Ant-plant mutualism: a dietary by-product of a tropical ant's macronutrient requirements

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Ant–plant mutualism: a dietary by-product of a tropical ant's macronutrient requirements

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Abstract. Many arboreal ants depend on myrmecophytic plants for both food and shelter; in return, these ants defend their host plants against herbivores, which are often insects. Ant–plant and other mutualisms do not necessarily involve the exchange of costly rewards or services; they may instead result from by-product benefits, or positive outcomes that do not entail a cost for one or both partners. Here, we examined whether the plant-ant *Allomerus octoarticulatus* pays a short-term cost to defend their host plants against herbivores, or whether plant defense is a by-product benefit of ant foraging for insect prey. Because the food offered by ant-plants is usually nitrogen-poor, arboreal ants may balance their diets by consuming insect prey or associating with microbial symbionts to acquire nitrogen, potentially shifting the costs and benefits of plant defense for the ant partner. To determine the effect of ant diet on an ant–plant mutualism, we compared the behavior, morphology, fitness, stable isotope signatures, and gaster microbiomes of *A. octoarticulatus* ants nesting in *Cordia nodosa* trees maintained for nearly a year with or without insect herbivores. At the end of the experiment, ants from herbivore exclosures preferred protein-rich baits more than ants in the control (i.e., herbivores present) treatment. Furthermore, workers in the control treatment were heavier than in the herbivore-exclusion treatment, and worker mass predicted reproductive output, suggesting that foraging for insect prey directly increased ant colony fitness. The gaster microbiome of ants was not significantly affected by the herbivore exclusion treatment. We conclude that the defensive behavior of some phytoecious ants is a by-product of their need for external protein sources; thus, the consumption of insect herbivores by ants benefits both the ant colony and the host plant.

Key words: ant–plant interactions; by-product benefits; diet balance; macronutrients; microbiome; mutualism; stable isotope analysis; trophic level.

Introduction

Mutualisms involve the exchange of goods or services between individuals of different species to their reciprocal benefit. These interactions are widely expected to be evolutionarily unstable if partners can "cheat" by taking the benefits of the mutualism without reciprocating (Sachs et al. 2004, Frederickson 2009, Jones et al. 2015). However, few studies have shown evidence of cheating, and theoretical and empirical work has described mechanisms that align the fitness interests of mutualistic partners, thereby preventing cheating (Sachs et al. 2004, Frederickson 2013, 2017). Previous research has emphasized partner fidelity feedback or partner choice as explanations for why mutualists rarely cheat (Sachs et al. 2004, Frederickson 2009, Weyl et al. 2010), but a simpler possibility is that reciprocating a mutualism is not always costly (Jones et al. 2015). Indeed, "by-product benefits" likely confer stability to many mutualistic interactions, including plant-animal and animal–animal mutualisms (Sachs et al. 2004, Mayer et al. 2014).

In plant–animal mutualisms, plants often benefit as a by-product of animal foraging. For example, animals pollinate flowers as they forage for nectar or pollen and disperse seeds as they forage for fruit (Leimar and Connor 2003). Most pollinators and seed dispersers are probably not under selection to cheat because foraging directly increases animal fitness, and the pollination and dispersal services animals provide to plants are simply "happy accidents" of their foraging behavior. Instead, the outcome of mutualism (i.e., the fitness benefits realized by both partners) strongly depends on the foraging behavior and nutritional needs of the animal partner (Leimar and Connor 2003, Mayer et al. 2014, Malé

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et al. 2017). This may be especially true in facultative interactions, but is also possible even in obligate symbioses, such as ant–myrmecophyte associations.

In protective ant–plant mutualisms, ants reduce herbivory on plants that provide them with food (extrafloral nectar or food bodies) or shelter (hollow structures called domatia on myrmecophytes; e.g. Janzen 1966, Fiala et al. 1994). Ant–plant mutualisms have been studied through the lens of cheating and exploitation since the publication of Janzen's classic paper on a *Pseudomyrmex* species that parasitizes swollen-thorn acacias (Janzen 1975, see also Gaume and McKey 1999, Tillberg 2004, Frederickson 2009, Mayer et al. 2014), but the idea that ants may benefit plants as a by-product of their foraging behavior has received less attention than other mechanisms that can stabilize mutualisms (e.g., Weyl et al. 2010). During the early evolution of ant–plant mutualisms, ants likely foraged for insect prey on plants, reducing insect damage to plants as a by-product. Gradually, many plants evolved extrafloral nectaries, food bodies, and domatia to attract greater numbers of ants, decreasing herbivory and perhaps making ants less dependent on insects for food (Mayer et al. 2014, Weber and Agrawal 2014). Most tropical arboreal ants are omnivorous to varying degrees and consume some combination of arthropod prey, extrafloral nectar, food bodies, and honeydew excreted from hemipterans (Blüthgen et al. 2003, Davidson 2003). They may additionally receive important nutritional inputs from microbes that may modify the costs and benefits of the ant–plant interaction (Davidson 2003, Russell et al. 2009, Mayer et al. 2014, Sanders et al. 2014). Ants that eat arthropod prey may still benefit plants simply as a by-product of their foraging (Frederickson et al. 2012). Alternatively, if the ants do not consume herbivores, as is the case for some *Pseudomyrmex* ants defending *Acacia* trees (Janzen 1966), they may depend heavily on their host plants for food. For these ants, plant defense may be costly to the colony, at least in the short term. In the long term (i.e., over the lifetime of an ant colony), however, plant defense should still be beneficial to these ants because ridding plants of herbivores promotes plant growth and the production of additional food and nesting sites for the ant colony.

In a previous study (Frederickson et al. 2012), we determined the costs and benefits of having an ant partner (*Allomerus octoarticulatus*) for a common Amazonian myrmecophyte (*Cordia nodosa*). Here, we determine costs and benefits of the mutualism for the ant partner. We investigated several lines of evidence linking changes in ant foraging behavior and diet (i.e., trophic level, compensatory foraging, microbiome) to ant fitness (i.e., ant mass and size, colony size, and number of reproductives). By experimentally excluding most herbivorous insects from saplings of *C. nodosa* for nearly a year, we investigated whether *A. octoarticulatus* ant colonies benefit directly from patrolling *C. nodosa* and potentially eating insect herbivores, or whether colonies of this species pay a short-term cost to defend their host plants against herbivores. We expand on previous analyses that showed that ant colony size did not differ between herbivore-excluded and control ant colonies after almost a year in the experimental treatments (Frederickson et al. 2012). We estimated colony biomass in both treatments and investigated how colony size and worker mass jointly influence the number of reproductives produced by *A. octoarticulatus* ant colonies, an important fitness component.

We also explored whether the experimentally imposed shift in diet (i.e., the presence or absence of insect prey) affected ant foraging for proteins vs. carbohydrates and ant trophic level, as measured by analysis of nitrogen and carbon stable isotope ratios. Insects do not forage indiscriminately for macronutrients, but instead adjust their foraging behavior to maintain appropriate protein:carbohydrate (P:C) ratios (Raubenheimer and Simpson 1999, Dussutour and Simpson 2009) and extremely unbalanced P:C ratios often have negative consequences for insect performance (Dussutour and Simpson 2009, 2012). Other studies on phytoecious ants have shown that carbohydrate-rich diets increase ant aggression toward insect herbivores (Ness et al. 2009, Pringle et al. 2011, but see Mcglynn and Parra 2016). We asked whether ants in *A. octoarticulatus* colonies that had been prevented from foraging for insect prey recruited in larger numbers to protein or carbohydrate baits, compared to control colonies. We expected colonies in the herbivore-exclusion treatment to be protein starved and to recruit more to protein baits. Alternatively, finding that herbivore-exclusion did not affect ant foraging for protein vs. carbohydrates might suggest that insects are a minor component of *A. octoarticulatus*'s diet, or that this ant species has other ways of overcoming nitrogen limitation, such as via symbiotic bacteria.

Microbial symbionts can perform important functions for their host and in some cases have coevolved with them (Moran 2001). If ants have bacteria that help them overcome nutritional deficiencies in their diets, they might be able to survive feeding only on extrafloral nectar or food bodies, even if these plant-derived foods are nutritionally imbalanced (Janson et al. 2008). There is also substantial interest in understanding how diet shapes the gut microbiomes of animals; here we describe how diet affected the bacterial communities in the *A. octoarticulatus* gaster. Across mammals, species with similar diets tend to have similar gut microbiomes (Ley et al. 2008, Muegge et al. 2011). For example, eating a predominantly animal- or plant-based diet changes the abundance of many taxa in the human gut microbiome (David et al. 2014). In ants, diet and the composition of the gut microbiome are correlated across lineages (Russell et al. 2009, Anderson et al. 2012). Some of these gut microbes may upgrade, recycle, or even fix nitrogen for their ant hosts (Davidson 2003, Russell et al. 2009, Anderson et al. 2012, Six 2013), although experimental demonstrations are few (but see Feldhaar et al. [2007] for details of how *Blochmannia* compensates for nitrogen-poor diets in *Camponotus*). To our knowledge, no

field study has yet investigated how microbes residing in ant gasters respond to experimental shifts in diet in the wild. We characterized the gaster microbiome of *A. octoarticulatus* using 16S amplicon pyrosequencing and quantitative PCR, and compared our results between ants that had foraged or not on insect prey over a year. We thus determined how much the *A. octoarticulatus* gaster microbiome varies across ant colonies and experimental treatments. The consistent presence of particular microbial taxa across ant colonies might suggest that they carry out important functions for their hosts, while a shift in bacterial assemblages in response to treatment could indicate that bacteria are directly affected by what their hosts eat, or that ants regulate their microbiome in response to changes to their diet.

Methods

Study site and system

We carried out this study at the Centro de Investigación y Capacitación Río Los Amigos (CICRA) in the Peruvian Amazon (12°34′07″ S, 70°05′57″ W, elevation 230–270 m). CICRA extends over three different habitats: upland forests, floodplain forests, and swamps. Mean monthly temperature at the station varies from 21°C to 26°C and annual rainfall ranges from 2,700 to 3,000 mm (Pitman 2008).

Cordia nodosa (Boraginaceae) is an abundant ant-plant at CICRA. It has distinctive domatia: hollow, swollen stems that house ants. *C. nodosa* also provides nutritional rewards to ants in the form of food bodies on the surfaces of young leaves. The ants that nest in *C. nodosa* (*A. octoarticulatus*, three species in the genus *Azteca*, and occasionally *Myrmelachista schumanni*) protect their host plants against herbivores, and this benefit outweighs the cost of producing domatia and food bodies (Frederickson 2005, Frederickson et al. 2012). In this study, we focused on the obligate ant symbiont *A. octoarticulatus* (Myrmicinae). With very few exceptions, only one colony of *A. octoarticulatus* lives in an individual *C. nodosa* plant at a time; workers exclusively forage on the host plant, defending against herbivores. *Allomerus octoarticulatus* colonies live for an average of 7.8 yr, although their *C. nodosa* host plants can live for much longer (~77 yr) and are repeatedly colonized by ants throughout their lives (Frederickson and Gordon 2009).

Experimental manipulation of insect herbivores/prey

Our experimental design is described in detail elsewhere (Frederickson et al. 2012, 2013). Briefly, we grew *C. nodosa* saplings with and without most insect herbivores (potential *A. octoarticulatus* prey) and with and without ants, in a full-factorial manner. Here, we analyze data from only the two treatments with ants: A+H+ (ants added, herbivores allowed) and A+H− (ants added, herbivores excluded). The *C. nodosa* saplings used in the experiment were grown from wild-collected seeds in outdoor cages until they first produced domatia. In 2009, 52 saplings were transplanted in blocks of 4–13 2 × 2 m plots in the rainforest understory and then assigned at random to treatments. Twenty-six ant colonies were collected from naturally occurring *C. nodosa* plants at the same site; the number of workers in each colony was counted and the colonies were transferred to plants in the A+ treatments. Insect herbivores were manipulated using mosquito nets, which were hung over all of the saplings. To exclude herbivores in the H− treatment, the nets were staked securely to the ground; in the H+ treatment, the nets were raised 30 cm off the ground. Plants and ant colonies spent 314–329 d in the experiment.

Frederickson et al. (2012) showed that the herbivore exclusion treatment was successful, except for coccids that remained inside the domatia; herbivore damage to leaves was significantly lower on H− than H+ plants. Therefore, ant colonies in the H− treatment were prevented from foraging for insect prey on plant surfaces, while ant colonies in the H+ treatment had access to insect prey as a potential proteinaceous food source.

Ant diet

In June 2010, we investigated whether the ant colonies in the H− treatment were balancing their diet by foraging for more protein than the ant colonies in the H+ treatments. We measured the recruitment of *A. octoarticulatus* workers in our experimental colonies to two bait types with either 3:1 or 1:3 ratios of protein (whey protein powder, InterACTIVE Nutrition International Inc.®, Canada) to carbohydrates (table sugar) (modified from Dussutour and Simpson 2008). We poured the baits into 1.5-mL centrifuge tubes and attached with wires to various domatia. Both baits were present on the same tree at the same time but on different domatia. After five hours, we recorded the number of ants at these baits.

At the end of the experiment, we collected and preserved all the ants in each colony in 96% ethanol. We analyzed the ratios of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) to investigate the trophic level of each colony (Blüthgen et al. 2003). We took a random sample of 20 ants per colony, removed their gasters, oven-dried them for 48 h at 60°C, and weighed them (Blüthgen et al. 2003, Feldhaar et al. 2010). Stable isotope measurements on individual ants were not possible due to their small sizes. We compared the stable isotope ratios of ants in this experiment to those of naturally occurring plants, ants, insect herbivores, and predatory spiders collected at the same site (Appendix S1: Table S1). Samples were analyzed at the UC Davis Stable Isotope Facility.

Colony performance

To assess colony performance, we first counted all adult worker ants present on branches, leaves, and inside

domatia of the host trees to determine ant colony size at the end of the experiment. Second, we measured eight morphological characters (head length, head width, scape length, mandible length, mesosoma length, mesosoma width, petiole and postpetiole length, and gaster length) on 20 worker ants per colony, following Fernández (2007). We then measured the dry mass (oven-dried for 48 h at 60°C) of five workers per colony. Finally, to determine if worker mass is related to ant colony fecundity, we collected eight naturally occurring colonies of *A. octoarticulatus* (i.e., not in our main experiment), counted the number of workers and reproductives (i.e., alate males and females, as well as reproductive pupae and larvae). We also dried and weighed 20 workers from each of these eight colonies.

Gaster microbiome

We investigated the microbiome of ant gasters from this experiment by sequencing bacterial 16S rRNA genes. We surface-sterilized 20 workers per colony with a 10% bleach solution for 15 s and rinsed them with phosphate-buffered saline (PBS) solution for 30 s before removing the gasters. We removed the 20 gasters with tweezers and we pooled all of them in one sample to perform a phenol: chloroform DNA extraction. We purified the DNA extractions with a silica column (DNeasy Blood & Tissue Kit, QIAGEN®, Hilden, Germany) and isopropanol precipitation. We sent the resulting extracted DNA for microbial diversity analysis to the Research and Testing Laboratory in Lubbock, Texas for bacterial tag-encoded FLX pyrosequencing (bTEFAP) using the primers Gray28F (5′ TTTGATCNTGGCTCAG) and Gray519r (5′ GTNTTACNGCGGCKGCTG). The sequencing library was generated by one-step PCR with 30 cycles and the bTEFAP was performed on a 454 FLX, Roche, Branford, Connecticut, USA instrument (Dowd et al. 2008).

In addition, we performed quantitative PCR (qPCR) to measure the abundance of bacteria in the same DNA samples that were used to analyze bacterial diversity. We prepared each 20-μL reaction to quantify 16S rRNA gene sequences with 10 μL of Perfecta SYBR Green FastMix (VWR), 6.5 μL of molecular grade water, 0.75 μL of 338F (5′ACTCCTACGGGAGGCAGCAG) and 0.75 μL of 518R (5′ ATTACCGCGGCTGCTGCTGG) primers (Lane 1991), and 2 μL of sample. The reactions were duplicated and we ran them in one cycle of 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 55°C, 30 s at 72°C, and a final cycle of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. Absolute quantities were estimated using standard curves of *Escherichia coli* 16S rRNA genes cloned into a plasmid and linearized, per Sanders et al. (2017).

Statistical analysis

For the *Ant diet* and *Colony performance* analyses, we omitted three blocks of the initial 13 because at least one ant colony in each of these blocks did not survive to the end of the experiment. We included block as a random factor while fitting the statistical models. We square-root transformed all count data and log-transformed all mass data to improve normality. To analyze the recruitment of ant workers to protein-rich baits, we used an ANCOVA model with herbivore treatment as a fixed factor, and recruitment of ant workers to carbohydrate-rich baits as a covariate, thereby incorporating colony activity levels into the model. We compared the carbon and nitrogen stable isotope ratios between herbivore treatments with a MANOVA test followed by linear mixed models, with block as a random factor, to clarify which variables differed between treatments.

We ran a principal component analysis on the morphological character data. Most of the variation in the data set was explained by the first and second principal components (PC1 explained 50% and PC2 explained 14%). We used PC1 and PC2 to compare worker size between treatments in an ANOVA. We also analyzed the worker mass and colony growth data in mixed-effects ANOVAs. We excluded one block because its ant colonies were outliers in all the analyses; during the experiment, a large tree fell near this block, creating a light gap that caused unusually rapid plant and ant colony growth. To determine if worker mass was correlated with the number of reproductives, we regressed the number of alates per colony against ant colony size (i.e., number of workers). We used the residuals of that regression to compute a second linear regression of worker mass on the number of alates.

For the molecular analyses of gaster microbiota, we included all surviving colonies in the experiment. It was not possible to amplify the samples from the H+ treatment in one of the blocks (I), thus we could not include this sample in the analyses (final number of blocks analyzed = 11; nine pairs and two singletons); the use of these singleton blocks (I and J) did not change any of the microbiome patterns found in the analyses. We used AmpliconNoise to denoise the pyrosequencing data, thereby removing most of the amplification errors and chimeras from the data set (Quince et al. 2009). Afterwards, using the Qiime platform (Caporaso et al. 2010), we aligned and clustered the sequences to produce a denoised operational taxonomic unit (OTU) table, assigning taxonomy using the RDP classifier and database (Wang et al. 2007). We omitted all OTUs that had fewer than two reads per sample to exclude rare bacteria more likely to be contaminants or errors. We computed a single rarefaction at a depth of 600 reads to account for the differences in the number of reads in each sample; blocks H, I, and the H+ sample from D were excluded with this procedure because of a low number of reads. On this rarefied OTU table, we analyzed alpha diversity with Faith's Phylogenetic Diversity (PD), which is based on the phylogenetic diversity of samples, calculated with the total branch length of the bacterial tree. We examined beta diversity in a principal coordinates analysis (PCoA). To perform the PCoA, we created a distance matrix using the unweighted UniFrac algorithm. This algorithm uses the branch lengths in a

phylogenetic tree generated with the sample sequences to test for the fraction of total branch length that is unique to one environment, providing information on presence and absence of different bacteria types. We also used a weighted UniFrac algorithm that uses the same principles as unweighted UniFrac, but weights the branches by the taxon abundance (Lozupone et al. 2007). We used PERMANOVA with the function Adonis in R (1,000 permutations, method = bray) to analyze the variance of the bacterial assemblages resulting from treatment and block effects. Counts of 16S sequences obtained with qPCR were standardized by DNA concentrations for each sample. Statistical models were calculated in JMP (10.0.0, SAS Institute Inc., Cary, North Carolina, USA) and R (3.2.1, R Development Core Team 2015).

Results

Ant diet

Ants in the H− treatment strongly preferred protein-rich baits; more than twice the number of ants recruited to the protein-rich baits in the H− (mean ± SE, 16.7 ± 7.613 workers) than in the H+ (7.2 ± 4.060 workers) treatment (ANCOVA, treatment effect, $F_{1,7} = 8.249$, $P = 0.024$). There was also a significant effect of the covariate (number of ants at C-rich baits, $F_{1,15} = 12.412$, $P = 0.003$), reflecting differences in activity levels among ant colonies.

Ants that developed in the presence of insect herbivores had different carbon and nitrogen isotopic signatures than ants that developed without the insect herbivores (Fig. 1; MANOVA $F_{1,15} = 4.7296$, $P = 0.025$). This difference was mostly due to $\delta^{13}C$ (H+, −32.78‰ ± 0.19‰; H−, −33.50‰ ± 0.21‰; $F_{1,8} = 9.70$, $P = 0.014$; Fig. 1A), as $\delta^{15}N$ did not differ significantly between treatments (H+, 11.01‰ ± 0.27‰; H−, 10.35‰ ± 0.32‰; $F_{1,8} = 2.3731$, $P = 0.162$; Fig. 1C). For all the samples in the experiment, $\delta^{15}N$ was enriched compared to those of the naturally occurring reference samples (Fig. 1D), while $\delta^{13}C$ values of ants in the experiment were closer to those of naturally occurring ants (Fig. 1B).

Colony performance

Elsewhere, we reported that there was no significant difference in ant colony size between treatments, although H+ colonies were larger on average than H− colonies at the end of the experiment (Frederickson et al. 2012). Here, we found that workers that developed in the presence of insect prey were significantly heavier than those that did not (H+, 0.056 ± 0.002 mg; H−, 0.047 ± 0.003 mg; $F_{1,8} = 8.013$, $P = 0.022$; Fig. 2A), suggesting that colonies achieved a larger total biomass when they had access to insect prey. Workers also tended to be larger in the H+ than in the H− treatment, although this difference was not statistically significant (PC1, $F_{1,8} = 2.192$, $P = 0.177$). Finally, independently of colony size (i.e., worker number), worker mass explained about 36% of the variance in the number of adult reproductives in the naturally occurring colonies that we sampled ($F_{1,7} = 5.433$, $P = 0.05$; Fig. 2B). Therefore, colonies with heavier workers make more reproductives.

Gaster microbiome

Treatment did not influence the bacterial assemblage in *A. octoarticulatus* gasters, in particular the alpha diversity of the gaster microbiome: H+ and H− gasters were similarly diverse (Fig. 3A). The bacterial assemblage was

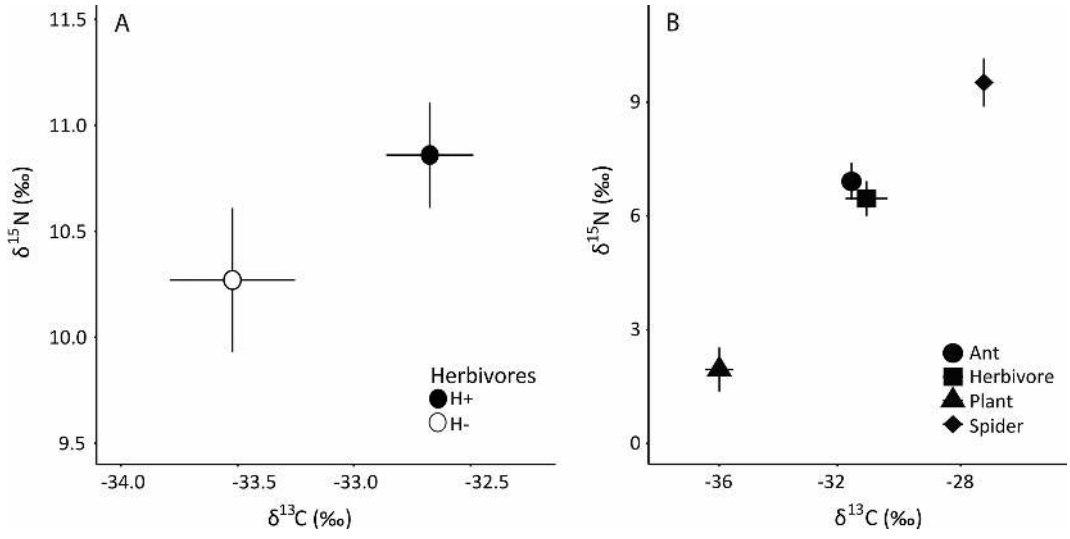

Fig. 1. (A) Carbon and nitrogen stable isotope ratios for ants in the H+ and H− treatments. Open circles indicate treatments with no access to prey (H−), while black circles indicate treatments with access to insect prey (H+). (B) Carbon and nitrogen isotope ratios for naturally occurring *C. nodosa* (plant), *A. octoarticulatus* workers (ant), insect herbivores (coleopterans, hemipterans, etc.), and predatory spiders found on *C. nodosa* are given for reference. All values are mean ± SE.

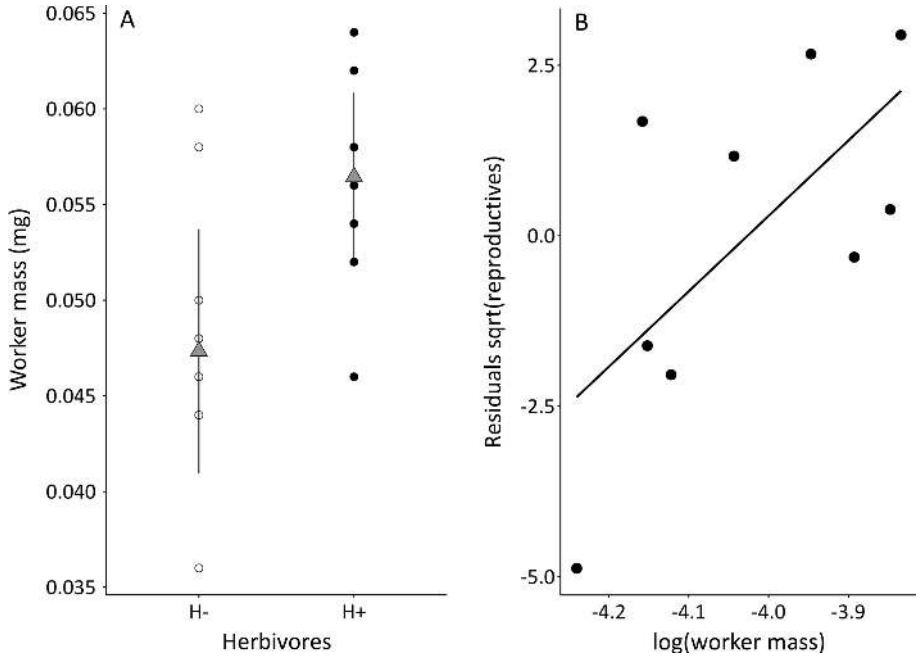

FIG. 2. (A) Worker mass (mean ± SE) for ants in the experimental manipulation (gray triangles). Ants with no access to prey in open circles (H−), while ants with access to insect prey in black circles (H+). Each circle is the mean mass of five workers per colony. (B) Relationship between worker mass (mean mass of 20 workers per colony in grams, log-transformed) and the residual values of the number of reproductives (i.e., alates; square-root transformed [sqrt]) per colony (adjusted $r^2 = 0.36$; intercept = 44.518, slope = 11.057).

dominated by Firmicutes (38.9%), Proteobacteria (18.4%), Bacteroides (12.5%), and Actinobacteria (9.7%; Fig. 3B). Unexpectedly, Fusobacteria were abundant in one ant colony (H−, block J); these bacteria, which are often pathogenic, accounted for 84.5% of the bacteria in that sample.

Analysis of beta diversity through PCoA also did not show any differences between treatments in the microbial assemblages in the ant gasters (Appendix S1: Fig. S1). The first three principal coordinates explained about 41% of the variation in the data with unweighted UniFrac and 72% with weighted Unifrac. The PERMANOVA test did not detect significant shifts in bacterial assemblages between treatments but suggested that environmental factors in different blocks might have influenced the structure of these assemblages when outlier blocks A and J were present in the analyses (Appendix S1: Table S2). However, no measured variables (e.g. number of scale insects, light, etc.) predicted changes in the bacterial assemblages (results not shown).

Similarly, bacterial abundance did not seem to be different between treatments. However, all samples had very few bacteria in the ants' gasters; as a result, 16S counts were at or below the detection limit when analyzed with qPCR making it unreliable to perform statistical analyses on these data.

DISCUSSION

Although *A. octoarticulatus* colonies can survive in the absence of insect prey, our results suggest that insect prey do contribute to colony nutrition and fitness. Thus, foraging for insect prey appears to be directly beneficial to *A. octoarticulatus* colonies, and plant defense may well be a by-product of this behavior. After a year excluding most potential animal prey, workers in the H− treatment recruited to protein-rich baits more often than their H+ counterparts. This result is consistent with the geometric theory of insect nutrition of Raubenheimer and Simpson (1999), House (1969), and Waldbauer and Friedman (1991). They suggest that insects should balance their diet to reach an optimal concentration of essential nutrients required for development and survival when these are scarce in the environment. *Allomerus octoarticulatus* workers foraged more frequently at protein baits when insect prey was absent, suggesting that *A. octoarticulatus* actively seeks insect herbivores as a food source. This is supported by observations that a closely related ant, *A. decemarticulatus*, builds traps with fungi and plant debris to capture insects (Dejean et al. 2005, 2013, Orivel et al. 2017). We observed similar carton-like structures built by *A. octoarticulatus* that might play an important role on food acquisition (Leroy et al. 2010, Dejean et al. 2013, Orivel et al. 2017).

We expected that the experimental manipulation of insects would result in lower $\delta^{15}N$ and $\delta^{13}C$ values for workers in the H− than the H+ treatment, because these workers should have been more dependent on plant-derived foods and therefore feeding at a lower trophic level. The stable isotope models showed that the treatment had a significant effect on $\delta^{13}C$ values, but not on $\delta^{15}N$

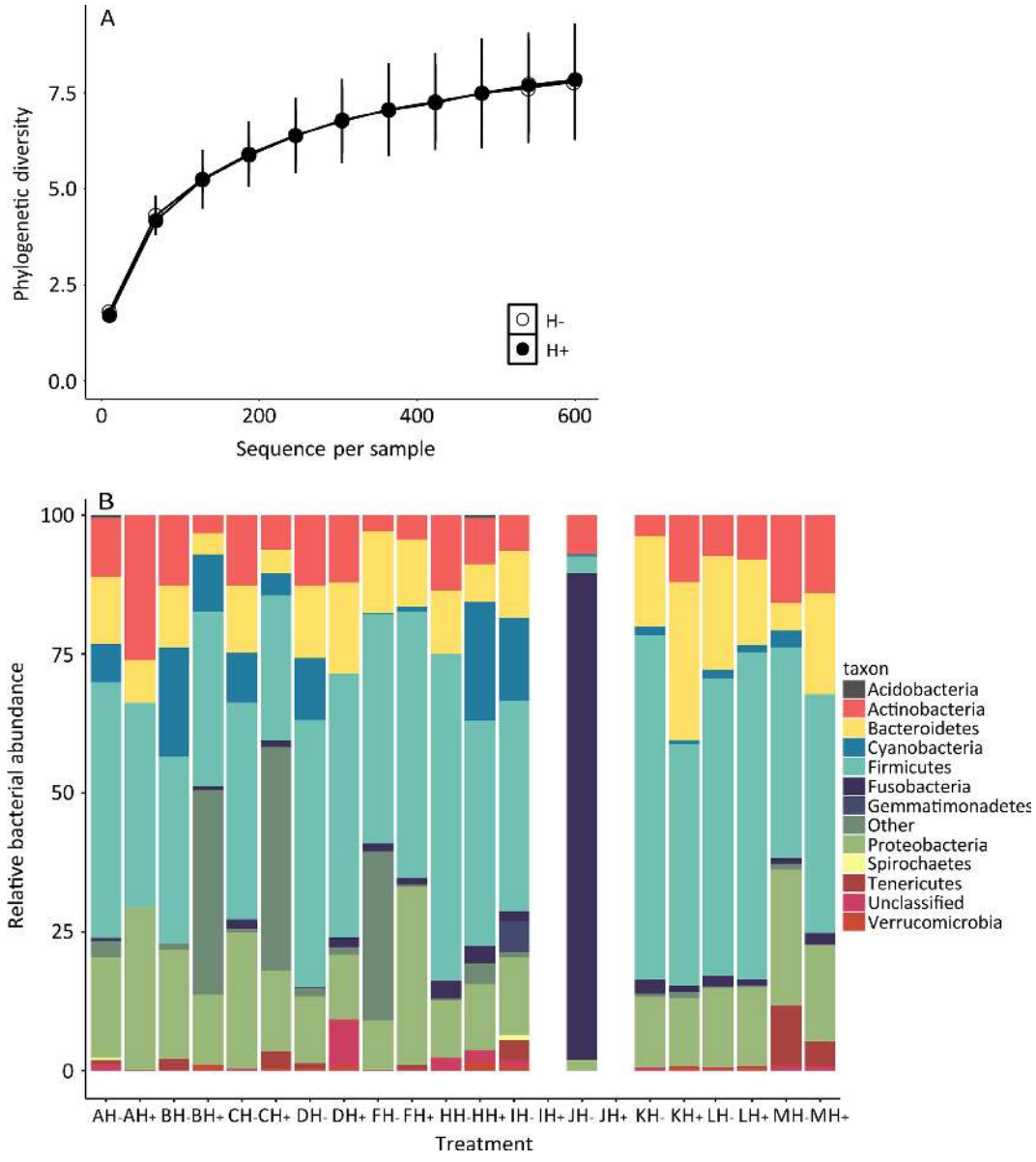

FIG. 3. (A) Phylogenetic diversity (PD) of bacteria in ant gasters with access to insect prey (solid circles) and without insect prey (open circles). Rarefaction plot obtained with a PD whole tree matrix. Values are mean ± SE. (B) Relative abundance of bacteria phyla in each sample. Colors in the bar graph represent the proportion of different operational taxonomic units (OTUs) clustered at 97% similarity. Samples are identified by their block letter followed by treatment (H+, access to insect prey; H−, no insect prey).

values. Overall, we observed an average decrease of 0.6‰ for $\delta^{15}N$ and 0.72‰ for $\delta^{13}C$ in H− ants compared to H+ ants (Fig. 1A). The average change in isotopic signature of ^{13}C is consistent with predictions that trophic level changes with differences of ~0.5–1‰ for ^{13}C. This is not the case for our values of ^{15}N, which are expected to change 3–5‰ for ^{15}N per trophic level (Minagawa and Wada 1984, Sagers et al. 2000, Michener and Kaufman 2007). Ants might be able to balance their carbohydrate to nitrogen intake very precisely; hence ants that do not have access to insect prey might consume less protein, making them smaller, but with similar ratios of ^{15}N relative to ^{14}N incorporated in the ants' tissues. In the absence of insect prey, ant workers may eat more plant-derived food, food bodies and honeydew, which can have different $\delta^{13}C$ signatures (Feldhaar et al. 2010, Brewitt et al. 2014, Parmentier et al. 2016), although this does not explain the similar $\delta^{15}N$ values between treatments. Alternatively, our experiment may not have had sufficient statistical power to detect treatment differences in $\delta^{15}N$ ratios.

All of the ants in the experiment were substantially ^{15}N enriched compared to workers from naturally occurring

A. octoarticulatus colonies from both the same site (Fig. 1B) and a nearby site (Davidson 2003). Since the experimental plants, ants, and soil were sourced from the same area as the reference samples, we did not expect the experimental manipulation to produce such a marked and general shift in $\delta^{15}N$. The ant colonies we studied range in size and age at the beginning of the experiment (Frederickson et al. 2012) but all of them had a similar ^{15}N enrichment, implying that these factors might not explain the shift in ^{15}N between experimental and naturally occurring ant colonies. It is possible that the season when the ants were collected or the specific location of the experimental plots drastically changed the isotopic values, as it has been demonstrated in other studies (e.g., Menke et al. 2010). This enrichment however does not invalidate the observed differences in isotope ratios between the experimental treatments.

Colony performance was consistent with the diet analyses; *Allomerus octoarticulatus* colonies had higher relative fitness (e.g., worker mass) when insects were part of their diet in this experiment. Although there was no statistically significant difference in colony growth or size between treatments, worker mass data showed that ants in the H− treatment were lighter than ants in the H+ treatment. Thus, ant biomass was higher when insect prey was available. In naturally occurring colonies from the same population, colonies with heavier workers made more reproductives; such colonies may have more energy to invest in producing reproductives. These results support a previous study by Dejean et al. (2013), in which supplementation with insect herbivores increased the reproductive output of *Allomerus decemarticulatus* colonies. Although more experimental work needs to be done to determine if worker mass causes an increase in reproductive output, numerous field studies and mathematical models have shown that body mass is positively related to reproductive success of individuals and colonies, in large part because body mass influences metabolism, growth rate, mortality rate, and egg size (Banse and Mosher 1980, Juliano 1985, Savage et al. 2004). For example, workers with higher body mass could live longer, reducing the need for continuous worker production in the colony and allowing more resources to be invested in the production of reproductives. The results of this experiment add to those of earlier studies of ants that found that colony fitness can be inferred by the mass of adult reproductives and colony size (Gordon 1992, Deslippe and Savolainen 1994, Wagner and Gordon 1999, but see Ingram et al. 2013).

The assemblages of symbiotic bacteria in *A. octoarticulatus* gasters were not influenced by our experimental manipulation of insect prey. We would have expected this result if the microbiome were so tightly associated with the ants that it does not vary among ant colonies; however, we found that the bacterial assemblages in *A. octoarticulatus* gasters were very variable among colonies, negating our assumptions of a core bacterial community. This result, in concert with a decrease in worker mass when insect herbivores are absent, support a scenario were the gaster microbiome cannot compensate for a low-nutrient diet. Phylogenetic diversity was equal in both treatments and the composition of bacteria seemed to vary independently in each colony (Fig. 3). Furthermore, the variation in taxa among all samples shows that most bacteria are not tightly associated with ant diet. Neither weighted (relative abundance of bacteria included) nor unweighted (only presence/absence of bacteria) UniFrac analyses showed strong patterns in dissimilarity among the bacterial assemblages in the two treatments, and this was supported by a PERMANOVA test (Appendix S1: Table S2, Fig. S1). The PERMANOVA test shows a marginally significant block effect on microbiome assemblages, suggesting some environmental impact. Bacterial communities may be affected indirectly by environmental interactions with the host. For example, ants in some of the blocks did poorly; in block G neither of the colonies survived and in block J, the H+ colony did not survive to the end of the experiment and the H− colony was probably infected with a bacterial pathogen from the phylum Fusobacteria. This common bacterium was never in the same relative abundance found in the sample from block J. Thus, the question of what factors structure bacterial assemblages in *A. octoarticulatus* gasters remains unanswered.

Similar to other studies of bacterial assemblages in other arthropods, we found a complex and diverse community of bacteria. The bacterial taxonomic units in *A. octoarticulatus*, including OTUs in phyla such as Firmicutes, Bacteroidetes, and Proteobacteria, are mostly common inhabitants of arthropod guts (e.g., bees [Cox-Foster et al. 2007], aphids [Oliver et al. 2010], ants [Van Borm et al. 2002, Seipke et al. 2013]). Bacterial groups such as γ-Proteobacteria and Bacteriodetes have been suggested to provide many benefits to ants (Zientz et al. 2005, Eilmus and Heil 2009). These two taxa make up over 20% of the bacteria found in *A. octoarticulatus*. One interesting finding was the high relative abundance of Actinobacteria, a taxon usually associated with soils, from which we found bacteria from the genus *Pseudonocardia*. This type of bacteria was also present in 37% of all *Allomerus* studied by Seipke et al. (2012*b*) and some of the Actinobacteria strains in this ant have antifungal properties (Seipke et al. 2012*a*, Gao et al. 2014). This is relevant because *Allomerus* ants need to maintain the carton trails they build with fungi and plant debris (Dejean et al. 2005). Pinto-Tomas et al. (2009) determined that in Attini ants, *Pseudonocardia* bacteria could produce antibacterial agents that help to defend their fungal gardens. Following Leroy et al. (2010), we emphasize that the association among fungi, ants, and bacteria might be more widespread and important that previously thought. The low absolute quantities of bacteria we measured in this system argue for caution in interpreting our results (Sanders et al. 2017); so while we do not expect highly conserved relationships between symbiotic bacteria and *A. octoarticulatus* workers or the

microbiome compensating for a low-nutrient diet, we acknowledge the possibility of indirect benefits from the gaster microbiome not tested in this study.

In conclusion, mutualisms are surprisingly evolutionarily robust, despite a widespread expectation that partners should experience selection for selfish, cheating behavior (Axelrod and Hamilton 1981, Bronstein 2001, Douglas 2008). However, many mutualisms are likely stabilized by by-product benefits, in which partners are not under selection to cheat because their mutualistic behavior is not costly, even in the short term (Sachs et al. 2004, Mayer et al. 2014, Jones et al. 2015). We hypothesized that ant–plant relationships can be stabilized through by-product benefits, especially when ants consume insect herbivores. We manipulated the presence of arthropod prey visiting *C. nodosa* plants to determine if the lack of an external protein source affects ant performance. We found that ant colonies actively forage for protein-rich food, and achieve higher colony biomass when they have access to arthropod herbivores. The mutualism between *C. nodosa* and *A. octoarticulatus* thus appears to have arisen through by-product benefits to the plant of the ants' foraging behavior. Ant colonies forage for and consume arthropod prey on their host plants, resulting in mutual benefits to host plants and ant colonies.

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Supporting Information

Additional supporting information may be found in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ecy.2036/suppinfo

Data Availability

Data associated with this paper are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.gf7bh.