The Role of Parabiotic Ants and Environment on Epiphyte Composition and Protection in Ant Gardens

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Abstract
Ant gardens (AGs) are a multi-partner specialized ant-plant interaction involving several ant and epiphyte species. Although studies on AGs have reported possible roles for some species in this system, there are unanswered questions regarding the process of epiphyte incorporation in the AGs and the role of less aggressive ant species in AG protection. In this study, we used AGs in the Brazilian Amazon forest formed by two parabiotic ant species to test a set of hypothesis regarding two main questions: 1) How is AG plant community composition affected by the surrounding environment? 2) Does Crematogaster levior play a role in the chemical detection of herbivory in the AGs? After identifying epiphytes occurring at AGs at the forest edge and in the interior, we found that ant gardens in each environment exhibited different compositions, and that plant species bearing oil or extrafloral nectar glands were more frequent in AGs located in the forest interior than in those at the forest edge. By performing experiments with volatile compounds emitted from injured epiphytes, we detected that only Camponotus femoratus was responsive, responding almost eight times faster in response to plant extracts than water treatments. Our results support the idea that environmental conditions affect ant preference for feeding resources provided by epiphytes and consequently shape the structure of the epiphyte community in AGs. On the other hand, the role of C. levior in AGs remains unknown, since it seems to play no direct or indirect role in AG protection.

Introduction

Mutualism is a ubiquitous interspecific interaction that occurs among a great diversity of living organisms (Janzen, 1985). Plants and insects, in particular, are frequently involved in three types of mutualisms: pollination, seed dispersal and protection against herbivores (Bronstein, 1994; Bronstein et al., 2006). In these interactions, plants attract and reward insects for their actions by offering shelter and/or food through specialized structures, while insects guarantee flower pollination, seed dispersal or protection against consumers (Bronstein et al., 2006).

Although the majority of plant-insect mutualisms are generalized and facultative (Stanton, 2003; Bronstein et al., 2006), some interactions can be very specialized (see Rico-Gray & Oliveira, 2007). One remarkable case of specialized interaction are ant gardens (AGs), mutualistic associations between epiphyte plants and ant species in Asian and Neotropical rainforests (Hölldobler & Wilson, 1990). Ant gardens are ant nests built on the branches of trees and on which aggregates of epiphyte plant species grow (Ule, 1901). The nest can hold one or, more frequently, more than one ant species and several phylogenetically distant epiphyte plant species (Orivel & Leroy, 2011). When more than one ant species occurs in the same nest, they show a parabiotic behavior, in which the ants live in close association foraging trials but do not exhibit obvious parasitic or exploitative interactions (Davidson, 1988; Forel, 1898; Vantaux & Leroy, 2007; Orivel & Leroy, 2011, but see Menzel et al., 2015).
Seeds from most AGs epiphytes are dispersed by the associated ants, which incorporate these seeds into carton nest continuously over the lifespan of the nest (Orivel & Leroy, 2011). Although seeds from AG epiphytes commonly bear aril or elaiosome, seed selection by ants seems not determined by the quantity or quality of such appendages as patterns of seeds selection by AGs ants remains the same after the removal of such seed structures (Orivel & Dejean, 1999). In fact, it seems that AG ant species are attracted by a set of specific volatile compounds released by the seed coat of some of epiphyte species commonly found on AGs (Youngstead et al., 2008). After germination, ants of at least one species protect plants against herbivores by patrolling on leaves (Vantaux et al., 2007), while the roots and stems of epiphytes increase the stability and moisture of ant nests (Yu, 1994). Some epiphytes also provide feeding resources for ants through extrafloral nectaries (EFN), oil glands and fruits (Kleinfeldt, 1978; Hölldobler & Wilson, 1990). Although previous studies has been able to identify the general benefits for both interacting sides (see Orivel & Leroy, and references therein), it has been very hard to identify the determinants of plant and ant species composition in AGs, and also the roles played by different partner species in this multispecific interaction. For this reason, here, we investigate two main processes that remain unclear: 1) the process of epiphyte incorporation in the AGs and 2) the role of less aggressive ant species in AG protection.

It is known that epiphyte species are not randomly distributed on AGs, but that instead there are some preferential associations between particular ants and some epiphyte species (Orivel & Leroy, 2011). These preferential associations can be explained by two non-mutually exclusive mechanisms: 1) preferential incorporation of seeds from epiphyte species that confer larger benefits to the ant colonies, e.g. plant species that provide food resources; and 2) niche filtering, i.e. local abiotic conditions determining the plant species capable to reach and establish in the nests. Since different habitats may restrict the plant species capable of surviving at specific environmental conditions, niche filtering may be the main force affecting which epiphyte species colonizing AGs that occur in habitats with contrasting environmental conditions. However, ant preferences for some plants may still play a secondary role. Therefore, it is likely that variation in epiphyte composition among AGs is primarily driven by species ‘filtering’ as a response to local abiotic conditions and, secondarily, by preferential incorporation of particular epiphyte species by the ants on AGs. Such epiphytes should be the ones that provide the greater benefits to ants. Consequently, it is possible that AGs located in contrasting habitats shelter a different pool of epiphyte species, but that the most common epiphyte species found in the AGs provide similar benefits (e.g. food resources), regardless of the environment.

In order to unravel the complete scenario related to the presence of some specific epiphyte species on AGs it is necessary to understand not only the incorporation process of epiphyte species on AGs, but also the roles that different ant species plays in these plants. Although it is often assumed that all ant species living on AGs benefit the epiphytes, ant species seem to differ in the efficiency with which they protect the epiphytes against herbivores. For example, among the ant species most commonly present in Amazonian AGs, Crematogaster levior Longino (2003) (Myrmicinae) and Camponotus femoratus Fabricius (1804) (Formicinae), only C. femoratus seems to protect the epiphytes (Vantaux et al., 2007; Vicente et al., 2014). However, most ant responses regarding epiphyte protection were measured based only on ant recruitment (Vicente et al., 2014) or after the simultaneous usage of physical and chemical stimuli to trigger their responses (Vantaux et al., 2007). Although ant recruitment may be a reliable measure of aggressiveness towards potential herbivores, it does not consider other behavioral responses that may occur between the initial stimulus and ant recruitment. For example, C. levior might be able to detect and communicate the presence of herbivores through chemical stimuli and consequently could improve the aggressive response exhibited by C. femoratus. Therefore, the study of such responses may reveal previously undetected interactions between ant species in their protective response in AGs.

Here we investigate AGs constructed by ant species Crematogaster levior and Camponotus femoratus in the Central Brazilian Amazon forest. We addressed two main questions: 1) How is AG plant community composition affected by the surrounding environment and plant traits? 2) Does C. levior play a role in the chemical detection of herbivory in the AGs? For the first question, we postulated two non-mutually exclusive hypotheses. First, the distribution of adult epiphyte species in AGs is determined not only by ant behavior, but also by local environmental conditions. Second, ants prefer incorporate seeds from epiphyte species that provide food resources. To evaluate these hypotheses, we used AGs located in two habitats with contrasting abiotic conditions within tropical forests: forest edges and interior (see Murcia, 1995). Then, by comparing AGs found in areas of forest edge and interior, we expect to find different epiphyte species associated to the AGs (first hypothesis), but with the most common species in each environment presenting extrafloral or oil producing glands (second hypothesis). To answer the second question, we hypothesize that C. levior is able to recognize compounds released by injured epiphytic plants, although it does not react aggressively to them. Consequently, we expect that C. levior and C. femoratus will show similar time responses to detect chemical stimuli related to herbivory, although only C. femoratus will exhibit recruitment behavior.

Methods

Study area

This study was conducted at the “Km 41” reserve of the Biological Dynamics of Forest Fragments Project (a collaborative initiative between INPA and STRI), located ca.
80 km north of Manaus, Central Amazon, Brazil (02°24' S - 59°22' W). We carried out the experiments during August 2006 and August 2010 in a continuous tropical rain forest at the km 41 reserve and at its border along the access road. Annual rainfall varies between 1900 and 2500 mm, with a moderately dry season (< 100 mm/month) from June to October (Gascon & Bierregaard, 2001). The forest is a dense evergreen forest, with a 2-12 m tall understory, a sub-canopy between 15 and 25 m tall, a canopy about 30-37 m tall and emergent trees up to 45 m - 55 m tall (Camargo & Kapos, 1995; Laurance et al., 2011).

Epiphyte occurrence in AGs

We sampled all AGs found up to 6m high along two 5 km transects, one located in the forest interior (more than 50 m from the forest edges) and the other transect at the edge of the same mature forest. The forest edge was created by the construction and maintenance of a 10 m wide dirt road that gives access to the study area. In each AG we collected and identified all plant species growing in it as well as the ant species present in the nests. The same researcher (Jakovac, C.C.) performed all observations. For every plant species collected, we looked for the presence of glands that indicated the production of extrafloral nectar or oil (Elias & Gelband, 1976). Other plant traits like the presence of fruit pulp or an elaiosome are also potential drivers of differential epiphyte recruitment to AGs after seed dispersal. However, we considered only the presence of glands associated with extrafloral nectar or oil as a predictor because these structures are temporally predictable. The other food resources offered by plants are normally seasonal, which should reduce their importance as drivers of the plant composition patterns we investigated here.

To evaluate if epiphyte species composition depends on the location of the AGs (forest interior and forest edge), we performed an Analysis of Similarity (ANOSIM) with 999 permutations and ordered the species composition using a non-metric multidimensional scaling analysis (NMDS) with a Sørensen index. To evaluate if the frequency of epiphytes in AGs is determined by the presence of extrafloral nectaries or oil producing glands, we fitted generalized linear models with Poisson error distribution, checking for overdispersion of the data. For that, we used each epiphyte species as a sample unit and the number of AGs where each epiphyte species occurred in each environment as the response variable. As explanatory variables, we considered the presence of extrafloral nectaries or oil producing glands (as binary variable) in each epiphyte species, the environment where each AG was found (forest edge or interior) and the interaction between the presence of glands and the environment. We predicted that, if ants select epiphytes that provide continuous food resources to compose their AGs, the presence of glands will have an effect on the frequency of epiphytes in both environments. We also compared epiphyte richness in AGs between both environments with a t-test. We performed all analyses with the base and the vegan (Oksanen et al., 2014) packages in R 3.0.2 (R Core Team, 2014).

Ant response to epiphyte volatile compounds

To analyze ant response to volatile compounds released by injured plants, we selected 20 ant gardens located up to 2 m high at the forest edge, and occupied simultaneously by the parabiotic ants Camponotus femoratus and Crematogaster levior. For each AG, we applied two treatments: (1) aqueous extract of leaves from Peperomia macrostachya (Piperaceae) (0.34 g/ml) (hereafter referred as extract treatment) and (2) water (control treatment). Peperomia macrostachya is one of the six most abundant epiphyte species in Neotropical AGs (Orivel & Leroy, 2011) and occurred in all AG selected for this experiment. We prepared the extracts from P. macrostachya leaves collected from AGs that were not used in the experiment. To avoid oxidation of the extracts, we prepared them immediately before conducting the experiments.

We applied the extracts to pieces of cotton and held them at a short distance (approximately 5 cm) from P. macrostachya leaves located near one of the nest entrances and without ants patrolling. We refrained from touching the leaves with the cotton pieces to make sure any subsequent response would be triggered only by volatile compounds and not by any physical stimulus. For each AG we applied one treatment at a time with minimal intervals of 20 min between treatments. The order in which treatments were applied to each AG was randomly assigned beforehand. We quantified ant response to treatments using three descriptors: ant attraction (binary), ant response time and ant recruitment. We considered ants to have been attracted by the extract if they exhibited exploitative behavior such as moving toward the tip of the focal leaf followed by inspection with the antennae and/or worker recruitment. We measured ant response time as the interval between bringing the cotton pieces close to the leaf and ant recruitment occurring on the focal leaf (up to a maximum of 10 min), and counted the number of ants recruited during this 10 min. period. We calculated ant recruitment by subtracting the number of ants initially present on the focal leaf from the number observed 10 min. after the treatments. We chose a 10 min. period because although ants still patrolled the leaves after this time interval, their number started to decline.

To evaluate ant response to each treatment, we looked for differences in ant attraction, ant response time and number of ants recruited between control and plant extract treatments. We fitted a generalized linear model with binomial error distribution, checking data overdispersion, to analyze differences in ant attraction between treatments. To evaluate if response time and ant recruitment differ between treatments we performed a factorial ANOVA. For every analysis, we included each
sampled AG as a grouping factor. For the analysis considering ant response time, we included all AGs. For the AGs in which we did not observe any ant response after 10 min observation, we considered the maximum time of observation (10 min) as the time until ant response. We performed all analyses using R3.0.2 (R Core Team 2014).

**Results**

Colonization of AGs by epiphytes

We sampled 26 AGs, 17 at the forest interior and nine at the forest edge. All AGs were occupied only by parabiotic ants *C. femoratus* and *C. levior*. In total, we identified 15 epiphyte species, eight of them bearing extrafloral nectaries or oil producing glands (Table 1). The most frequent family was Araceae, which was found in 42% of the AGs, followed by Gesneriaceae (28%) and Piperaceae (24%).

The number of epiphytic species ranged from 1 to 5 per AG and did not differ between environments (*t* = 0.25; *df* = 24; *p* = 0.80). On the other hand, the composition of epiphyte species was different between edge and forest interior (ANOSIM, *R*=0.52, *p*=0.001; NMDS, stress=0.09; Fig 1). Regarding epiphyte frequency distributions, AGs in both environments exhibited the same structure: one or two species were common to more than 70% of the AGs, while the other species were less frequent (Fig 2). The epiphytes *Codonanthe calcarata* (Gesneriaceae) and *Anthurium trinerve* (Araceae) were both found in 77.8% (*n* = 7) of the AGs at the forest edge but in only 35.3% (6) and 23.5% (4) of the AGs in the forest interior, respectively (Table 1). In the forest interior, the most common epiphyte species were *Peperomia macrostachya* (Piperaceae) and *Philodendron megalophillum* (Araceae), occurring in 88.2% (15) and 41.2% (7) of the AGs, respectively (Table 01). Those species were rarely found at the forest edge (Table 01). The presence of glands that indicated production of extrafloral nectary or oil did not by itself explain the high frequency of a few epiphyte species on AGs in either environment (Fig 3).

However, we found that the frequency of epiphytes in the AGs was determined by an interaction between the presence of glands and environment. The frequency of plants bearing glands capable of producing oil or extrafloral nectar was more than threefold higher in AGs located in the forest interior than in forest edge. Accordingly, epiphytes not bearing such glands were almost two times more frequent in AGs located in the forest edge than in the forest interior (*χ²* glands*environment* = 13.36, *df* = 17, *p* < 0.001; Fig 3).

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**Table 1.** Relative frequency of epiphyte species occurring in association with ants in ant gardens (AG) in forest interior and forest edge in Central Amazonian forest, Brazil.

<table>
<thead>
<tr>
<th>Epiphytic species</th>
<th>Acronym</th>
<th>Extrafloral nectaries</th>
<th>Florest interior (%)</th>
<th>Forest edge (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aechmea sp.</td>
<td>Asp</td>
<td>X</td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Anthurium gracile</td>
<td>Agra</td>
<td></td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Anthurium sp.1</td>
<td>Ansp1</td>
<td></td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Anthurium sp.2</td>
<td>Ansp2</td>
<td></td>
<td>5.8</td>
<td>44.4</td>
</tr>
<tr>
<td>Anthurium trinerve</td>
<td>Atri</td>
<td></td>
<td>23.5</td>
<td>77.8</td>
</tr>
<tr>
<td>Codonanthe calcarata</td>
<td>Ccal</td>
<td>X</td>
<td>35.3</td>
<td>77.8</td>
</tr>
<tr>
<td>Codonanthe crassifolia</td>
<td>Cera</td>
<td>X</td>
<td>0</td>
<td>11.1</td>
</tr>
<tr>
<td>Codonanthespis sp.</td>
<td>Csp</td>
<td>X</td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Codonanthe opsulsei</td>
<td>Cule</td>
<td>X</td>
<td>17.6</td>
<td>11.1</td>
</tr>
<tr>
<td>Ficus sp.</td>
<td>Fsp</td>
<td></td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Oedematopmus sp.</td>
<td>Osp</td>
<td></td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Peperomia macrostachya</td>
<td>Pmac</td>
<td>X</td>
<td>88.2</td>
<td>11.1</td>
</tr>
<tr>
<td>Philodendron megalophillum</td>
<td>Pmeg</td>
<td>X</td>
<td>35.3</td>
<td>0</td>
</tr>
<tr>
<td>Philodendron sp.</td>
<td>Psp</td>
<td>X</td>
<td>11.8</td>
<td>22.2</td>
</tr>
<tr>
<td>Psychotria sp.</td>
<td>Pssp</td>
<td></td>
<td>5.8</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig 1.** Epiphyte species composition in ant gardens in forest edges sites (empty circles) and interior sites (black circles) in Central Amazonian Forest. (forest edge, *n* = 9; forest interior, *n*=17).
Ants response to epiphytes volatile compounds

Only *C. femoratus* workers were able to detect and respond to the leaf extract treatment. Although *C. levior* workers were observed foraging during the experiment, they did not exhibit any exploitative behavior (approximation and/or contact attempts using antennae, for example) and did not recruit workers in response to any treatments.

The response of *C. femoratus* to volatile compounds released by *P. macrostachya* was greater than the response to water for every tested descriptor. *Camponotus femoratus* were attracted by the extract in 95% of the cases in which it was offered, but were attracted by water in only 40% of the cases ($\chi^2 = 15.64; df=1; p < 0.001$; Fig 2). This same species responded four times faster ($F_{(1,196)} = 47.94; p < 0.001$; Fig 4) and recruited nine times more workers ($F_{(1,19)} = 71.28; p < 0.001$; extract: $5.17 \pm 0.6$, water: $0.6 \pm 0.16$) in response to the plant extracts than to the water treatment.

Discussion

Our results indicate that ant gardens at the forest edge and in its interior are occupied by plant communities with different species compositions, but following a similar structure. In both environments, AG-epiphyte communities...
were characterized by a few common species present in most AGs and several less frequent species. Interestingly, we found that the frequency of species with extrafloral nectars or oil glands was higher at the forest interior than at the forest edge, indicating that the probability of plants offering such resources to occur in the AGs change according to the environment. We also showed that epiphytes able to establish and develop on AGs seem to benefit from *C. femoratus* protection against herbivores. Although this effect has already been reported (Vantaux et al., 2007; Vicente et al., 2014), our results further suggest that *C. levior* does not play any role, even an indirect one, in epiphyte defense.

Our results suggest that the environment determines the composition of epiphyte species in AGs, and may affect the selection of epiphytes by ants due to differential resource availability. Nest carton is considered a very suitable substrate for epiphyte establishment because it is rich in organic matter and at the same time allows for good root aeration (Orivel & Leroy, 2011). Therefore, many epiphyte species could potentially germinate and develop on AGs. Because extrafloral nectaries are especially prevalent in plants with high growth rates and with affinity for full-light habitats as the ones found in the forest edge (Schupp & Feener, 1991; Blüthgen & Reifenrath, 2003), it seems unlikely that the low occurrence of epiphytes in the AGs in the forest edge was due to unsuitable local conditions for such plants. Instead, because plants with nectar and oil glands are common at the forest edge, it should be easy for ants to find those resources outside of their AGs. Consequently, the relative benefit for ants of recruiting and caring plants with extra food resources to the AGs should be relatively lower at the edge than in the forest interior. Thus, epiphytes bearing food-providing glands may not be preferentially incorporated by ants into AGs located at the forest edge due to their lower relative importance to ants occurring in this habitat. However, because *C. femoratus* responded aggressively to epiphyte volatile stimulus in the AGs located at the forest edge, it seems that any epiphyte will be defended once incorporated into the AG. Perhaps this occur because they also provide additional benefits to ants such as nest support aid.

Our finding that *C. levior* do not participate in any step of the aggressive response is curious. *Crematogaster* species are known to preferentially feed on carbohydrate rich resources such as those present in extrafloral nectaries (Blüthgen & Fiedler, 2004). Hence, these ants consequently may benefit from the presence of epiphyte species bearing this trait in the AGs. Since individuals of *C. femoratus* defend the epiphyte species, *C. levior* may increase its benefits by not paying the costs associated to plant defense (see Archetti et al., 2011). But, if this is true, why do individuals of *C. femoratus* allow the presence of individuals of *C. levior*? If colonies of *C. femoratus* do not obtain any benefits from this association, theory predicts that they should avoid interactions with *C. levior* (Edwards et al., 2010). It is important to note that, although workers of *C. femoratus* forage exclusively on the forest understory for short periods during the day (e.g. Vantaux et al., 2007), *C. levior* individuals are able to forage both on the forest understory and on the forest ground, over larger distances from the nest and under more extreme weather conditions (such as at forest edges) in comparison with *C. femoratus* (Vantaux et al., 2007). Those differences in foraging habits might limit the extent of interspecific competition between the two ant species for resources provided by their shared AG. Additionally, *Camponotus* individuals can get food from *Crematogaster* workers through trophallaxis (Menzel et al., 2014). Therefore, *C. femoratus* may co-occur with *C. levior* due to the additional resource input provided by the latter.

In summary, it seems that *C. femoratus* is the most important partner for epiphytes due to its protective services in the AGs. The plants, on the other hand, are important as resource providers to both ant species, at least in areas with low light availability. If *C. femoratus*, in fact, benefit from *C. levior* by increasing food acquisition, it may be that that the multi-partner interaction found in the AGs is maintained by a series of two-way mutualisms: *C. femoratus* and epiphyte plants; *C. femoratus* and *C. levior* and the indirect effect of *C. levior* on epiphyte plants through the maintenance of *C. femoratus*. It is important to note, however, that the identity of partners in these two-way interactions, especially the identity of epiphytes, might vary due to changes in local species communities.

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