Urea Concentrations in Ant Feces

The lowest urea concentrations were detected for colonies 1 and 2 (1.98–8.65 µg urea/mg ant feces), whereas colonies 3–6 displayed notably higher concentrations of urea in the feces (18.39–31.05 µg urea/mg ant feces) (Table 1). The statistical comparison of colonies 1–2, maintained on Diet B, with colonies 3–6, maintained on diet C, revealed significantly higher concentrations of urea per milligram feces per day in colonies 3–6 ($p = 0.003$). The urea concentrations calculated per fecal spot produced more uniform results, with values in the range of 0.24–1.66 µg urea per fecal spot for all colonies/samplings, and no significant differences between the colonies maintained at the two different locations were observed (1–2 vs. 3–6; $p \gg 0.05$). Thus, these results suggested that colonies 3–6 produced smaller fecal spots with higher urea concentrations than colonies 1 and 2. At the time of writing, we did not have any explanation for this result.

To confirm that urea was deposited directly from the ants and not formed exclusively after defecation, the urea content of the rectal fluid collected from major worker ants of colonies 1 and 2 was also investigated. The urea concentrations were in the range of 0.63–3.70 µg urea/mg rectal fluid, or 0.12–0.35 µg/rectal fluid drop (Table 2). These concentrations were comparable to the values detected in the fecal spots collected on the glass slides from the same colonies but at the low end of the concentration range (Tables 1, 2).

To evaluate the chemical similarity between the rectal fluid and feces samples collected on the glass slides, the chromatograms of the two sample types were compared. Relatively, the same chromatographic peaks were present in both the rectal fluid and fecal spot samples, with three clusters of peaks, eluting at approximately 10.5, 14, and 16 min, dominating the chromatograms (data not shown). Although the peak areas were different, the high degree of qualitative similarity between the two sample types suggested that the rectal fluid samples were representative of *O. smaragdina* fecal depositions.

Total Nitrogen and Carbon Content in *O. smaragdina* Feces

The total nitrogen and carbon analysis of *O. smaragdina* feces revealed 2 % nitrogen and 39 % carbon, corresponding to 20 and 390 µg of nitrogen and carbon, respectively, per mg feces. These values corresponded to 4.1 µg of nitrogen and 80.1 µg of carbon per fecal spot. In the sample in which the total nitrogen and carbon content was measured, the urea concentration was 4.92 µg per mg feces, or 1.01 µg per fecal spot (pooled sample used to validate the quantification).

$^{15}N_2$-Urea Uptake and Translocation in *C. arabica* Leaves

The quantities of ^{15}N taken up and translocated in *C. arabica* plants are shown in Table 3. Based on these

Table 2 Quantification of urea in ant feces directly obtained from major workers of colonies 1 and 2 as rectal fluid (five drops obtained from a total of five workers per sample; three replicate samplings)

Colony	Urea (µg/mg rectal fluid)	Total dry weight of five rectal fluid drops (mg)	Urea (µg)/rectal fluid drop
1	2.62	0.60	0.12
1	2.18	0.93	0.19
1	3.70	1.45	0.29
2	0.63[a]	0.30	0.06
2	1.51[a]	1.73	0.35
2	1.07[a]	0.81	0.16

The rectal fluid samples were collected during a period in which these colonies were maintained on the sucrose/cockroach diet (Diet B)

[a] Below LOQ

Table 3 ^{15}N taken up and exported in the *C. arabica* plants treated with a high and a low quantity of urea representing estimates of urea quantities deposited from *O. smaragdina* feces onto a host plant leaf (low-urea treatment: 13.28 µg per leaf and high-urea treatment: 66.4 µg urea per leaf)

Plant group	^{15}N assimilated (µg) (source + sink)	^{15}N exported to sink (µg)	% ^{15}N assimilated	% of ^{15}N applied exported to sink	% of ^{15}N assimilated exported to sink
Low-urea treatment					
4 days	5.74 ± 1.51 aA	0.27 ± 0.04 aA	45.61 ± 11.97	2.14 ± 0.31	4.80 ± 0.62
11 days	8.62 ± 0.87 bA	0.87 ± 0.31 bA	68.47 ± 7.51	6.94 ± 2.45	10.03 ± 3.01
High-urea treatment					
4 days	32.95 ± 4.39 aB	0.91 ± 0.28 aB	52.32 ± 6.97	1.45 ± 0.45	2.75 ± 0.69
11 days	46.06 ± 2.65 bB	1.81 ± 0.38 bB	73.14 ± 4.21	2.88 ± 0.60	3.97 ± 0.91

The treated source leaves and the uppermost developing leaves (sink) were sampled from individual plants after 4 and 11 days (Fig. 2). The numbers represent average ^{15}N quantities per leaf pair (dry weight; $N = 5$) ± the standard deviation. Within the columns for each of the urea treatments (high and low), different small letters indicate significant differences between the quantities of ^{15}N assimilated or exported at 4 days compared with 11 days ($p < 0.05$). Within each column, different capital letters indicate significant differences between the quantities of ^{15}N assimilated or exported in plants exposed to the low-urea treatment compared with the high-urea treatment after 4 and 11 days, respectively ($p < 0.05$)

results, the urea concentrations used in the treatments of the *C. arabica* plants at both time points (days 4 and 11) significantly affected the total amounts of urea assimilated (^{15}N in source + sink leaves). After 4 days, an average of 5.74 µg ^{15}N was taken up in leaves treated with the low urea concentration, whereas significantly more ($p < 0.0001$) ^{15}N (32.95 µg ^{15}N) was taken up in leaves treated with the high urea concentration (Table 3). After 11 days, significantly ($p < 0.0001$) higher amounts were assimilated in plants treated with high urea concentrations (8.62 and 46.06 µg ^{15}N, respectively; Table 3). Furthermore, time was observed to have a significant effect on the amount of urea uptake (Table 3). Significantly more ^{15}N was taken up at 11 days after the urea application in the plants treated with both high (46.06 µg; $p = 0.0004$) and low (8.62 µg; $p = 0.068$) urea concentrations compared with the 4-day sampling (high: 32.95 µg; low: 5.74 µg).

For the amounts of ^{15}N exported to the developing sink leaves located directly above the source leaves, analogous results were obtained (Table 3). After 4 days, an average of 0.91 and 0.27 µg ^{15}N was exported to the sink leaves of plants treated with high and low urea concentrations, respectively (Table 3). Hence, significantly more urea was exported in plants supplied with the high urea concentration ($p = 0.010$) (Table 3). Similar effects were observed after 11 days, where significantly more ^{15}N was translocated in the plants treated with the high urea concentration than in plants treated with the low urea concentration (1.81 µg ^{15}N vs. 0.87 µg ^{15}N, respectively, $p = 0.0026$; Table 3). Time was also observed to have a significant effect on the amounts of ^{15}N exported to the developing leaves. A comparison of the 4- and 11-day samplings within high- and low urea-treated plants revealed that after 11 days significantly higher amounts of ^{15}N were exported in the plants treated with both urea concentrations (high: $p = 0.0028$; 4 days: 0.91 µg; 11 days: 1.81 µg and low: $p = 0.0026$; 4 days: 0.27 µg; 11 days: 0.87 µg) (Table 3).

The total foliar nitrogen concentrations of the dried leaves were not significantly affected after treatment, except for *C. arabica* plants treated with the high urea

concentration, which had significantly lower ($p < 0.05$) concentrations of total nitrogen (mg/g) compared with control plants (Table 4). Plants typically respond to an enhanced supply of nitrogen fertilizer through an increase in the total nitrogen concentration in the leaves. However, in the present study, an increase in the total foliar nitrogen concentrations was not expected because of the low amounts of urea applied. The decrease in total nitrogen in the leaves subjected to the high-urea treatment might reflect an increase in biomass of the plants treated with the high urea concentration, warranting a dilution of the nitrogen during the course of the experiment. This effect was not investigated in detail because the experimental setup did not supply the necessary data. However, these differences diminished and became non-significant when differences in the dry weight of the leaves were considered and the total nitrogen content for the sampled leaf pairs was calculated instead as mg per dry leaf pair (data not shown).

Discussion

Urea Concentrations in Ant Feces

Urea was detected in both the fecal spots collected on glass slides and the rectal fluid samples collected directly from the major workers (Tables 1, 2), demonstrating that urea was directly deposited from the ants. For colony 1, comparable urea concentrations per milligram feces were observed between the rectal fluid samples and the fecal spots collected on glass slides (Diet B; $p = 0.092$), suggesting that most of the urea in the fecal spots was directly deposited from the ants. For colony 2, we could not compare the urea content in the feces from the glass slides and the rectal fluid samples because of the insufficient amounts of feces produced during four of the diet periods (Table 1; Fig. 1). Although comparable urea quantities were detected for colony 1, based on the results of the present study, we could not exclude the possibility that some proportion of the urea detected in the feces collected on the glass slides was formed after defecation, for example, via microbial decomposition of other nitrogen-containing compounds. These aspects were not investigated further because they were beyond the scope of the experiment.

As previously stated, the concentrations of urea in *O. smaragdina* feces primarily varied between the colonies maintained at the two different locations (1–2 and 3–6), and significantly ($p = 0.003$) higher concentrations were observed in colonies 3–6 compared with colonies 1–2, maintained on Diet B (Table 1). The higher concentrations of urea in the feces from colonies 3–6 might be associated with the addition of house flies to the diet of these ants. In contrast, the presence or absence of dietary protein did not significantly affect the urea concentration in the feces. Statistical comparison of the feces of colony 1 collected while this colony was maintained on the sugar-only diet (Diet A) with feces collected in periods during which colony 1 was maintained on the protein-containing diet (Diet B) did not reveal a significant difference in the urea concentration per mg feces or of the urea concentration per fecal spot ($p = 0.164$ and $p = 0.159$, respectively). We could not compare the influence of dietary protein on the urea concentration for colony 2 due to insufficient amounts of feces deposited from this colony during four of the six diet periods (Table 1; Fig. 1). Nonetheless, these results indicate that the source, but not the presence, of protein may have significantly affected the urea content. These aspects give rise to interesting questions that are beyond the scope of the present study but should be addressed in future experiments.

Foliar *C. arabica* Uptake and Translocation of $^{15}N_2$-Urea in Amounts Corresponding to the Fecal Urea Deposited from *O. smaragdina*

Considerable amounts (45.61–73.14 %; Table 3) of the applied urea were taken up in the source leaves and the results demonstrated that a smaller proportion of the applied urea (1.45–6.94 %) was translocated to the neighboring developing leaves (Table 3). The majority of the

Table 4 Total nitrogen concentrations in the dried leaves collected from *C. arabica* plants treated with two different quantities of $^{15}N_2$-urea representing a high and a low estimate for the amounts of fecal urea deposited from *O. smaragdina* ants (low-urea treatment: 13.28 µg per leaf; high-urea treatment: 66.40 µg per leaf) and deionized water as a control

	Leaf treatment	4 days, source leaves	4 days, sink leaves	11 days, source leaves	11 days, sink leaves
Total N (mg/g)	Control	43.65 ± 3.90a	44.38 ± 2.01a	41.81 ± 3.46a	41.18 ± 3.55a
	Low urea	42.56 ± 5.18a	42.65 ± 1.58a	38.09 ± 3.09a	39.25 ± 1.93a
	High urea	41.06 ± 3.71a	40.77 ± 1.70b	35.01 ± 2.34b	35.38 ± 1.69b

The treated source leaves and the uppermost developing sink leaves were sampled from individual plants after 4 and 11 days. The numbers represent average values ($N = 5$) ± standard deviations. Within the columns representing the four sampling occasions, average values followed by different letters are significantly different ($p < 0.05$)

urea was taken up in the first 4 days, as the additional 7 days only showed an approximate 20 % increase in urea assimilation (Table 3). These percentages were consistent with other studies of foliar urea uptake. Stiegler and others (2011) reported that the highest level of assimilation occurs within the first hour after urea application, and after 24 h 50–60 % of the urea was taken up in the leaves of two turf grasses. In apple trees, Dong and others (2002) reported an uptake percentage of 35 % of the applied urea, whereas peach trees assimilated 48–58 % of the foliar-applied urea (Rosecrance and others 1998a). In general, fruit trees take up approximately 60 % of the foliar-applied urea (Weinbaum 1988). Other experiments have demonstrated that leaf age and leaf nitrogen status influence the assimilation of urea in leaves considerably, with significantly less urea absorbed in older leaves compared with younger leaves and nitrogen-deficient leaves compared with nitrogen-sufficient leaves (Bondada and others 2001). Both observations were associated with increased levels of epicuticular waxes on older and nitrogen-deficient leaves that complicate urea assimilation (Bondada and others 2001).

Approximately 2.75–10.03 % of the urea nitrogen taken up was exported to the developing leaves directly above the source leaves (Table 3). Zilkah and others (1987) showed that in avocado, approximately 12 % of the assimilated urea was translocated to younger shoots after 6 days, whereas a reduction in the export ratios to approximately 10 % was evident after 14 days. Dong and others (2002) did not investigate the translocation to developing leaves but demonstrated that urea applied to the leaves of young apple trees was rapidly distributed to stem bark and roots. The highest export rate was observed in the first days after urea application, and this export steadily declined during the course of the experiment: after 20 days, 63.6 % of the absorbed urea was exported (Dong and others 2002). Thus, in the present study, some proportion of the applied urea was likely exported to other plant organs. Investigations of these aspects were beyond the scope of the present study but could be addressed in future trials.

Nutritional Value of Ant Feces

In a previous untargeted metabolomics study (Vidkjær and others 2015), we compared the metabolic changes of ant-hosting *C. arabica* plants with those of control plants for 7 months. In the ant-hosting plants, increased levels of total nitrogen and several amino acids were established, thereby suggesting an increased nitrogen supply to ant-hosting plants (Dong and others 2002; Merigout and others 2008). The present study established a nitrogen content of 4.1 μg N/fecal spot, and in the same sample the urea concentration was 1.01 μg per fecal spot. Although further screening of ant feces revealed the presence of additional nitrogen-containing compounds, such as putrescine, uric acid, and amino acids (data not shown; manuscript in preparation), the results of the present study revealed that approximately 11.5 % of the feces nitrogen was urea. This result suggests that urea is an abundant nitrogen-containing compound in weaver ant feces and a prime candidate for increased nitrogen supply to ant-hosting plants. As previously stated, urea is a foliar nutrient widely used in agriculture and in spray fertilization of fruit trees (Rosecrance and others 1998b; Bondada and others 2001; Furuya and Umemiya 2002; Toselli and others 2004). Urea is considered the best form of nitrogen for foliar applications and is considered more suitable than most inorganic nitrogen-containing compounds, reflecting that the physico-chemical properties of urea result in rapid absorption into leaf tissue (Yamada and others 1965; Knoche and others 1994; Bondada and others 2001).

The details regarding the metabolic fate and mechanisms of urea assimilation, particularly for foliar urea uptake, are not currently known (Witte 2011). Nonetheless, it has been established that the increased supply and use of urea instigate metabolic changes similar to those observed in the ant-hosting plants of the former metabolomics study (Foyer and others 2003; Amtmann and Armengaud 2009). The uptake of urea in plant roots and leaves, without prior hydrolysis to inorganic nitrogen-containing compounds, has also been reported in several independent experiments (Merigout and others 2008). It is also known that plants possess dedicated urea transporters, effectively hydrolyze urea, and are capable of exclusively utilizing urea as a nitrogen source. Most plants absorb foliar and root-supplied urea rapidly, and studies suggest that upon uptake, ureases hydrolyze urea to NH_3 (and CO_2), which can subsequently be incorporated into the plants' nitrogen metabolism (Lam and others 1996; Merigout and others 2008; Witte 2011). The foliar uptake of urea in *C. arabica* has previously been reported by Cain (1956) but without the application of isotopically labeled urea. Nazario and Lovatt (1993) demonstrated urease activity, which is necessary for urea assimilation, by establishing that the foliar application of ^{14}C-urea results in the subsequent release of $^{14}CO_2$. These observations all suggested that the urea from ant feces could be absorbed, assimilated, and translocated in *C. arabica* leaves.

Collectively, the results of the present study demonstrated that fecal urea from *O. smaragdina* likely initiated the metabolic responses of ant-hosting plants, displaying characteristics of enhanced nitrogen availability, as observed in the previous study (Vidkjær and others 2015). Because these responses were observed in developing leaves without spots of ant feces, the nitrogen nutrients were not only absorbed but also translocated within the host plants of *O. smaragdina*. Thus, these effects could

only have been instigated by urea if this nutrient was both absorbed and translocated, as clearly demonstrated in the present study.

In contrast to the previous experiment (Vidkjær and others 2015), in which increased levels of total nitrogen were observed in the young developing leaves of *C. arabica* plants hosting *O. smaragdina*, the present study, as expected, did not reveal increased concentrations of total nitrogen in the leaves as a result of the urea treatment compared with the controls. This result does not contradict the notion that the nitrogen supply increased via urea in ant-hosting plants. Instead, this observation was consistent with the low quantities of urea applied to the leaves in the present study. Notably, Bondada and others (2001) treated the leaves of *Citrus sinensis* L. with 2.76 mg of nitrogen per leaf (as $^{15}N_2$-urea), whereas the *C. arabica* leaves in the present study were treated with 12.59 and 62.97 µg, respectively. In plants hosting weaver ants, new fecal depositions constantly contribute more urea, and multiple leaves are provided with feces. Thus, the precise quantities of urea supplied to the entire plant might greatly exceed the quantities used in the *C. arabica* plant treatments in the present study, thereby increasing the amounts of nitrogen exported to young developing leaves and eliciting an increased total nitrogen concentration in the young leaves of ant-hosting *C. arabica*.

Both the present study and the previous independent metabolomics study were conducted under laboratory conditions. Based on the quantification of fecal urea in the present study, Pinkalski and others (2015) assessed the contribution of urea from *O. smaragdina* feces in Australian mango orchards. The results showed a urea nitrogen contribution of 0.1–0.4 kg/ha in one year or a total nitrogen contribution of 1.0–3.3 kg/ha (based on a total nitrogen content in *O. smaragdina* feces of 4.1 µg per fecal spot) (Pinkalski and others 2015). These numbers were considered conservative estimates, corresponding to 9–30 % of the nitrogen required for commercial mango production. Thus, the urea/nitrogen deposited via weaver ant feces might not supply nitrogen in the amounts normally supplied to a commercial mango orchard. However, commercial fertilizers are often distributed on the plantation floor, where a large proportion of the nitrogen might be lost to soil microbes and ground vegetation or via leaching. In contrast, the urea from ant feces is directly deposited onto the foliage and can be assimilated without prior modification. In young developing leaves and fruits, the demand for nitrogen nutrients is particularly high, reflecting the growth processes of these plant parts. Interestingly, the activity of *O. smaragdina* in these areas is notably higher, resulting in enhanced fecal deposition rates onto these parts of the tree (Hölldobler and Wilson 1978; Offenberg and others 2006; Offenberg 2007). Thus, the increased fecal contribution in these areas might have a profound impact on the nutritional status of ant-hosting trees and augment fruit production. Furthermore, in areas with low soil nitrogen and in third-world countries where the farmers cannot apply expensive commercial fertilizers, the effects of the fecal depositions of weaver ants might greatly enhance the numbers and quality of fruits produced from tree crops.

In conclusion, to our knowledge, the present study is the first to quantify the urea content in the fecal depositions of weaver ants. The results clearly demonstrated that urea, in the amounts estimated to be deposited from weaver ants onto a single host plant leaf, was not only assimilated but also translocated to the developing leaves of *C. arabica* plants under laboratory conditions. These observations established that fecal urea uptake and translocation, as a prerequisite for the metabolic responses of ant-hosting plants observed in the previous metabolomics study, did indeed occur. Furthermore, these results support the hypothesis that urea is a primary source of the increased nitrogen supply to plants hosting *O. smaragdina*, established by the previous metabolomics study.

In future studies, further investigations of the nutritional aspects of weaver ant interactions with host plants will be conducted through experiments in which the ants are fed an isotopically labeled diet and the label is followed as it is absorbed and distributed within the host plants to facilitate a more detailed understanding of the nutrient flux from weaver ants to plants. Further studies are also needed to elucidate whether an increased nutrient supply via a weaver ant colony affects plant growth rate and fruit production and quality.

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Compliance with Ethical Standards

Conflict of Interest None of the authors have any potential conflicts of interest.

Human and Animal Rights This article does not contain any studies with human or animal subjects.

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