Leaf damage induces ant recruitment in the Amazonian ant-plant *Hirtella myrmecophila*

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Abstract: *Allomerus octoarticulatus* is a plant-ant that colonizes domatia of the understorey tree *Hirtella myrmecophila* in the Central Amazon and forages for invertebrates, including leaf herbivores, on the host plant. We conducted manipulative experiments to study the ant's recruitment response to damaged leaves and leaf extracts of the host and to extracts of *Protium hebetatum*, a non-myrmecophytic sympatric tree species. Artificial damage to leaves of *H. myrmecophila* caused an increase in the number of recruits to the leaf. Ant response was stronger in young than in mature leaves. Recruitment was restricted to damaged leaves. No increment in recruitment rates was observed in undamaged, adjacent leaves. Different levels of leaf damage did not elicit differences in recruitment rates. Aqueous extract of leaves, placed on undamaged leaves of the host plant, also led to increased recruitment compared with water (control), and more ants were recruited to extracts from young than from mature and old leaves. Extracts of both *H. myrmecophila* and *Protium hebetatum* induced recruitment. We discuss the evolutionary importance of plant leaf components for maintenance of the ant-plant mutualism.

Key Words: *Allomerus*, Amazon, induced resistance, mutualism, plant resistance

INTRODUCTION

Associations between ants and plants provide one of the best examples of mutualistic interactions, and have contributed much to understanding the evolution of mutualisms (Bronstein 1998). Most of the associations among these partners seem to be occasional and not specialized (Beattie 1985). However, in some plants called myrmecophytes or ant-plants, the relationship tends to be more specialized (Benson 1985). At least 250 myrmecophytes are known in the Neotropics. Most inhabit primary forests, and many of them do not offer food resources to the ants (Benson 1985). These plants have hollow structures (domatia), which, depending on the species, may be located in the root, trunk, petiole or leaf blade, and which provide nest sites and shelter for the ants (Beattie 1985, Davidson & McKey 1993a, b). In exchange, the ants suppress herbivores (Fonseca 1994, Itioka *et al.* 2000, Vasconcelos 1991), prune competing plants (Davidson & Mckey 1993b, Federle *et al.* 1998,

Janzen 1966) and provide nutrients to the host (Fischer *et al.* 2003, Janzen 1966, Treseder *et al.* 1995). Some researchers suggest that herbivory is the key selective pressure responsible for evolution of these ant-plant mutualisms (Beattie 1985, Fonseca 1994).

Studies demonstrated that ants residing in ant-plants (e.g. *Macaranga* spp.) aggressively recruited to leaf damage or plant sap (Fiala & Maschwitz 1990), but only recently have researchers begun to investigate the proximate cues that induce aggressive ant responses, as well as quantify the dynamic nature of ant defence (Agrawal 1998, Agrawal & Dubin-Thaler 1999, Brouat *et al.* 2000, Lapola *et al.* 2003). Agrawal & Rutter (1998) proposed an analogy between the herbivore-induced behaviour of ants and the herbivore-induced chemical resistances of plants. When a herbivore damages a leaf, the plant produces compounds that can negatively affect the herbivore (Karban & Baldwin 1997). Similarly, damaged leaves or extracts of myrmecophytic plants, such as *Cecropia obtusifolia*, *Leonardoxa africana* and *Macaranga* spp., produce volatiles that induce recruitment of ants to that leaf, to combat a possible herbivore presence (Agrawal 1998, Agrawal & Dubin-Thaler 1999,

Brouat *et al.* 2000, Fiala & Maschwitz 1990). These plants provide rewards (food bodies and extrafloral nectar) besides nesting space (domatia) that ensure long-term interactions and enhance stability for the ant-myrmecophilous plant mutualism (see Heil & McKey 2003).

Izzo & Vasconcelos (2002) showed that the Amazonian plant-ant *Allomerus octoarticulatus* substantially decreases herbivory on young leaves of the myrmecophyte *Hirtella myrmecophila* (Chrysobalanaceae), a plant that does not provide food rewards to the ants. It is not yet known whether ant recruitment is involved in the protection of this ant-plant, as well as in other Amazonian ant-plants, or how such recruitment might be induced in myrmecophytic plants that do not provide food rewards. In this study, we experimentally caused damage to leaves and placed aqueous extracts of leaves onto undamaged leaves of *H. myrmecophila* to determine the effects on recruitment to the site by *A. octoarticulatus*. The principal questions were: (1) Does leaf damage induce ant recruitment? (2) Is the intensity of recruitment dependent on the age of the leaf? (3) Is recruitment intensity dependent on the level of damage? (4) Is the recruitment stimulus chemical in nature? (5) Does the chemical stimulus vary with leaf age? (6) Is the chemical stimulus specific to *Hirtella myrmecophila*? We also describe the recruitment behaviour.

METHODS

Study area and species

This study was conducted in July–August and November–December 2002 in an 800-ha forest preserve administrated by the Biological Dynamics of Forest Fragments Project, a collaborative project between the National Institute for Amazonian Research (INPA) and the Smithsonian Institution. Located 70 km north of the city of Manaus, in the Brazilian state of Amazonas (02° 24′ S, 59° 52′ W), the preserve is a *terra firme* (upland) forest surrounded by largely undisturbed forest. The climate is humid tropical monsoonal (Köppen type Am), with rainy season between November and May and dry season from June to October (Lovejoy & Bierregaard 1990).

Hirtella myrmecophila Pilg. (Chrysobalanaceae) is a small tree very common in the understorey of *terra firme* forests of the Central Amazon. This plant presents a characteristic unique among ant-plants: all leaves are produced with a pair of domatia fully integrated in the base. However, in old leaves the domatium dries and falls off, while the leaf remains on the branch for more than 2 y. In the vicinity of Manaus this plant is almost exclusively associated with the ant *Allomerus octoarticulatus* Wheeler (Myrmicinae) (Fonseca 1999,

Izzo & Vasconcelos 2002). *Allomerus octoarticulatus* can also inhabit *Hirtella physophora* Mart. & Zucc. and *Duroia saccifera* (Schult. & Schult. f.) K. Schum. (Rubiaceae), but these plant species are present at much lower density in the study area. Their relationship with *A. octoarticulatus* remains unstudied. *Hirtella myrmecophila* does not produce any food for the ant; *A. octoarticulatus* feeds mainly on insects that venture onto the leaves (Izzo & Vasconcelos 2002).

Experiment 1. Leaf damage

Ants might recruit to a leaf stimulated by leaf damage, and this behaviour could vary according to age of the damaged leaf, since mature leaves do have domatia, but typically support fewer ants than young leaves (Izzo & Vasconcelos 2002). Moreover, the recruitment induced by leaf damage might extend to neighbouring leaves. We tested these two hypotheses in 17 plants of 0.5–2.5 m height, bearing at least two branches with both young and mature leaves. Young leaves were defined as recently expanded with light green colour which are only found at branch tips. Mature leaves are dark green and tougher than young leaves. In each plant we randomly selected two branches: experimental and control. On the experimental branch we randomly selected one young and one mature leaf and cut off the distal half ($\sim$ 30–40 cm^2) of both to simulate herbivory. In the control branch we disturbed (gently shook) but did not cut, one young and one mature leaf randomly chosen. We counted the number of ants on the proximal half of experimental and control leaves, and on neighbouring leaves, just before cutting or disturbance (time 0) and at 1, 3, 5, 10 and 15 min after the treatments.

Experiment 2. Level of leaf damage

Greater levels of leaf damage might induce more ants to recruit. To test this hypothesis, we selected 10 plants with the same conditions of the first experiment and randomly chose two branches. On one branch, a young leaf received two cuts of 0.5 cm length, removing a triangular piece of 0.12 cm^2 ($\sim$ 0.1–0.2% of the leaf area) from the leaf edge. A larger triangle, of 4.5 cm^2 ($\sim$ 5–7% of the leaf area) with 3.0 cm sides, was removed from the young leaf of a second branch. We counted the number of ants recruited as in the first experiment.

Experiment 3. Chemical stimuli from *Hirtella myrmecophila*

To test whether recruitment is stimulated chemically, we selected 10 plants with the same conditions as in the first experiment with two branches of each plant being randomly selected as experimental or control. The young

leaf on the experimental branch received three drops (drop *c.* 50–80 ml) of aqueous extract of *H. myrmecophila* leaf, while the young leaf on the control branch received three drops of only water. Leaf extracts were obtained by macerating 5 g of fresh leaves in 80 ml of water. We applied both substances directly on the experimental leaves using syringes. Aqueous extract was employed successfully by Fiala & Maschwitz (1990) to recruit plant-ants. We used the same aqueous extract for no more than 1 h to avoid changes in its chemical nature. We counted the number of ants recruiting to the leaf at different times after treatment, as in the first experiment.

Experiment 4. Leaf age

To compare ant recruitment in the presence of aqueous extracts from *H. myrmecophila* leaves of different ages, we selected 13 plants, as in the first experiment. On each plant, a single young leaf was randomly selected on each of three branches. A leaf on the first branch was treated with extract from young *H. myrmecophila* leaves; the leaf chosen on a second branch was treated with extract from mature leaves; the third leaf was treated with extract from old leaves. Young and mature leaves were identified as above; old leaves are those that lack domatia or have completely dried domatia, i.e. no shelter is available for ants. We counted the number of recruiting ants as above.

Experiment 5. Chemical stimuli from a non-myrmecophytic plant

Allomerus octoarticulatus might recognize and be induced to recruit only under stimulus of compounds produced by damaging leaves of the host plant (*H. myrmecophila*). To test this hypothesis, we conducted an experiment similar to experiment 3, with the difference that the young host leaves on the experimental branches received three drops of aqueous extract of young leaves of *H. myrmecophila*, while young host leaves on the control branches received three drops of aqueous extract of young leaves of *Protium hebetatum* Daly (Burseraceae). We chose this plant because it contains aromatic resin in its leaves, is common in the study area and is phylogenetically distant from the Chrysobalanaceae (Judd *et al.* 1999).

Recruitment behaviour

Ant recruitment was defined by Hölldobler & Wilson (1990) as a special assemblage, where the ants are conducted to a specific point where food or another resource is available. According to these authors, chemical compounds are usually involved in this behaviour. Behaviours of *Allomerus* workers were observed and carefully recorded during the experiments on leaf damage, chemical stimuli, and when models of herbivores (termites) were placed on the leaves.

Statistical analysis

All the experiments were conducted using a randomized block design (Hurlbert 1984), in which each plant (sample unit) received two (in one case three) treatments. We compared the number of ants between two treatments over time using a randomized block, repeated-measures ANOVA, in which plants were considered as blocks and the time as the factor of repetition. The probabilities were corrected with the Greenhouse-Geisser (G-G) approximation procedure to avoid sphericity (Zar 1996). We did not re-use plants in the different experiments. The number of ants recruited to leaves that received extracts of young, mature and old macerated *H. myrmecophila* leaves (three treatments) over time were compared using the Tukey *a posteriori* test, with $\alpha = 0.05$.

RESULTS

Induced recruitment of *Allomerus octoarticulatus*

Simulated herbivory (leaf damage) led to an increase of 100–400% in the number of the ants compared to pre-damage densities, suggesting that ants will recruit in the event of a herbivore attack. These recruited ants remained active on the damaged leaf at least for 10–15 min. At the end of our 15-min observation period, ant numbers were still elevated above pre-damage levels. Young leaves that were merely disturbed (control) showed no increase in ant numbers over time (Figure 1). Interaction between treatment and time was significant (Table 1), indicating an increase in the number of ants only on damaged leaves. The same pattern was found when comparing mature leaves that were damaged and disturbed (control) (Figure 1, Table 1). However, the recruitment response was weaker for mature damaged leaves compared with young damaged leaves (randomized block, repeated-measures ANOVA, $F_{1,16} = 10.5$, P = 0.005, Figure 1). Undamaged young leaves adjacent to young damaged leaves did not present enhanced recruitment (Figure 1, Table 1).

Recruitment intensity was not affected by the two levels of experimental damage to the leaf, (Figure 2, Table 2). Ants recruited to leaves that received aqueous foliar extracts of *H. myrmecophila*, but did not recruit when only water was applied (Figure 3a, Table 2). The difference in recruitment intensity between these two treatments over time was significant (time × treatment effect, Table 2). Extracts of young leaves induced 2–3.5-fold more ants
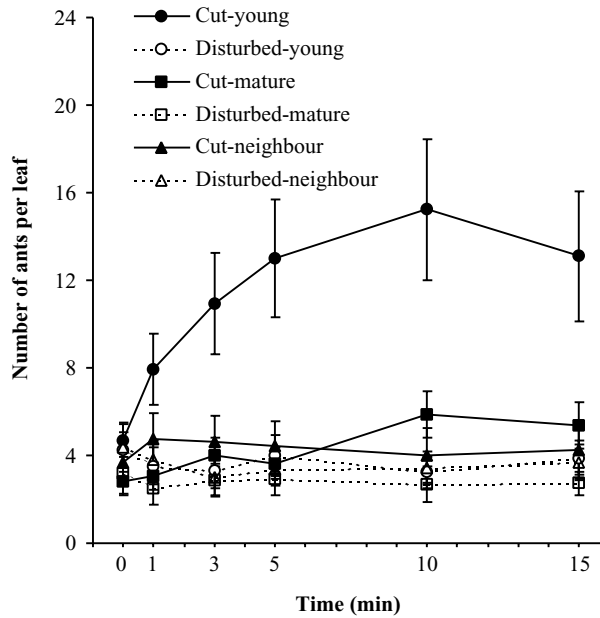
Figure 1. Mean number ($\pm 1\,$SE) of ants *Allomerus octoarticulatus* on young and mature leaves of *Hirtella myrmecophila* damaged (cut) and disturbed (control) and neighbouring to those cut and disturbed.

Table 1. Randomized-block, repeated-measures ANOVA examining the recruitment of ants (number/leaf) on (1) young leaves cut vs. control (disturbed) and (2) mature leaves cut vs. control, and on (3) neighbouring leaves of those cut and control. Plants were considered as blocks and time as the factor of repetition.

Source of variation	df	F	P	G-G
(1) Leaf cut (young leaves)				
Plant	16	1.94	0.098	
Treatment	1	16.4	0.001	
Error	16			
Time	5	5.78	0.000	0.006
Time × Plant	80	0.99	0.509	0.506
Time × Treatment	5	7.07	0.000	0.002
Error	80			
(2) Leaf cut (mature leaves)				
Plant	16	2.15	0.068	
Treatment	1	11.8	0.003	
Error	16			
Time	5	4.63	0.001	0.018
Time × Plant	80	1.07	0.370	0.422
Time × Treatment	5	3.67	0.005	0.039
Error	80			
(3) Neighbouring leaves				
Plant	16	2.09	0.076	
Treatment	1	0.39	0.538	
Error	16			
Time	5	0.50	0.773	0.717
Time × Plant	80	0.75	0.896	0.858
Time × Treatment	5	1.80	0.122	0.146
Error	80			

to recruit relative to extracts of mature and old leaves (Figure 3b) as indicated by the significant time, treatment, and time × treatment effects (Table 2). Young-leaf extract
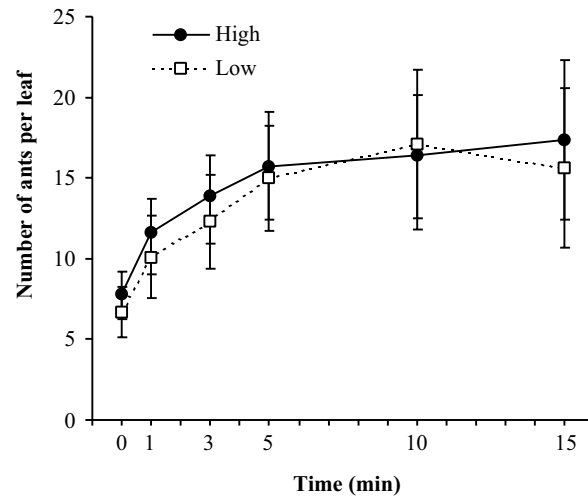

Figure 2. Mean number ($\pm 1\,$SE) of ants *Allomerus octoarticulatus* on young leaves of *Hirtella myrmecophila* that received high and low levels of damage. (High: triangle of $3.0 \times 3.0 \times 3.0\,$cm; Low: triangle of $0.5 \times 0.5 \times 0.5\,$cm).

Table 2. Randomized-block, repeated-measures ANOVA examining the ant recruitment (number/leaf) on (1) leaves with different levels of damage (see Methods), and after applications of (2) leaf extracts vs. water, (3) young vs. old leaves and (4) extracts of *Hirtella myrmecophila* vs. *Protium hebetatum*. Plants were considered as blocks and time as the factor of repetition.

Source of variation	df	F	P	G-G
(1) Damage level				
Plant	9	8.21	0.002	
Treatment	1	0.26	0.624	
Error	9			
Time	5	34.90	0.000	0.000
Time × Plant	45	6.30	0.000	0.000
Time × Treatment	5	0.54	0.748	0.663
Error	45			
(2) Leaf extract vs. water				
Plant	9	4.29	0.020	
Treatment	1	13.53	0.005	
Error	9			
Time	5	8.81	0.000	0.001
Time × Plant	45	1.32	0.176	0.264
Time × Treatment	5	8.14	0.000	0.002
Error	45			
(3) Extracts of young, mature and old leaves				
Plant	12	2.62	0.022	
Treatment	2	13.51	0.000	
Error	24			
Time	5	19.11	0.000	0.000
Time × Plant	60	2.19	0.00	0.010
Time × Treatment	10	5.28	0.000	0.001
Error	120			
(4) Extracts of *H. myrmecophila* vs. *P. hebetatum*				
Plant	9	4.22	0.022	
Treatment	1	1.34	0.276	
Error	9			
Time	5	11.43	0.000	0.001
Time × Plant	45	1.35	0.160	0.283
Time × Treatment	5	1.74	0.144	0.209
Error	45			

Figure 4. Mean number (± 1 SE) of ants *Allomerus octoarticulatus* on young leaves of *Hirtella myrmecophila* that received extracts of young leaves of *H. myrmecophila* and of *Protium hebetatum*.
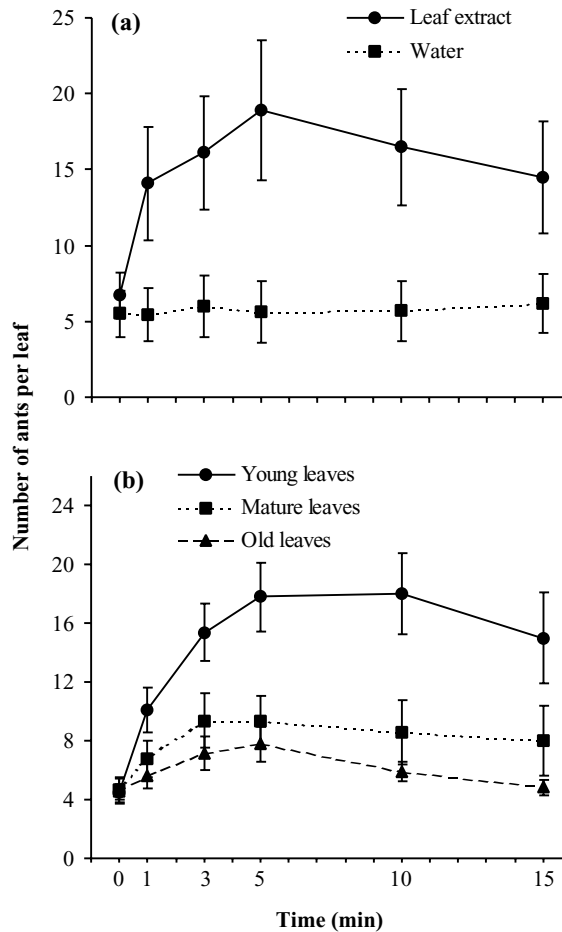

Figure 3. Mean number (± 1 SE) of ants *Allomerus octoarticulatus* on young leaves of *Hirtella myrmecophila* that received (a) extracts of young leaves of *H. myrmecophila* (experimental) and water (control), and (b) extracts of young, mature and old leaves of *H. myrmecophila*.

always induced a greater recruitment response, while there was no statistically significant difference between mature- and old-leaf extracts (Tukey's test: young vs. mature: $P = 0.009$; young vs. old: $P = 0.001$; mature vs. old: $P = 0.588$). The ants recruited under chemical stimuli of the aqueous leaf extracts from both *H. myrmecophila* and *P. hebetatum* (Figure 4), according to the significant time effect (Table 1). Surprisingly, this recruitment occurred with the same intensity, independent of the origin of extract (treatment effect, Table 2).

Recruitment behaviour

We observed recruitment behaviour during the experiments. After cutting, the few (1–3) ants located close to the damage site were attracted to the cut, touching it with their antennae and mandibles. A few seconds later they moved quickly to the domatia and after 3–4 s more
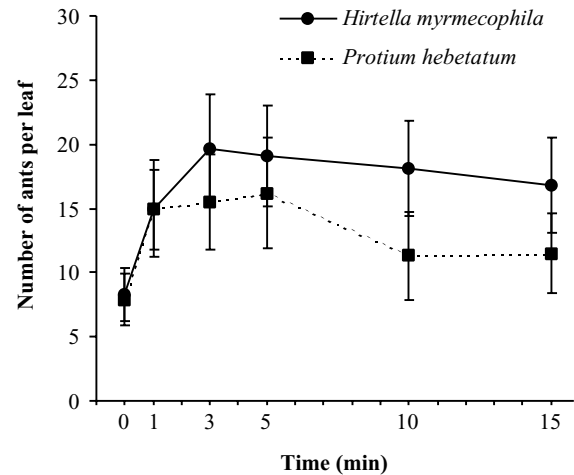
several ants left the domatia and moved rapidly to the cut or near this area. Prior to cutting, the few ants present on the leaf blade had been nearly immobile, but after the cut they became more active. After 5–10 min the number of ants active on the blade stabilized (Figure 2) or began to decrease (Figures 1a, 3–5) and between 20–30 min several ants had re-entered the domatia, while those remaining on the leaf surface again became nearly static. In the experiments with leaf extracts, when pure water was applied to the leaves, the ants did not recruit nor approach the liquid. When old-leaf extract was applied a few ants recruited, approached the drops, touched their antennae, but did not remain for long. In contrast, after application of young-leaf extract, the ants recruited and behaved as on damaged leaves. Several ants approached the drop, and touched it with antenna and mandible, and remained in contact for several seconds.

When termites were individually placed on leaves, the first and second ants that found the insect immediately seized a leg and pulled it backwards immobilizing the prey. The recruitment of other workers to kill and transport these termites to the domatia is only made by the third or fourth ant.

DISCUSSION

In this study we showed that the ant *A. octoarticulatus* was quickly stimulated to recruit other ants to the site of leaf damage on its myrmecophyte host. Prior studies have reported similar ant behaviour on other ant-loving plant species, as a response to leaf damage and presence of herbivores (Cronin 1998, Fiala & Maschwitz 1990, Lapola *et al.* 2003, Rocha & Bergallo 1992, Young *et al.* 1990). Such recruitment behaviour can be induced by biotic

factors (e.g. presence of herbivores) or chemical factors (compounds released by the damaged plant). Plants have also been reported to quickly reward/attract ants by inducing production of nutritive substances in extrafloral nectaries after an herbivore attack (see Heil & McKey 2003, Heil *et al.* 2001).

Simulated herbivore damage to young and mature leaves of *H. myrmecophila* recruited more ants than the respective control leaves. However, the ant's response was stronger on the young leaves relative to mature ones. This suggests that young leaves may contain higher concentration of some chemical compound which induces recruitment. Furthermore, the number of ants patrolling mature leaves is smaller than on young leaves, probably related to the smaller number of ants occupying mature domatia (T. J. Izzo, *pers. obs.*). This places a limit on the recruitment response measured here that is unrelated to the chemical stimulus per se. Rocha & Bergallo (1992) also reported that the recruitment response of *Azteca* ants was positively correlated with (i.e. limited by) the colony size on the host plant *Cecropia pachystachya*.

Allomerus octoarticulatus ants recruited with the same intensity, irrespective of the two levels of leaf damage employed in this experiment. These results differ from those of Agrawal & Dubin-Thaler (1999) for the *Cecropia obtusifolia* – *Azteca* system, obtained at La Selva, Costa Rica. They found a strong positive relationship between the level of leaf damage and number of ants that were recruited, probably because this ant can detect volatile compounds, which are produced in greater quantity and concentration with higher levels of leaf damage. The system studied here diverges from those studied by Agrawal & Dubin-Thaler (1999) or by Fiala & Maschwitz (1990) (*Macaranga* – *Crematogaster* system, in Malay Peninsula), that also recorded ant recruitment after leaf damage, in an important characteristic: whereas *Cecropia* and *Macaranga* offers rewards (food bodies) to the ants, *H. myrmecophila* does not provide any apparent food source to the ants. Hence, *A. octoarticulatus* feeds primarily on invertebrates captured on leaves and must recruit as a result of certain stimuli coming from the leaf. The herbivory damage should be a reliable signal of the presence of food on the leaf. Whenever an ant does not find the herbivore, but only the damaged area (as observed here), it immediately goes into the domatia to recruit other workers. A better strategy when the herbivore is not found is to call for help (workers) to find the prey, maximizing effort and minimizing time of encounter. Since *A. octoarticulatus* is a relatively slow ant, workers would probably lose their prey if they spent time recruiting instead of capturing it upon encounter.

Our data showed that the ants recruited after exposure to leaf extracts from the host plant *H. myrmecophila*, indicating that they are induced to recruit under stimuli of chemical inductors. Similar results were also obtained by Lapola *et al.* (2003) in the *Maieta guianensis* (Melastomataceae) – *Pheidole minutula* (Formicidae) system. In the *Cecropia* – *Azteca* system, volatile compounds of the leaf extracts induced ants to recruit (Agrawal & Dubin-Thaler 1999). In the present system, *A. octoarticulatus* had to touch the antenna and mandibles on the damaged area, suggesting that liquids in addition to volatile compounds may be involved in this recruitment. Similar results were also observed by Brouat *et al.* (2000) for the *Leonardoxa africana* – *Petalomyrmex phylax* system, in Cameroon. If there are volatile effects, these must be weak and with action at short distance. In addition, *A. octoarticulatus* did not recruit in neighbouring leaves to those damaged, reinforcing the hypothesis that volatile compounds are not involved in recruitment. This suggests that the recruitment induced by chemical compounds is local, affecting only ants present on damaged leaves.

Extracts of both young, mature and old leaves of *H. myrmecophila* induced ants to recruit, but extracts of young leaves were much more effective. Young leaves of *H. myrmecophila* are more susceptible to herbivore attack (Izzo 2002) and a higher concentration of some compounds in these leaves can guarantee rapid ant recruitment. Plants can maintain constitutive resistance more strongly in more vulnerable tissues, such as young leaves (optimal defence hypothesis) (McKey 1974), or immediately induce the production or reallocate higher concentration of chemicals to young leaves (see Iwasa & Kubo 1996, Karban & Baldwin 1997) in response to herbivore attack. In contrast, mature and old leaves have fewer foraging ants per leaf and, consequently, the probability of a herbivore being found on it is lower (in old leaves, the ants, as the domatia, are not even present). However, these leaves are lignified, resistant to herbivore attack and do not rely on ants for resistance (Izzo & Vasconcelos 2002). From the plant's perspective, the presence of ants and the induction of recruitment in these leaves seems to be much less important than in expanding and recently expanded leaves.

Allomerus octoarticulatus responded to young-leaf extracts from their host plant, as well as to those of *P. hebetatum*. Fiala & Maschwitz (1990) and Agrawal & Dubin-Thaler (1999) found similar results in other plant–ant systems and proposed that the ants can recruit to volatile compounds from heterospecific plants in order to prune them, since these plants can be competitors or parasites of their host plant. In the present system, this hypothesis is unsupported, since *A. octoarticulatus* has not been observed pruning neighbouring plants or lianas (T. J. Izzo, *pers. obs.*). The lack of specificity in the response of ants, however, suggests that very general cues are used in inducing ant recruitment to *Hirtella*. In contrast, Fiala & Maschwitz (1990) reported that *Crematogaster* ants recognize their host plant species (*Macaranga*) and

discriminated other plants, and suggested that chemical cues are involved in this process. It remains unclear why *Allomerus* responds to extracts from a plant species with which it is not typically associated.

The processes responsible for the origin of the mechanisms of induced responses in ant-plants are poorly known (Brouat *et al.* 2000). In the studied system, the constant necessity of *A. octoarticulatus* to obtain prey, its main diet, can provide a clue. Our interpretation is that the ants evolved to recognize common chemical compounds discharged after herbivore damage. This evolutionary process, that generates mutual benefit, was probably an important factor in the maintenance and stability of mutualism in this system.

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