

Epiphytic ant-plant obtains nitrogen from both native and invasive ant inhabitants

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Abstract

Ant-plants have been extensively used as model systems in the study of the evolution and ecology of mutualisms. Using a ¹⁵N isotope labeling experiment, we found that both a native ant mutualist (*Philidris cordata*) and an invasive ant (*Pheidole megacephala*) provide nitrogen to the Australian ant-plant *Myrmecodia beccarii*.

KEYWORDS

¹⁵N, invasive species, mutualism, myrmecophyte, myrmecotrophy, nitrogen, stable isotope, tropical Australia

1 | INTRODUCTION

Ant-plants, or myrmecophytes, are plants that have evolved to house ants in modified structures known as domatia. In return, ants may protect their host-plant from herbivory, remove encroaching vegetation, or provision the plant with nutrients from their waste (myrmecotrophy) (Bronstein et al., 2006; Heil & McKey, 2003). The benefit received by the plant depends on its ecology; epiphytes, with no access to soil nutrients, are thought to be more reliant on ant-derived nutrients (Mayer et al., 2014).

Substantial partner quality variation has been recorded among ants inhabiting ant-plants, particularly regarding patrolling behavior and defensive activity (Bruna et al., 2004; Frederickson, 2005; Gaume et al., 2005; Palmer & Brody, 2007). Although benefits provided by different ant inhabitants may be context dependent, some ant species are clearly suboptimal. Species that accept resources

while providing little or no benefit in return are referred to as “cheaters” if they have evolved from mutualistic ancestors (Segraves et al., 2005), or “exploiters” if they are third-party species that share no co-evolutionary history with the plant (Bronstein, 2001).

Invasive species are frequent exploiters of mutualisms. Invasion by non-native ants often alters ecological interactions because the invaders displace native ants and fail to emulate their functional roles (Lach, 2003). However, the effect of invasive ants in mutualisms varies based on ecological context and the species of ant invader (Ness & Bronstein, 2004). Few studies have examined the effects of invasive ants in myrmecophyte mutualisms. The invasive electric or little fire ant, *Wasmannia auropunctata*, from South America displaces the native *Tetraponera aethiops* from the domatia of *Barteria fistulosa* in Gabon, resulting in an increased incidence of lianas encroaching on host trees (Mikissa et al., 2013). Similarly, the African big-headed ant *Pheidole megacephala* displaces the native

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ant inhabitants of *Vachellia drepanolobium* (Visitacao, 2011) and causes a marked increase in the level of elephant herbivory to the plants by failing to defend plants (Riginos et al., 2015) even resulting in a decrease of tree-level photosynthesis (Milligan et al., 2021). To the best of our knowledge, there is no published research examining how an invasive ant affects a nutrient-provisioning ant-plant mutualism.

The ant-plant *Myrmecodia beccarii* (Rubiaceae) is an epiphyte endemic to northeastern Australia, found in *Melaleuca* woodlands and mangrove forests, and listed as vulnerable (Department of the Environment, 2022). As *M. beccarii* seedlings develop, their hypocotyl swells to form a tuber in which domatia form as a series of cavities (Figure 1). There are two types of cavities used by ants: light smooth-walled chambers where the ants keep their brood and dark rough-walled chambers where ants deposit their waste (Greenfield et al., 2021; Huxley, 1978). *M. beccarii* appears to display a high degree of partner specificity with the ant *Philidris cordata* (Dolichoderinae) (Volp & Lach, 2019), which is consistent with the partner specificity observed in other *Myrmecodia* species (Chomicki & Renner, 2017). A preliminary study revealed that *Phi. cordata* provides *M. beccarii* with nitrogen and reported no evidence that *Phi. cordata* protects *M. beccarii* from herbivory (Sommer, 1990). Rather, *Phi. cordata* presence increased herbivory, purportedly due to ants tending larvae of *Hypochrysops apollo* (Lepidoptera: Lycaenidae). The invasive African big-headed ant, *Phe. megacephala*, has also been recorded inhabiting *M. beccarii* with records as far back as 1928 (Barrett, 1928; Common & Waterhouse, 1981). However, whether *Phe. megacephala* can provide nutrients to *M. beccarii* or acts as an exploiter in this nutrient-provisioning mutualism is unknown.

We examined nitrogen provisioning in the *M. beccarii* ant-plant mutualism. We aimed to determine if the native and invasive ant species differed in the amount of nitrogen they provide to *M. beccarii*. We predicted that the native mutualist ant (*Phi. cordata*) would provide more nitrogen than the invasive ant (*Phe. megacephala*).

To determine whether *M. beccarii* can obtain nitrogen from ant inhabitants we conducted a stable isotope labeling experiment in a shadehouse located at James Cook University's Cairns campus (16°48'58.3"S, 145°41'13.48"E). We purchased two-year-old *M. beccarii* plants from a licensed commercial nursery (Takahar Gardens, Mackay) where they had been growing from seed and regularly fertilized. Plants had not been treated with pesticides, nor to our knowledge had they been inhabited by any ant species. We grew plants in plastic pots with a composted pine bark potting mix (Debco™ orchid: 8–18mm grade) until moving them to the field to enable ant colonization.

We randomly assigned unoccupied ant-plants to be placed in locations inhabited by either the native or invasive ant and allowed the plants to be naturally colonized by the ants. *Philidris cordata* colonies were obtained by attaching unoccupied *M. beccarii* plants to trees among a population of *M. beccarii* just north of Cairns (16°46'50.24"S 145°41'34.66"E). *Pheidole megacephala* colonies were obtained from James Cook University's Cairns campus (16°48'58.3"S, 145°41'13.48"E) by placing plants at the base of trees with *Phe. megacephala* nesting in the soil. After colonization by the respective ant species, we returned plants to the shadehouse and placed them in plastic containers (29 × 21 × 10 cm) with walls coated with Fluon® and Tanglefoot®. Ant colonies were provided with cotton wool balls soaked in 10% sucrose solution in a 50mL plastic centrifuge tube *ad libitum* and a protein source (mealworms and crickets) 2–3 times per week.

To examine nitrogen transfer from ants to plants, we provided ant colonies with a 50% sucrose solution labeled with ¹⁵N-enriched glycine (98 atom% ¹⁵N, Sigma-Aldrich). ¹⁵N labeling provides a simple way to track short-term changes in nitrogen flux and has repeatedly been used to study myrmecotrophic mutualisms (Mayer et al., 2014). We provisioned each ant colony with a total of 5mg of ¹⁵N-enriched glycine over a 14-day period by providing ants 100μl of labeled solution provided in 5mL plastic test tubes on alternating days. Prior to labeling, we took samples for both ants and plant leaves to

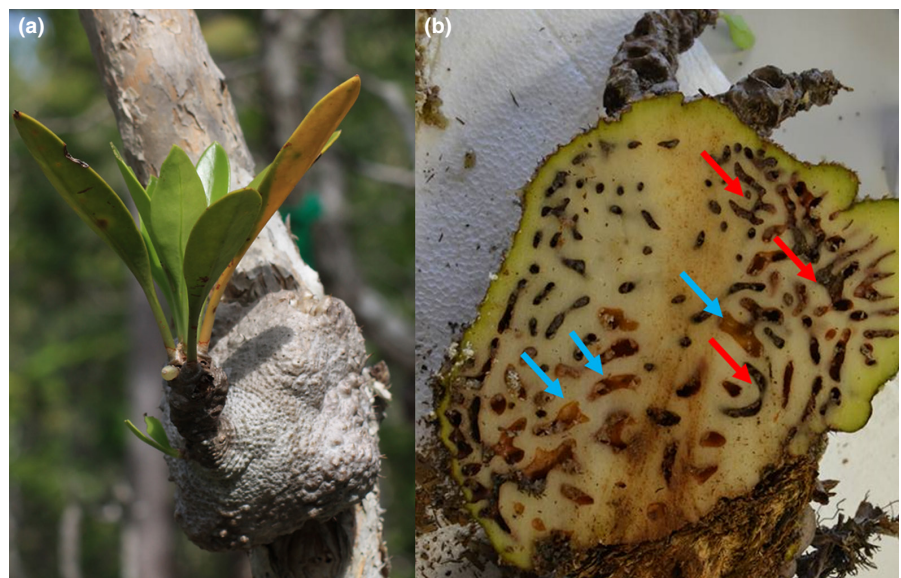


FIGURE 1 (a) A young *Myrmecodia beccarii* and (b) a dissected *M. beccarii* showing domatia with smooth- (blue arrows) and rough- (red arrows) walled cavities

obtain background levels of ^{15}N . Three weeks after the isotope label was first fed to the ants, we collected samples of approximately 20 worker ants to ensure the ^{15}N label was present in the ant colonies. Three and six weeks after the label was fed to the ants, we sampled one 15mm diameter leaf disc from the youngest fully expanded leaf to determine ^{15}N transfer to plants. Samples were dried in an oven at 70°C and ground into a fine powder. We conducted this experiment twice in two separate rounds, each of which had five replicates for each ant species. Each replicate consisted of an ant colony within a single plant. One *Phi. cordata* ant colony died during the experiment; so, the plant it inhabited was removed from our study. Therefore, we had $n = 9$ plant replicates with *Phi. cordata* and $n = 10$ plant replicates with *Phe. megacephala*. One plant inhabited by *Phe. megacephala* died between the 3- and 6- week sampling points, preventing us from obtaining a 6-week sample.

Ant and leaf samples were analyzed using an elemental analyzer (EA1110; Carlo Erba) coupled to a continuous flow isotope-ratio mass spectrometer (Micromass; Isochrom) at the Australian National University Stable Isotope Laboratory. Nitrogen has two stable isotopes ^{15}N and ^{14}N . The stable isotope composition of a sample was expressed as a δ (delta) value in per mil (‰), calculated as follows:

$$\delta^{15}\text{N} = \frac{(R_{\text{sample}} - R_{\text{std}})}{R_{\text{std}}} \times 1000,$$

where R_{sample} is the molar ratio of the heavy isotope (^{15}N) to the light isotope (^{14}N) of the sample, and R_{std} is that of the standard (atmospheric N_2).

We used a linear mixed-effects model to test if ant colonies were able to uptake the labeled nitrogen and if there was any difference in uptake between the ant species. We used a second model

to examine if the ^{15}N label was transferred from ants to plants and if there was any difference in transfer between species. In both models, we used $\delta^{15}\text{N}$ values of either ants or plant leaves as the response variable, with species and sample time as fixed effects, and the plant identity as a random effect. The variances of post-pulse leaf $\delta^{15}\text{N}$ values of *Phe. megacephala* occupied plants were greater than those of plants inhabited by *Phi. cordata* according to Bartlett's test ($K\text{-squared} = 67.98$, $p < 0.001$). Therefore, we log-transformed ($\log(x+2)$) leaf $\delta^{15}\text{N}$ values to account for the unequal variance and the negative δ values. We used the `lmer()` function in the R package `lme4` (Bates et al., 2015) to create the models, and `lmerTest` (Kuznetsova et al., 2015) to obtain p-values and F-statistics. All statistical analyses were performed in R version 3.6.2 (R Core Team, 2019).

Both ant species were able to uptake the ^{15}N label ($F_1 = 123.13$, $p < 0.001$), and there was no significant difference in the ^{15}N uptake between the species ($F_1 = 2.79$, $p = 0.11$) (Table S1). The ^{15}N pulse substantially increase ant $\delta^{15}\text{N}$ values for both *Phi. cordata* (4.5 ± 3.8 , pre-pulse; 4119.0 ± 1084.2 , 3-weeks post-pulse) and *Phe. megacephala* (6.1 ± 1.6 , pre-pulse; 3041.1 ± 1231.5 , 3-weeks post-pulse). The ^{15}N label was transferred from ants to plants for both ant species, seen as the significant increase in plant $\delta^{15}\text{N}$ values over time ($F_1 = 43.37$, $p < 0.001$) (Figure 2, Table S2). There were no detectable differences in the log-transformed mean $\delta^{15}\text{N}$ change in plants between the two ant species ($F_1 = 2.71$, $p = 0.12$) (Figure 2, Table S2).

We showed that *M. beccarii* plants can obtain nitrogen from the native mutualistic ant *Phi. cordata* and the invasive ant *Phe. megacephala*. However, although both species provided N to the plant, the variance of our untransformed leaf $\delta^{15}\text{N}$ data differed between species, indicating that the ant species may vary in their reliability in providing N to the plant. Previous work on invasive ants

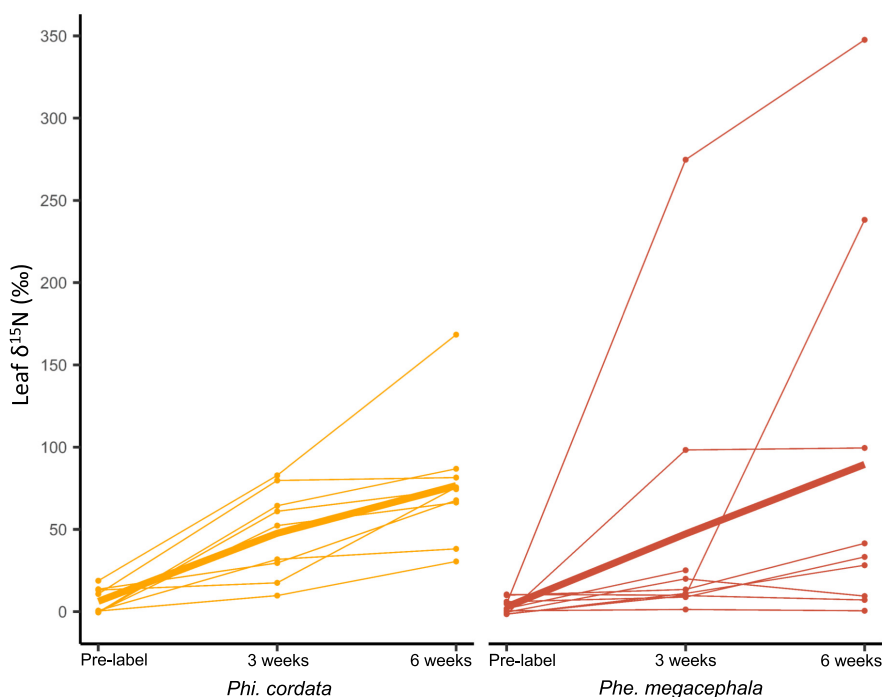


FIGURE 2 Effect of ant occupant species on *M. beccarii* leaf $\delta^{15}\text{N}$ values at three and six weeks after ant colonies were fed the ^{15}N label. Thin lines represent individual plants and bold lines represent the mean for each ant species over the respective sampling points. There was a significant increase in plant ^{15}N values over time ($F_1 = 43.37$, $p < 0.001$) indicating transfer of N from ants to plants, but ant species did not differ in their contribution of ^{15}N to *M. beccarii* ($F_1 = 2.71$, $p = 0.12$)

in ant-plants has revealed the invasive ant's inability to defend host plants from herbivory (Riginos et al., 2015) and encroaching vegetation (Mikissa et al., 2013), possibly because they have not coevolved with the ant-plants or their antagonists.

Other ant-plants obtain nutrients from a range of partners that may share an evolutionary history including interloping earthworms (Chanam et al., 2014), multiple facultative species of ant inhabitants (Dejean et al., 2017), and even carton-nesting "social parasite" ants that deposit their waste at the base of ant-plants (Dejean et al., 2021). Thus, some myrmecotrophic ant-plants can obtain nutrients from multiple different partners. The mechanisms determining partner quality in myrmecotrophic ant-plant mutualisms are less clear than those of plant-protection mutualisms. In plant-protection symbioses, ant behavior, particularly aggression, is the primary factor determining ant partner quality (Bruna et al., 2004). In myrmecotrophic ant-plant systems, there are at least two mechanisms that may determine the partner quality of nutrient-providing ants: ant behavior and the relationship between plant-ants and microbes and/or fungi.

Ant behavior may play an important role, as nutrient-provisioning ants may preferentially deposit waste material on absorptive surfaces (Chomicki & Renner, 2019; Huxley, 1978). In the related Hydnophytinae ant-plant, *Squamellaria huxleyana*, *Philidris nagasau* ants defecate and deposit detritus on the hyper-absorptive warty surfaces inside domatia, whereas generalist *Pheidole knowlesi* ants do not target their defecation or detritus to particular domatia surfaces in *Squamellaria wilkinsonii* (Chomicki & Renner, 2019). In other ant-plant systems, fungi (Leroy et al., 2011) and bacteria (Lucas et al., 2018) mediate the transfer of nutrients from ants to their host plant. Fungi likely play an important role in *M. beccarii* nutrient acquisition as the fungal communities differ between rough-walled ant waste chambers and smooth-walled brood chambers (Greenfield et al., 2021; Huxley, 1978).

Our findings imply that both ant species investigated did not differ in partner quality to *M. beccarii* in the context of our experiment. This outcome raises several questions about the *M. beccarii* ant-plant mutualism: as *Myrmecodia-Philidris* mutualisms appear to be specialized and the mutualisms conserved across their range (Chomicki & Renner, 2017), why is such specificity maintained if other ants may be acceptable mutualists? Do ant inhabitants differ in how much nitrogen they provide plants over longer periods of time, or are there other benefits provided by *Phi. cordata* that makes it an optimal partner? Finally, what are the mechanisms that enable partner selection? Answers to these questions will advance our understanding of the evolutionary ecology of this system as well as provide more general insights into ant-plant mutualisms.

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CONFLICT OF INTEREST

The corresponding author, on behalf of all authors, confirms that there are no conflicts of interest that may influence the objectivity of the research presented in this manuscript.

AUTHOR CONTRIBUTIONS

T.M.V, L.A.C, and L.L conceived and designed the study. T.M.V performed the experiments, analyzed the data, and wrote the first version of the manuscript. L.A.C. and L.L edited the manuscript.

DATA AVAILABILITY STATEMENT

The data for this study are openly available at: <https://doi.org/10.25903/5ef53d362b066>.

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