Interspecific variation in the defensive responses of ant mutualists to plant volatiles

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In ant–plant mutualist systems, ants patrol their host plants and search for herbivores. Such patrolling can be inefficient, however, because herbivore activity is spatio-temporally unpredictable. It has been proposed that rapid and efficient systems of communication between ants and plants, such as volatile compounds released following herbivory, both elicit defensive responses and direct workers to sites of herbivore activity. We performed bioassays in which we challenged colonies of two Amazonian plant-ants, Azteca sp. and Pheidole minutula, with extracts of leaf tissue from (1) their respective host-plant species (Tococa bullifera and Maieta guianensis, both Melastomataceae), (2) sympatric ant-plants from the Melastomataceae, and (3) two sympatric but non-myrmecophytic Melastomataceae. We found that ants of both species responded dramatically to host-plant extracts, and that these responses are greater than those to sympatric myrmecophytes. Azteca sp. also responded to non-myrmecophytes with an intensity similar to that of sympatric ant-plants. By contrast, the response of P. minutula to any non-myrmecophytic extracts was limited. These differences may be driven in part by interspecific differences in nesting behaviour; although P. minutula only nests in host plants, Azteca sp. will establish carton satellite nests on nearby plants. We hypothesize that Azteca sp. must therefore recognize and defend a wider array of species than P. minutula. © 2008 The Linnean Society of London, Biological Journal of the Linnean Society, 2008, 94, 241–249.


INTRODUCTION

Ants that defend plants from herbivores in exchange for rewards such as food or shelter are a defining characteristic of tropical forests, with over 100 plant genera and 40 ant genera participating in these mutualisms (Benson, 1985; Davidson & McKey, 1993). These ants are often obligately associated with their host plants, and establish colonies solely in swollen thorns, leaf pouches, hollow stems, and other specialized structures known as domatia. Following Janzen's (1966; 1967) pioneering studies of Acacia–Pseudomyrmex interactions, empirical work has demonstrated that the loss of ant colonies can have major consequences for host plants. Plants from which colonies are experimentally removed can suffer increased rates of herbivory, reduced fecundity, and an elevated probability of mortality relative to those in which colonies are left undisturbed (Heil & McKey, 2003). Because of these consequences, and because both ants and plants are often associated with a limited suite of partner taxa (Fonseca & Ganade, 1996), these interactions have become model systems with which to study the evolutionary ecology of mutualisms (Bronstein, 1998).
In many ant–plant systems, resident ants patrol the leaves of their host plants and search for herbivores. Such patrolling can be inefficient, however, as herbivores are often at low density and their activity is unpredictable both spatially (i.e. the location on a plant where herbivores will be found) and temporally (i.e. during what time of the day or season they will feed). As a result, it has been argued that there should be strong selection for rapid and efficient systems of ant–plant communication that would both elicit defensive responses and direct workers to sites of herbivore activity (Brouat et al., 2000; Heil & McKey, 2003). Such systems would allow ants to allocate workers to activities that directly enhance colony fitness, such as caring for brood or foraging for food, until they are needed for host-plant defense. Enhanced ant colony fitness stemming from more efficient worker allocation would also benefit plants indirectly because colony success and plant condition are closely related.

Because ants use elaborate systems of chemical communication (Hölldobler & Wilson, 1990), several studies have suggested that the volatile chemical compounds emitted by damaged plants may be an important means of ant–plant communication (Agrawal & Rutter, 1998; Agrawal, 1998; Brouat et al., 2000). Although work identifying the particular compounds that serve as ant cues remains limited (Brouat et al., 2000), the results of field experiments are consistent with this hypothesis. For example, Agrawal (1998) found that the number of Azteca workers patrolling the leaves of the Neotropical tree Cecropia obtusi-folia increased dramatically when solutions of the leaf volatile hexanal were applied to leaf surfaces. Subsequent experiments in other ant–plant systems using similar methods have documented large increases in ant patrolling following exposure to host-plant extracts (Lapola, Bruna & Vasconcelos, 2003; Bruna, Lapola & Vasconcelos, 2004; Christianini & Machado, 2004; Romero & Izzo, 2004).

It has also been hypothesized that ants use volatile cues to discriminate between plant species, thereby allowing workers to identify potential competitors to prune (Frederickson, Greene & Gordon, 2005; Janzen, 1969) or aiding dispersing queens in the identification of putative host plants (Edwards et al., 2006; Jürgens et al., 2006). Although a number of carefully designed laboratory studies have addressed this issue (Fiala & Maschwitz, 1990), field experiments evaluating the potential for differential responses to plant volatiles remain limited (Agrawal & Dubin-Thaler, 1999; Romero & Izzo, 2004). Furthermore, to our knowledge, no studies have compared the responses of different ants present in a community with the extracts of multiple plant species. This makes it difficult to determine whether the responses observed to date are unique to the ant species under investigation, or are a function of the particular plant species with which the experiments were conducted. Ant responses to extracts from a diversity of plant species may indicate stimulatory compounds are ubiquitous and serve physiological functions beyond recruitment (e.g. hexanal), whereas responses limited to certain plant species (e.g. host plants or other myrmecophytes) may indicate that stimulatory compounds are highly specialized or reflect the ability of ants to discriminate among the volatile profiles of plant species.

We challenged colonies of two ant species associated with Amazonian ant-plants with leaf-tissue extracts from a suite of related sympatric plant taxa. Our experiments addressed the following questions: (1) Do the ant species studied respond similarly to extracts made from their host plants as they do to other sympatric myrmecophytic plant species from the same plant family? (2) Do ants respond more strongly to extracts made from myrmecophytes than to those made from related non-myrmecophytes? (3) Do taxonomically distinct plant-ants differ in their response to volatiles from the same plant species?

**MATERIAL AND METHODS**

All fieldwork was conducted from August to September 2003 in Reserve #1501 of Brazil's Biological Dynamics of Forest Fragments Project (BDFFP) (2°30'S, 60°W). This 800-ha reserve is embedded in a large expanse of nonflooded lowland forest; annual rainfall is in the range 1900–3500 mm, and there is a distinct dry season from June to December (for details on the BDFFP sites, see Bierregaard et al., 2002). Tecomella bullifera and Maieta guianensis (both Melastomataceae) are understory shrubs that grow to approximately 3 m and 1 m in height, respectively. Leaves of both species have two pouches at their base in which ant queens establish colonies (Michelangeli, 2000; Vasconcelos & Davidson, 2000). Although up to six putative species of ants have been found inhabiting the domatia of T. bullifera in our field sites, most individuals (> 70%) are colonized by a single undescribed species, hereafter referred to as Azteca sp. (Fonseca & Ganade, 1996; Bruna, Vasconcelos & Heredia, 2005). Maieta guianensis, which is also colonized by multiple ant species in our sites, is most commonly associated with Pheidole minutula (Mayr) (> 90% of plants). Ants forage for insects on the host plant’s leaves and tend scale insects for honeydew (Vasconcelos & Davidson, 2000).

We conducted three bioassays in which we simultaneously challenged ant colonies with different types of leaf-tissue extracts. In the first bioassay, we challenged colonies of P. minutula and Azteca sp. with extracts made from their respective host-plant species (hereafter HP), a sympatric myrmecophyte from the Melas-
tomataceae (hereafter SM), and a control solution (water). In the second bioassay, we simultaneously challenged colonies with extracts made from a sympatric and closely-related non-myrmecophyte (CRNM) from the Melastomataceae, the host plant, and the control solution. The third bioassay was identical to the second, except we used a more-distantly related and non-myrmecophytic member of the Melastomataceae (DRNM) instead of the CRNM. Recent phylogenetic analysis indicates that Tococa is nested within Miconia, whereas Maieta is nested within Clidemia (Michelangeli et al., 2004). We therefore used the following species combinations for our bioassays: P. minutula colonies inhabiting M. guianensis were challenged with M. guianensis (HP), T. bullifera (SM), Clidemia japurensis (CRNM), and Miconia albicans (DRNM); Azteca sp. colonies inhabiting T. bullifera were challenged with T. bullifera (HP), M. guianensis (SM), M. albicans (CRNM), and C. japurensis (DRNM).

Extracts were made by soaking approximately 5 g of fresh leaf tissue in 60 mL of distilled water for 24 h; all leaves were washed with water and a non-abrasive sponge prior to extraction to remove epiphylls, ant chemical trails, or other contaminants. Although the extraction of some types of volatile compounds (e.g. terpenoids) might be superior in solvents such as hexane or chloroform, we used water to compare our results with those of previously published experiments. For each day’s bioassays, we collected leaves from a plant that had not been previously used in experiments. Both Azteca sp. and P. minutula patrol young and old leaves with equal intensity (Vasconcelos & Davidson, 2000; Bruna et al., 2004), suggesting that patterns of ant activity are independent of potential age-related differences in leaf chemistry. Nevertheless, we made all extracts with leaves of intermediate age (i.e. not newly expanded nor those at the base of branches) to avoid the potentially confounding effects of leaf-age, chemical composition, and act activity (cf. Brouat et al., 2000; Romero & Izzo, 2004).

A total of 75 T. bullifera colonized by Azteca sp. and 60 M. guianensis colonized by P. minutula were used. These plants were divided into three size classes based on the number of domatia they had (T. bullifera: < 25, 26–75, > 75; M. guianensis: < 20, 21–40, > 40); experimental treatments were evenly distributed among size classes to minimize potential effects of colony size (Fonseca, 1993). Each ant colony was simultaneously challenged with one of the three combinations of extracts: (1) HP, SM, water; (2) HP, CRNM, water; or (3) HP, DRNM, water. Each plant received only one of the treatment combinations (N = 25 and N = 20 T. bullifera and M. guianensis per treatment, respectively); we avoided applying the same treatment to nearby plants to reduce potential local effects.

Bioassays were conducted by placing five drops of each extract on the upper surface of three randomly selected, fully expanded leaves. One extract was placed on each leaf, and each leaf was located on a different branch. Because the movement of ants between neighbouring leaves or branches on which extract has simultaneously been placed is limited (M. R. Darrigo, unpubl. data), ant abundance on each leaf reflects a response to that extract, rather than a preference among the simultaneously presented extracts. To verify that there was no difference in the number of patrolling workers on leaves receiving the extracts, we counted worker numbers at three 5-min intervals prior to the start of the experiment (~10, ~5, and 0 min). We then counted worker numbers an additional five times (5, 10, 15, 30, and 60 min after treatments were applied). The effect of extract type on the abundance of patrolling workers (square-root transformed) was analysed using repeated measures analysis of variance (ANOVA). Throughout the text, we present back-transformed values of ant abundance. Each plant had three treatments, with no replication within plants. Extract type was considered a fixed effect, with each plant considered a random effect (Sokal & Rohlf, 1995). When a significant main effect of treatment was found, we conducted three pairwise repeated measures ANOVA tests to identify the extract types that were significantly different (i.e. HP versus SM, HP versus water, and SM versus water); the value for significance in these tests was adjusted to a = 0.017 to correct for multiple comparisons.

To directly compare the responses of ant colonies to myrmecophytic and non-myrmecophytic plants, we combined results from the different experiments in the following analysis. We first calculated the percentage difference between a colony’s response to plant extracts 5 min after initiating the bioassay and the response to the control solution at that time period. This new response variable was then rank-transformed (Conover & Iman, 1981) and used as the dependent variable in one-way ANOVAs comparing the four plant categories (HP, SM, CRNM, and DRNM), with Fisher’s protected least significant difference post-hoc tests used to compare the category means. To evaluate the responses of P. minutula and Azteca sp. to extracts of the same plant species, we compared the rank-transformed percentage difference values using t-tests. All analyses were conducted using Systat, version 8.0 (SSI, 2001).

RESULTS

Ants responded rapidly and dramatically to the application of host-plant extracts. Within 5 min of applying T. bullifera solutions, the mean ± SE number of

Azteca sp. workers patrolling leaves was 11-fold higher than on control leaves to which water had been applied (18.74 ± 1.76 versus 1.75 ± 0.34, respectively, all experiments combined, Fig. 1A, C, E). Similar results were observed with P. minutula colonies: the response on leaves to which Maieta guianensis extract was added was four-fold greater than on control leaves (13.29 ± 0.85 versus 3.12 ± 0.36, respectively, all experiments combined, Fig. 1B, D, F).

**Figure 1.** Number of *Azteca* sp. and *Pheidole minutula* workers patrolling leaves of *Tococa bullifera* and *Maieta guianensis* from 10 min prior to 60 min after colonies were challenged with leaf-tissue extracts and a control solution (water). Colonies of each species were challenged with the following combinations of extracts: (A, B) their host plant, a sympatric myrmecophyte, and water; (C, D) their host plant, a closely-related non-myrmecophyte, and water; (E, F) their host plant, a distantly related non-myrmecophyte, and water. Significant differences between treatments (evaluated with post-hoc repeated measures analysis of variance) are denoted with different lower-case letters. For bioassays conducted with colonies of *Azteca* sp., the host plant is *T. bullifera*, the sympatric myrmecophyte was *M. guianensis*, the closely-related non-myrmecophyte was *Miconia albicans*, and the distantly-related non-myrmecophyte was *Clidemia japurensis*. For bioassays conducted with *P. minutula* colonies, the host plant in *M. guianensis*, the sympatric myrmecophyte was *T. bullifera*, the closely-related non-myrmecophyte was *C. japurensis* and the distantly-related non-myrmecophyte was *M. albicans*.
tively, Fig. 1B, D, F). Worker abundance remained significantly higher 60 min after the leaf extract was applied.

Repeated measures ANOVA and subsequent post-hoc tests indicated ant responses to extracts of the host plant, a sympatric myrmecophyte, and water were significantly different from each other (Azteca sp. $F_{2,48} = 82.66$ for main effect of treatment, $P < 0.001$; P. minutula: $F_{2,36} = 61.08$ for main effect of treatment, $P < 0.001$), with the intensity of these responses changing over time (Table 1). The number of Azteca sp. workers patrolling leaves to which T. bullifera extract was applied was approximately twice that on leaves to which extracts of the sympatric myrmecophyte M. guianensis (Fig. 1A), whereas, for P. minutula, the host-plant extract elicited a response one-third greater than to an extract of T. bullifera (Fig. 1B). Post-hoc repeated measures ANOVA indicated that both of these differences were significant ($P < 0.017$ in all comparisons; results not shown).

Both Azteca sp. and P. minutula also responded significantly to extracts of non-myrmecophytic plants (Fig. 1). Although, for Azteca sp., this response was two- to three-fold less intense than that to the host-plant extract (Fig. 1A, C, E), it was still up to 4.5-fold greater than the response to control the solution ($P < 0.017$ in all comparisons; results not shown). By contrast, the response of P. minutula to non-myrmecophytic plants at the peak of worker response was only 1.5–2.2-fold greater than that to control solutions (Fig. 1D, F).

For Azteca sp., the proportional increase in recruitment differed significantly among species (MS = 3358.19, $F_{3.94} = 4.63, P = 0.005$) with the response to host plants significantly greater than that to all other species (HP versus SM: $P = 0.04$, HP versus CRNM: $P = 0.001$, HP versus DRNM: $P = 0.002$, all other pairwise comparisons not significant; Fig. 2A). Similarly, the relative responses of P. minutula also differed among species (MS = 1922.02, $F_{3.73} = 4.36, P = 0.007$); however, the response to host plants was only significantly greater than the response to non-myrmecophytic species (HP versus CRNM: $P = 0.01$, HP versus DRNM: $P = 0.001$, all other pairwise comparisons not significant; Fig. 2A).

### Table 1. Repeated measures analysis of variance for the effect of leaf-tissue extract source on ant worker recruitment

<table>
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<th>Experiment</th>
<th>Source</th>
<th>d.f.</th>
<th>MS</th>
<th>$F$</th>
<th>Source</th>
<th>d.f.</th>
<th>MS</th>
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<tr>
<td></td>
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<td>Error</td>
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<td>Time</td>
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<td>2.17</td>
<td>8.87***</td>
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<td>0.72</td>
<td>3.67***</td>
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<td>0.41</td>
<td>8.87**</td>
<td>Time × Plant</td>
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<tr>
<td></td>
<td>Error</td>
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<td>Error</td>
<td>144</td>
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<td></td>
<td>Error</td>
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<td>2.00</td>
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<td>Error</td>
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<td>Error</td>
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Significance values are denoted with asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; probabilities corrected with Greenhouse–Geisser and Huynh–Feldt statistics were nearly identical; results not shown).

C, control; CRNM, closely-related non-myrmecophyte; d.f., degrees of freedom; DRNM, distantly-related non-myrmecophyte; HP, host plant; SM, sympatric myrmecophyte.

Fig. 2B). Finally, the proportional responses of *Azteca* sp. and *Pheidole minutula* to extracts of *M. guianensis* were not significantly different (*t* = 1.06, d.f. = 42, *P* = 0.30), but *Azteca* sp. responded more intensely to *C. japurensis* (*t* = 2.60, d.f. = 41, *P* = 0.01), *M. albicans* (*t* = 2.01, d.f. = 42, *P* = 0.05), and *T. bullifera* (*t* = 4.45, d.f. = 42, *P* < 0.001; Fig. 3).

**DISCUSSION**

Plants that have been damaged by herbivores emit an array of terpenoids, fatty-acid derivatives, and other volatile compounds (Pichersky & Gershenzon, 2002), and numerous studies have demonstrated that ants respond to this bouquet of host-plant chemicals (Agrawal, 1998; Lapola et al., 2003; Christianini & Machado, 2004; Romero & Izzo, 2004). However, few studies have directly compared the intensity of ant responses to volatile phytochemicals from multiple plant species found in the community. Our results are consistent with those of Fiala & Maschwitz (1990), who found that *Crematogaster borneensis* responds more strongly to extracts of its own host plant than it does to other *Macaranga* species. Although additional studies are clearly needed, the results of our bioassays suggest that ants can both recognize the volatile profiles of their host plants and discriminate among different ant-plant species in the same plant family. Several studies have screened Melastomataceae species for chemical constituents (Isaza, Ito & Yoshida, 2004; Motta et al., 2005). However, few have simultaneously compared the composition and quantity of these compounds in multiple species of the Melastomataceae (but see also Mimura, Salatino & Salatino, 2004; Michelangeli & Rodriguez, 2005), and none of them have attempted to link these compounds with insect behaviour. More studies that integrate phytochemistry with phylogenetic reconstructions, such as the comprehensive work of Jürgens et al. (2006), Mimura et al. (2004), and Michelangeli & Rodriguez (2005), are needed to determine how ants discriminate among related ant-plant species based on the presence or abundance of different volatile compounds.
Although both focal ant species recruited to extracts made from ant-plants, they differed notably in their responses to non-myrmecophytes. The number of patrolling Azteca sp. increased by two- to three-fold in response to volatiles from both closely- and distantly-related non-myrmecophytes, a result similar to that observed by Romero & Izzo (2004) when comparing the responses of Allomerus octoarticulatus to extracts of its host plant [Hirtella myrmecophila (Chrysobalanaceae)] and a non-myrmecophytic species [Protium hebetatum (Burseraceae)]. By contrast, the response of P. minutula to extracts of the same species was far less dramatic. Why does Azteca sp. respond so strongly to heterospecific volatiles whereas P. minutula does not? Unlike the Azteca studied by Agrawal & Dubin-Thaler (1999), the Azteca residents of T. bullifera do not exhibit pruning behaviour (E. M. Bruna, H.L. Vasconcelos, unpubl. data), suggesting that this is an unlikely mechanism underlying heterospecific recognition. Instead, we hypothesize that it reflects the need to recognize and defend a broader array of plant species from attacks by herbivores. Large Azteca colonies establish satellite nests on neighbouring plants in which they both rear brood and tend coccids (Vasconcelos & Davidson, 2000), but the low density of host plants in these forests (Bruna et al., 2005) makes it unlikely these neighbours are conspecific. By contrast, P. minutula never establishes satellite nests in other plant species and never forages off of its host plant. Colonies therefore have little need to respond defensively to heterospecific stimuli, even if they are capable of detecting them.

Experimental work has found that plants attacked by herbivores release volatile signals that initiate defensive responses in neighbouring plants (Baldwin, Kessler & Halitschke, 2002), even when neighbours are not conspecifics (Karban & Maron, 2002). Although these studies have focused primarily on the induction of chemical defenses, it is possible that a similar mechanism could also elicit biotic ones. Agrawal (1998) observed results consistent with this hypothesis: holding a damaged Cecropia leaf next to an undamaged one on a different tree resulted in increased patrolling activity by Azteca workers. Although our experiment was not specifically designed to test for interplant communication, the fact that extracts from species other than host plants resulted in increased patrolling by Azteca sp. suggest that some ant genera may be receptive to airborne volatiles from neighbouring plants. However, the results of our bioassays with P. minutula also indicate that the likelihood of observing biotic responses will depend not only on the identity of neighbouring plants, but also on that of ant occupants.

Although the strong response of ants to extracts of myrmecophytes could suggest the evolution of novel stimulatory compounds, an alternative hypothesis is that only the relative abundance of chemical constituents has changed. Indeed, Brouat et al. (2000) proposed that volatiles that elicit ant responses did not evolve specifically for that purpose, but rather were compounds already present in leaf tissue used for alternative physiological processes (i.e. exaptation rather than adaptation). In perhaps the only comprehensive test of this hypothesis, Jürgens et al. (2006) conducted a phylogenetically controlled analysis of the volatile profiles of 11 Macaranga species. They found no obvious differences in the scent profiles of plants that do and do not have mutualistic interactions with ants, and there were no obvious groupings according to plant phylogeny. They therefore concluded there was no evidence for the evolution of novel compounds specifically designed to elicit ant responses. It is our hope that an increasingly well-resolved phylogeny (Michelangeli et al., 2004; Stone, 2006), coupled with chemical analyses of volatiles, will soon allow us to conduct similar tests with the Melastomataceae.

Ample empirical work has demonstrated that the defensive responses of plant-ants and other carnivorous insects can be stimulated by volatile compounds, and that these behaviours can lead to reduced herbivore abundance or plant damage (Thaler, 1999; Kessler & Baldwin, 2001; Bruna et al., 2004). However, our comparisons of sympatric ant–plant systems suggest that even though stimulatory chemicals are ubiquitous, the presence and intensity of ant responses is not, and will depend on both plant and ant identity in ways that have previously not been explored. Our results also emphasize that exploring the evolution of these responses will require the challenging integration of disciplines ranging from biochemistry to animal behaviour. Fortunately, promising approaches for doing so, such as the ability to manipulate genes encoding the biosynthesis of volatiles (Baldwin et al., 2006), the ability to identify biologically active compounds (Jürgens et al., 2006), and high-resolution macro videography for detailed observations of ant behaviour (Ehmer & Gronenberg, 1997), are becoming increasingly accessible.

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