

## Short Original Article

# Nutrient provisioning of its host myrmecophytic tree by a temporary social parasite of a plant-ant

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One of the most advanced ant–plant mutualisms is represented by myrmecophytes sheltering colonies of some plant-ant species in hollow structures called domatia. In turn, the myrmecophytes benefit from biotic protection and sometimes nutrient provisioning (myrmecotrophy). Furthermore, over the course of evolution, some ant species have become social parasites of others. In this general context, we studied the relationship between its host trees and *Azteca andreae* (Dolichoderinae), a temporary social parasite of the plant-ant *Azteca ovaticeps*, and, as such, obligatorily associated with myrmecophytic *Cecropia obtusa* trees (Urticaceae). A first experiment showed that the  $\delta^{15}\text{N}$  values of the young leaves of *Cecropia* sheltering a mature *A. andreae* colony were very similar to those for trees sheltering *Azteca alfari* or *A. ovaticeps*, two typical *Cecropia* mutualists for which myrmecotrophy is known. In a second experiment, by injecting a  $^{15}\text{N}$ -labelled glycine solution into locusts given as prey to *A. andreae* colonies, we triggered an increase in  $\delta^{15}\text{N}$  in the young leaves of their host *Cecropia*. Thus,  $^{15}\text{N}$  passed from the prey to the host trees, explaining the outcomes of the first experiment. We discuss these results in light of the notion of ‘by-product benefits’.

**ADDITIONAL KEYWORDS:** ant–plant mutualisms – *Azteca* – *Cecropia obtusa* – myrmecotrophy – social parasite – stable isotopes.

## INTRODUCTION

Ants diversified from ground-dwelling predatory taxa alongside the rise of angiosperms. They incorporated plant-based food such as sap into their diet while, through their aggressiveness and predatory behaviour, they began to provide biotic protection to the plants they patrolled (Borowiec *et al.*, 2020). After this stage, ants and plants evolved diffuse defensive mutualisms. For instance, the evolution of extrafloral nectar (i.e. a sugary-rich ant reward offered by plants for biotic

protection) helped to develop direct relationships between ants and plants. Also, because the loss of sap is generally compensated for by the protection provided against defoliators, plants obtain an indirect benefit through the mutualism between ants and sap-sucking, honeydew-producing hemipterans (Styrsky & Eubanks, 2007; Heil, 2015; Nelsen *et al.*, 2018).

The first arboreal nesting ants sheltered in pre-existing tree cavities, this behaviour preceding the evolution of nests made of carton or silk. Plants developed cavities specifically to shelter ant colonies, called ‘domatia’, around 23.03 Mya, between the last part of the Palaeogene and the Neogene (Nelsen *et al.*,

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2018). Domatia characterize ‘myrmecophytes’ that, in exchange for sheltering the colonies of only certain plant-ant species, are protected from phytophagy, competitors and pathogens. Most myrmecophytes provide their guest ants with food (e.g. extrafloral nectar and/or food bodies), whereas some of these ant species also tend hemipterans (González-Teuber & Heil, 2015).

For some myrmecophytes the ant wastes accumulate in the domatia where humid conditions favour their degradation by fungi and/or bacteria, releasing nutrients that are absorbed by the host plant through specific tissues (e.g. the walls of the domatia, rhizomes, protuberances and roots) or via fungi cultivated by the ants. This process, called ‘myrmecotrophy’, probably evolved in response to the absence of soil for epiphytes or nutrient-poor soils for tropical phanerophytes (Beattie, 1989; Treseder *et al.*, 1995; Solano & Dejean, 2004; Leroy *et al.*, 2011; Chanam *et al.*, 2014; Dejean *et al.*, 2020).

In the Neotropics, emblematic, pioneer myrmecophytic *Cecropia* (Urticaceae) are associated with plant-ants of the genus *Azteca* (subfamily Dolichoderinae) in a complex mutualistic relationship that enhances plant fitness. The ants protect their host plants from different kinds of enemies, mostly defoliating insects, and provide them with nitrogen-rich nutrients (myrmecotrophy) (Sagers *et al.*, 2000; Davidson, 2005; Longino, 2007; Dejean *et al.*, 2012; Oliveira *et al.*, 2015). In turn, the ants are lodged in hollow trunks and branches (domatia) and provided with food in the form of pearl bodies and, especially, Müllerian bodies produced specifically by the trichilia, a tissue situated at the base of the leaf petioles (Fig. 1). These *Azteca* tend hemipterans in the host tree domatia where founding queens also feed their first larvae with Ascomycota fungi. Founding queens and workers access their host *Cecropia* domatia by chewing entrance holes through the prostomata or preformed thinner zones of the stem walls that, in this case, are recognizable only to certain plant-ant species (Davidson, 2005; Longino, 2007; Mayer *et al.*, 2018).

This ant–plant mutualism can be supplanted by *Azteca andreae*, a temporary social parasite of the mutualistic *Azteca ovaticeps* with which incipient colonies share domatia, forming mixed colonies. While their host trees develop, *A. andreae* colonies grow, become completely independent, build a succession of larger and larger external carton nests (Fig. 1), and no longer inhabit the domatia. Relatively large *A. andreae* colonies occasionally move and install a new nest on a non-*Cecropia* neighbouring tree. Workers do not exploit Müllerian bodies but tend different hemipteran taxa on the foliage of the host tree and surrounding plants. They hunt by ambushing along the leaf margins and attack as a group all insects landing on the leaves, seizing them by their appendages while holding on to

the leaf surface thanks to the ‘Velcro’ effect between their claws and the leaf trichomes. This enables them to capture a wide range of insects, including very large and/or agile ones (Fig. 1) and efficaciously protect their host trees from herbivores (Dejean *et al.*, 2010a, b).

Because *Cecropia obtusa* trees of all sizes (up to 25 m in height) sheltering an *A. andreae* colony seemed very healthy, we hypothesized that in addition to biotic protection they benefit from nutrient provisioning. Yet, typical myrmecotrophy might be excluded as large *A. andreae* colonies no longer shelter in their host *Cecropia* domatia. Instead, workers throw their wastes from their carton nests. These wastes mostly fall at the base of the host tree where, by decaying, might provide nutrients to the host tree roots.

## MATERIALS AND METHODS

### STUDY AREA AND FOCAL TAXA

This study and the preliminary surveys that permitted us to develop the experimental protocol were conducted during the rainy seasons between 2013 and 2016 in the ‘coastal plain’ of French Guiana. The climate is tropical moist with ≈2500 mm of yearly rainfall distributed over 210 days. The rainy season extends from December to mid-July, and minimum and maximum monthly temperatures average 25.2 and 30.6 °C.

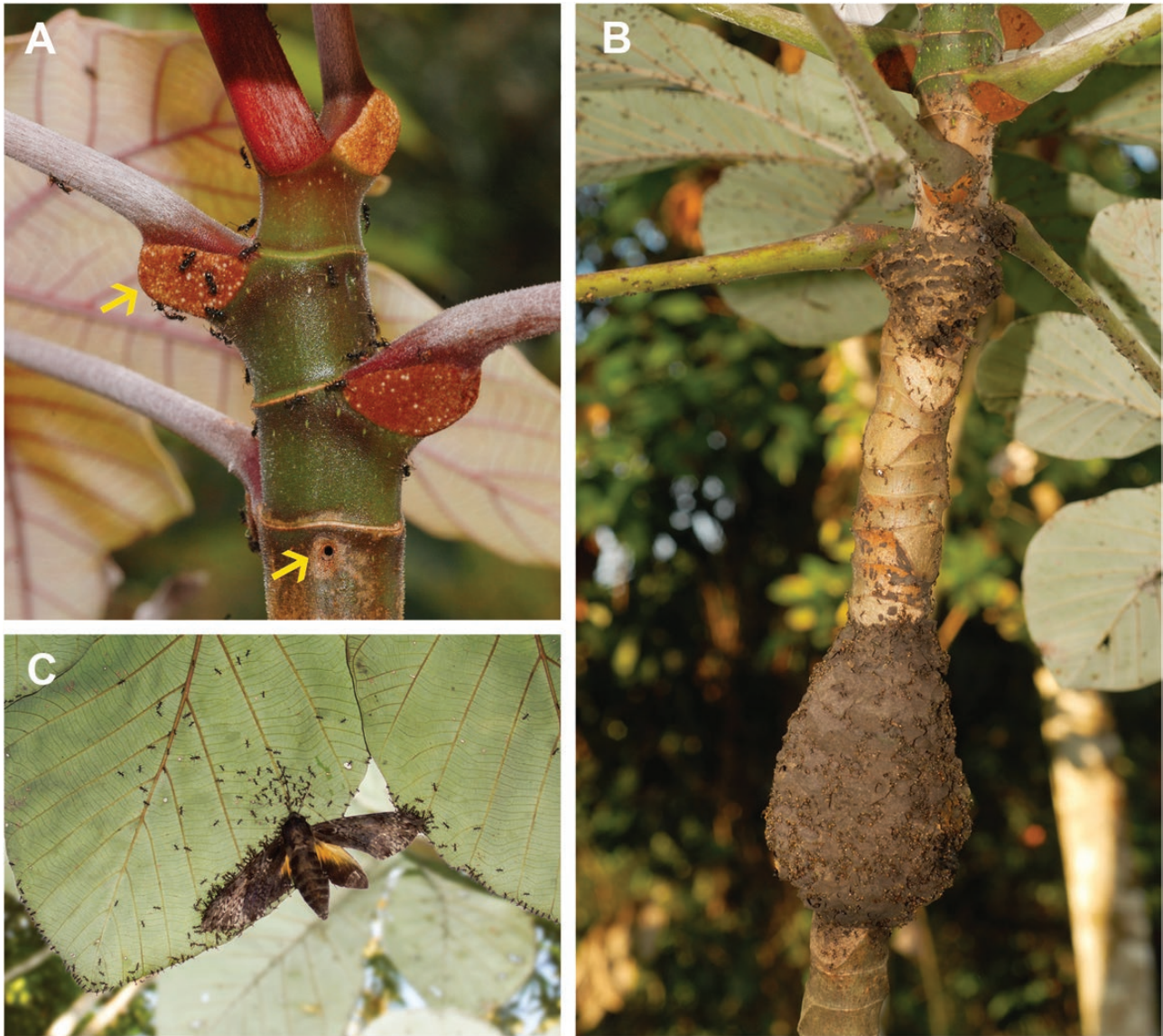
In the area studied two *Cecropia* species (i.e. *C. obtusa* and *C. palmata*) share two mutualistic *Azteca* species (i.e. *A. alfari* and *A. ovaticeps*); *A. andreae* parasitizes *A. ovaticeps* regardless of the host *Cecropia* species.

### NATURAL VALUES OF $\delta^{15}\text{N}$ IN *CECROPIA OBTUSA* LEAVES

During a field survey conducted around the city of Sinnamary (05°03′30.0″N, 52°58′34.6″W), we aimed to quantify the natural abundances of  $\delta^{15}\text{N}$  in *C. obtusa* leaves based on the absence or presence of *Azteca* spp. ant colonies. We proceeded in two stages due to the scarcity of trees devoid of any associated ants as well as trees sheltering *A. andreae* colonies. We first searched for *C. obtusa* not associated with ants (control lot: 23 trees 4–5 m in height) and then defined a zone with a radius of 15 m around these trees (similar soil). Within these zones the *C. obtusa* trees of a similar size sheltering an *A. alfari* or an *A. ovaticeps* colony (29 and 27 trees, respectively; 23 zones) were selected.

The same kind of sampling was conducted by selecting trees sheltering *A. andreae* colonies (18 trees) whose large carton nests permitted us to deduce that the host *Cecropia* tree’s youngest leaves developed only in their presence (38 and





**Figure 1.** A, extremity of a *Cecropia obtusa* tree sheltering a colony of *Azteca alfari* in its hollow internodes. Some workers are gathering Müllerian bodies (upper arrow). The lower arrow indicates a nest entrance chewed by workers in a prostomata. B, an *Azteca andreae* nest with the beginning of the construction of a new nest in the shade of young leaves. C, group-ambushing *A. andreae* workers hiding side-by-side under the *C. obtusa* leaves; they have captured a sphingid moth.

42 trees, respectively for trees sheltering *A. alfari* and *A. ovaticeps*; 18 zones). An  $\approx 5\text{-cm}^2$  piece of the youngest, most well-developed leaf from each *C. obtusa* was harvested for isotopic analysis. We were careful to compare trees that were relatively close to each other because differences in the composition of the plant's substrate can affect  $\delta^{15}\text{N}$  levels in the leaves (Vanderklift & Ponsard, 2003).

#### EXPERIMENTAL $^{15}\text{N}$ ENRICHMENTS

To verify the feasibility of an experimental  $^{15}\text{N}$  enrichment via the ants' prey, we conducted a first survey around Sinnamary on ten *C. obtusa* trees (i.e. 4–8 m in height) sheltering a large *A. andreae* carton nest. We also verified if the size of trees, a surrogate of sap volume and number of leaves, plays a role in that enrichment. Once weekly during 6 weeks we provided the *A. andreae* colonies with three  $\approx 4\text{-cm}$ -long  $^{15}\text{N}$ -enriched locusts. Using a micro-syringe, we injected 20  $\mu\text{L}$  of a 5%  $^{15}\text{N}$ -labelled glycine (98 at.%  $^{15}\text{N}$ , Isotec) solution into the locusts

just prior to furnishing them to the ants that seized them immediately. The absence of contact between the glycine and the host tree prevented the direct transfer of  $^{15}\text{N}$ . An  $\approx 5\text{-cm}^2$  piece of the youngest most well-developed leaf was harvested from each *C. obtusa* tree for isotopic analysis just before and 1 week after the enrichment.

Taking into account information from the previous survey, we conducted a second  $^{15}\text{N}$  enrichment survey on  $\approx 5\text{-m}$ -tall *C. obtusa* trees sheltering a large *A. andreae* colony. They were situated along the 27 km of road leading to Petit Saut ( $5^\circ 9' 53''\text{N}$ ,  $52^\circ 54' 20''\text{W}$ – $5^\circ 4' 10''\text{N}$ ,  $53^\circ 3' 1''\text{W}$ ) and along Route 1 between Kilometric points 85 ( $5^\circ 7' 48''\text{N}$ ,  $52^\circ 43' 27''\text{W}$ ) and 105 ( $5^\circ 17' 10''\text{N}$ ,  $52^\circ 54' 56''\text{W}$ ). Again, once weekly during 6 weeks, we provided the *A. andreae* colonies of nine experimental trees with five  $\approx 4\text{-cm}$ -long  $^{15}\text{N}$ -enriched locusts, whereas the *A. andreae* colonies of the 12 control trees received non-enriched locust prey. In both cases, as previously, a piece of young leaf was harvested for isotopic analysis just before and 1 week after the enrichment.

#### ISOTOPIC ANALYSIS

The leaf samples were cleaned and freeze-dried before being sent to the Colorado Plateau Stable Isotope Laboratory (Flagstaff, AZ, USA) where they were ground into a homogeneous powder. Stable isotope analyses were conducted on  $\approx 200$  mg of each sample using a Thermo-Finnigan Delta<sup>plus</sup> Advantage gas isotope-ratio mass spectrometer interfaced with a Costech Analytical ECS4010 elemental analyser. The  $\delta$  value for nitrogen, which has two stable isotopes,  $^{14}\text{N}$  and  $^{15}\text{N}$ , is expressed as:  $\delta^{15}\text{N}\text{‰} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1.000$  ( $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the molar ratios  $^{15}\text{N}/^{14}\text{N}$  of the samples and the standard, the latter corresponding to atmospheric nitrogen; Peterson & Fry, 1987).

#### STATISTICAL ANALYSIS

For the survey concerning the natural  $\delta^{15}\text{N}$  values, we assessed the effect of ant species on  $\delta^{15}\text{N}$  values for *C. obtusa* leaves using a linear mixed model on the two data sets pooled. The ant species was the fixed effect and the zones corresponding to a radius of 15 m around selected *C. obtusa* devoid of associated ants or sheltering an *A. andreae* colony was the random effect. We differentiated the effect of ant species by conducting post-hoc Tukey tests for all pairwise comparisons between *C. obtusa* devoid of associated ants or those sheltering one of the three *Azteca* species.

In the first survey of the experimental  $^{15}\text{N}$  enrichment, we used a linear model to assess the effect of tree height on  $^{15}\text{N}$  transfer by modelling the

$\delta^{15}\text{N}$  values of young leaves after enrichment using tree height and their leaf  $\delta^{15}\text{N}$  values obtained before enrichment. In the second survey we compared the differences in  $\delta^{15}\text{N}$  during the experimental period (i.e.  $\delta^{15}\text{N}$  after minus  $\delta^{15}\text{N}$  before the enrichment) between the experimental and the control trees using Student's *t*-test (Shapiro–Wilks normality test passed; Welch's correction due to unequal variances).

We conducted all statistical analyses with R Software (R Core Team, 2020); we used the packages 'lme4' (Bates et al., 2015) for calibrating the linear mixed model and 'multcomp' (Hothorn et al., 2008) for the post-hoc test.

## RESULTS

The natural  $\delta^{15}\text{N}$  values for leaves from *C. obtusa* trees sheltering colonies of the three *Azteca* species were significantly higher than were those devoid of associated ants (control). Interestingly, the differences between trees sheltering *A. andreae* and the two other *Azteca* species were not significant (Fig. 2A).

In the first survey of the experimental  $^{15}\text{N}$  enrichments, all the linear model coefficients were significantly non-zero (*t*-tests;  $P < 0.05$ ) and showed that a  $^{15}\text{N}$  enrichment occurred ( $\delta^{15}\text{N}$ :  $2.86 \pm 1.23\text{‰}$  vs.  $1.23 \pm 1.34\text{‰}$ ;  $N = 10$ ; intercept = 5.53;  $P < 0.001$ ). Because their coefficient, at 1.05, was close to one, the initial  $\delta^{15}\text{N}$  values had no effect on the enrichment ( $P < 0.001$ ), while the effect of tree height ( $-0.44$ ) mitigated the effect of enrichment as the taller the trees the lower the enrichment ( $P < 0.05$ ).

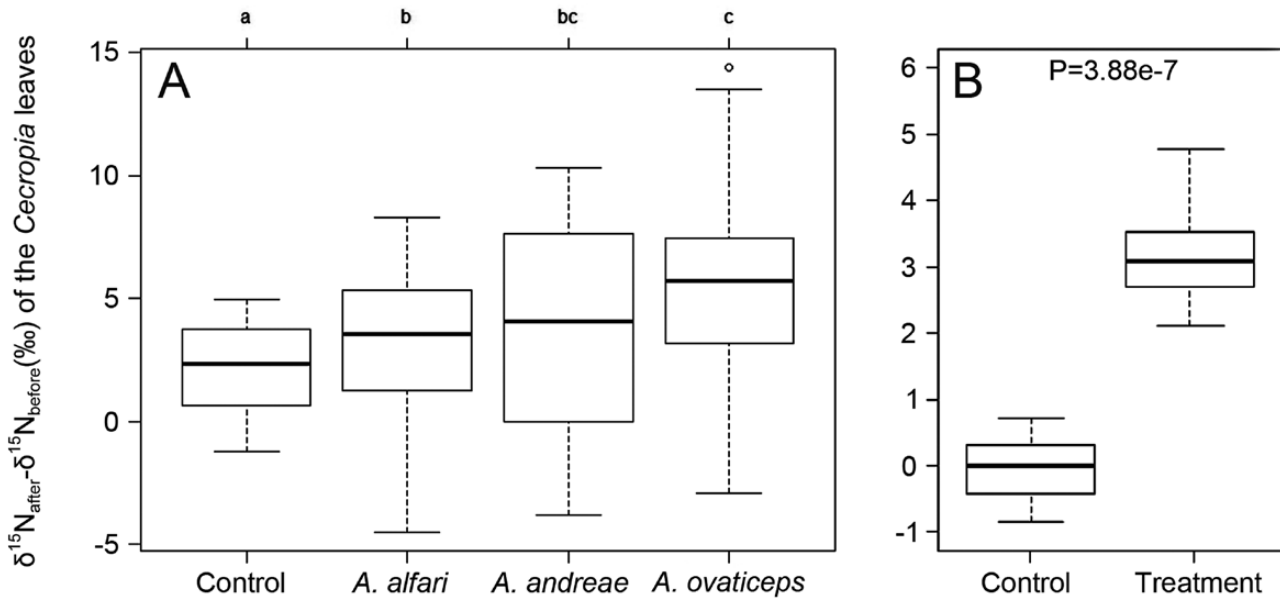
Again, in the second survey of the experimental  $^{15}\text{N}$  enrichments, the difference in the  $\delta^{15}\text{N}$  values of young *Cecropia* leaves before and after we provided their guest *A. andreae* ants with  $^{15}\text{N}$ -enriched prey (treatment) was significantly higher than that of the control trees harvested during the same time period (values centred around zero; Fig. 2B). Indeed, an increase in  $\delta^{15}\text{N}$  was noted for each experimental tree ( $\delta^{15}\text{N}$  of young leaves after vs. before enrichment:  $4.11 \pm 1.26\text{‰}$  vs.  $0.84 \pm 1.22\text{‰}$ ), but not for the leaves of the control trees ( $1.83 \pm 0.99\text{‰}$  vs.  $1.85 \pm 0.98\text{‰}$ ). These results indicate that some  $^{15}\text{N}$  passed from the  $^{15}\text{N}$ -enriched locust prey to the leaves of the host trees.

## DISCUSSION

Through two approaches this study shows that, in addition to the known very efficacious biotic protection provided thanks to their predatory behaviour (Dejean et al., 2010a, b), *A. andreae* colonies also provide their host *Cecropia* trees with nutrients.

First, in natural situations, the  $\delta^{15}\text{N}$  values of young leaves from trees sheltering *A. andreae* colonies did





**Figure 2.** A,  $\delta^{15}\text{N}$  values (‰) for young leaves from *Cecropia obtusa* trees devoid of associated ants (control) or sheltering an *Azteca* spp. colony (minima, maxima, medians, 25% and 75% percentiles). Statistical comparisons (linear mixed model): different letters indicate significant differences at  $P < 0.05$ . B, comparison of the difference in  $\delta^{15}\text{N}$  values of young *C. obtusa* leaves before and after we provided their guest *Azteca andreae* ants with  $^{15}\text{N}$ -enriched prey (treatment) or not (control). Statistical comparisons: Welch's adjusted  $t = 10.22$ ,  $df = 11.56$ ,  $P = 3.88e-07$ .

not differ from those sheltering *A. alfari* or *A. ovaticeps* for which myrmecotrophy was shown (Dejean *et al.*, 2012), which might be explained by taking into consideration the ecological contexts. For both *A. alfari* and *A. ovaticeps* myrmecotrophy is direct as their wastes are deposited in the host plant domatia where they decay and so produce nutrients that are directly absorbed by internal tissues. This process favours nitrogen absorption, but exogenic food is rare because these ants, which feed mostly on the host plant food bodies and hemipteran honeydew, are only occasional predators (Dejean *et al.*, 2009). By contrast, because *A. andreae* is a strong predator, each colony captures numerous insects daily, so that the hard parts of these prey exoskeletons constitute the majority of the colony wastes. Because insect exoskeletons are rich in nitrogen due to the presence of chitin and resilin (Wiesenborn, 2011), after being thrown away by the workers (A.D., pers. observ.) they decay on the ground and produce nitrogen-rich nutrients available to the host *Cecropia* roots. The daily rejection of insect prey carcasses by *A. andreae* colonies is probably equivalent to the direct myrmecotrophy noted for *A. alfari* or *A. ovaticeps*, explaining the results noted in Figure 2.

Second, through the  $^{15}\text{N}$  enrichment experiments we demonstrated that  $^{15}\text{N}$  passed from *A. andreae* prey to their host *Cecropia* leaves. The first survey showed that these enrichments were independent of the variations in the  $\delta^{15}\text{N}$  values of the trees before the enrichment,

a consequence of the variations in the values of their substrate (see Vanderklift & Ponsard, 2003). Because this survey revealed that the level of  $^{15}\text{N}$  enrichment is lower the taller the tree, we deduced that the low level of enrichment noted in this study compared to that obtained for *Cecropia* sheltering *A. alfari* or *A. ovaticeps* (Dejean *et al.*, 2012) can be due partly to different tree sizes (i.e.  $\approx 5$  m vs. 2–3 m in height). The lower frequency in  $^{15}\text{N}$  provisioning (i.e. six times vs. 15 times) probably also played a role and, compared to direct myrmecotrophy, the complex cycle of nitrogen provisioning described above for *A. andreae* probably includes losses. Here, the injected  $^{15}\text{N}$ , diluted in the prey haemolymph, was imbibed by the ants and later rejected with their faeces and corpses that decay on the ground with the rest of the wastes, the produced  $^{15}\text{N}$ -rich nutrients being absorbed by the host *Cecropia* roots.

Two other cases of  $^{15}\text{N}$ -based plant nutrient provisioning from ant wastes other than through direct myrmecotrophy involving myrmecophytes have been noted. *Vachiella constricta* is an extrafloral nectary-bearing shrub that, thanks to this food reward, incites ground-nesting ant species to install their nests at its base. Thus, its roots obtain nutrients derived from ant nests via decaying wastes deposited in specific subterranean nest chambers (Wagner & Nicklen, 2010). Also, due to their territoriality, workers of the arboreal weaver ant *Oecophylla smaragdina* deposit

landmarks on their host tree foliage. These landmarks are nitrogen-rich faecal droplets on twigs, leaves and fruits, so that the nitrogen is absorbed directly through the epidermis and translocated inside the plants (Pinkalski *et al.*, 2017).

These mutualisms are stable and ubiquitous in nature because the benefits for one partner are ‘by-products’ produced at almost no cost by the other partner for which they are intrinsic traits (Sachs *et al.*, 2004; Leimar & Hammerstein, 2010). In the relationship between *A. andreae* and *C. obtusa*, both biotic protection (due to the ants’ territoriality and predation) and myrmecotrophy (related to the ants’ wastes) correspond to by-product benefits for the host trees. Reciprocally, *A. andreae* takes advantage of the hollow *Cecropia* trunks and branches that shelter its incipient colonies and the structure of the leaf surfaces with which the workers’ claws mesh perfectly, allowing the ‘Velcro’ effect to come into play to capture large prey (Dejean *et al.*, 2010b), this time representing by-product benefits for the ants.

In conclusion, in the association between *A. andreae* and *C. obtusa* both partners can obtain by-product benefits, a situation known as a ‘by-product mutualism’ (Sachs *et al.*, 2004). Myrmecotrophy, or more generally the nutrient provisioning of plants by ants, a by-product benefit, might be very common, explaining why ants and plants developed mutualistic associations.

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