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ORIGINAL PAPER

Variation in the composition and activity of ants on defense of host plant *Turnera subulata* (Turneraceae): strong response to simulated herbivore attacks and to herbivore's baits

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Abstract Plants with extrafloral nectaries attract a variety of ant species, in associations commonly considered mutualistic. However, the results of such interactions can be context dependent. *Turnera subulata* is a shrub widely distributed among disturbed areas which has extrafloral nectaries at the base of leaves. Here, we evaluated whether the ants associated with *T. subulata* (i) vary in space and/or time; (ii) respond to simulated herbivory, and (iii) reduce herbivory rates. For this, we quantified the abundance and species richness of ants associated with *T. subulata* throughout the day in six different sites and the defensive capability of these ants under simulated herbivory in the leaves and stems of *T. subulata* plants ($N = 60$). We also checked the proportion of the lost leaf area and quantified leaf damage by chewing herbivores in the host plant. We found that a total of 21 ant species associated with the host plant. Species composition showed significant variation across the sampled sites and throughout the day. Visitation rates and predation by ants were higher in plant stems than in leaves. In general, herbivory rates were not correlated with ant association or activity, with the exception of the proportion of leaf area consumed; there was a significant lower herbivory rate on plants in which ants defended the leaves. Our results suggest that the benefits of association may depend on the ecological context. This context dependence may mask the correlation between the defense of ants and herbivory rates.

Keywords Extrafloral nectary · Herbivory · Indirect defense · Protection

Introduction

Plants express a wide variety of direct and indirect defense mechanisms known to minimize herbivory (Strauss and Zangerl 2002). Indirect mechanisms are represented by the emission of herbivory-induced plant volatiles that attract natural enemies of herbivores (Dicke et al. 1990) or by defense provided by natural enemies (e.g., predators and parasitoids) that are attracted to resources offered by the host plant (Moreira and Del-Claro 2005; Byk and Del-Claro 2011; Heil 2011). Some plant species, for example, produce extrafloral nectaries (EFNs), which provide attractive resource for potential defenders (Moreira and Del-Claro 2005).

Ants are among the most abundant and diverse macroarthropods in tropical terrestrial environments (Wilson 1971), and are the most frequent visitors to plant EFNs (Oliveira and Brandão 1991). Such associations can benefit the host plant directly (through effective predation) or indirectly (by their presence or patrolling, which may repel potential herbivores). Ant–plant associations are

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commonly considered mutualistic. However, the results of these interactions can be context dependent when there are low specificity between the species involved (Heil 2008; Bronstein 2009). The resources offered by plants may also attract individuals that do not offset the energy investment of the host, resulting in low contribution to increasing plant fitness. In some cases, ants may also harm other positive associations for the host plant (e.g., pollinator, predator, and parasitoid visitors) (Ness 2006; Assunção et al. 2014). Some studies have suggested that the effectiveness of defense depends on the species identity (Stanton and Palmer 2011) and quantity of ants associated with host plants (Rico-Gray and Oliveira 2007). In the shrub *Turnera ulmifolia*, for example, studies have reported a total of 25 species of associated ants (Cuautle et al. 2005) and found that their relationships with the host plant are not always mutualistic (Torres-Hernández et al. 2000; Cuautle et al. 2005; Salazar-Rojas et al. 2012).

The genus *Turnera* L. (Turneraceae) consists of shrub species occurring in a range of countries in the Latin America (Piacente et al. 2002). *Turnera subulata* is a widely distributed ruderal plant that is abundant in different Brazilian biomes (Arbo 2005). It occurs in natural environments, but is more frequently found in disturbed areas. In this species, the petiole of each leaf has a pair of EFNs which are typically associated with ants (Arbo 2013). However, the nature of these ant–plant associations has not been studied.

Here, we evaluated whether *T. subulata* ant assemblages (i) vary across sampled sites and/or throughout the day; (ii) respond to simulated herbivory and the damage to different structures of the host plant (stem and leaf); and (iii) reduce herbivory rates (for sucking and chewing insects) via defense of the host plant.

Methods

Study area

The study was conducted at the campus of the Federal University of Sergipe (UFS) (10°55′35″S, 37°6′14″W), located in São Cristóvão, Sergipe, Brazil. The climate is classified as tropical wet and dry (Aw) according to the Köppen system, with an average temperature of 25.3 °C and an average annual rainfall of 1,372 mm. The experiments were conducted from March to April, 2015 (“dry season”).

Associated ant assembly

To assess whether there is temporal and spatial variations in the composition of plant-associated ants, these insects were collected from 60 plants (10 plants from each sampled site) containing at least five main branches. The plants were randomly selected in six different areas (e.g., “sampled sites”), with a minimum distance of 80 m from each other (designed to test for variation across sampled sites). Ants were collected using the beating tray technique—a white tray placed beneath tree branches to catch falling insects after three vigorous hits (Herms et al. 1990; Prado et al. 2016)—over three periods of the day: 10:00–12:00 a.m.; 1:00–3:00 p.m., and 4:00–6:00 p.m. (to test variation throughout the day). Specimens were collected with forceps, placed in vials with 80% alcohol, and identified using published identification keys (Bolton 2003; Baroni-Urbani and Andrade 2007). Specimens were compared to those at the Padre Jesus Moure Entomological Collection at the Federal University of Paraná, Brazil. Ants were later classified into feeding guilds according to Brown Jr (2000).

Ant responses to *T. subulata*-simulated herbivory and plant damage

Experiments were conducted to test the indirect defense of plants by ants, via (i) recognition mechanisms and aggressiveness against potential herbivores (i.e., herbivore cues) and (ii) perception and response to odors emitted by damaged plant tissue (i.e., simulated herbivory cues). To isolate these responses, two separate experiments were performed.

The first experiment measured ant responses to immobilized herbivores (i.e., without plant damage), and the second experiment measured responses to simulated herbivory cues (i.e., plant tissue damage) in the absence of herbivores. Treatments were applied to arbitrarily selected plant branches for both experiments, and ants were allowed to choose between cues from the stems or leaves (see Fig. 1). Treatments were applied 15 cm from the apical end of the branches. In all cases, the behavioral experiments were conducted between 7:00 and 10:00 a.m. in the absence of rain.

For the first experiment, *Nasutitermes macrocephalus* worker termites were fixed to host plants as proposed by Oliveira et al. (1987). A single live termite ‘bait’ was adhered to the leaf or stem of each plant using a 1-cm strip of double-sided tape. We randomly selected three branches of each plant, in which the leaf and the stem on the same branch were always subjected to the same treatment (Fig. 1). The treatments were as follows: (i) taped termite “herbivore bait” (termite + double-sided tape); (ii) control (only tape) (double-sided tape controls); or (iii) control (no-treatments) (Fig. 1a). Observations were carried out for all treatments during ten consecutive minutes simultaneously by two researchers. During the observation period, we measured the following: (i) time spent for the first ant

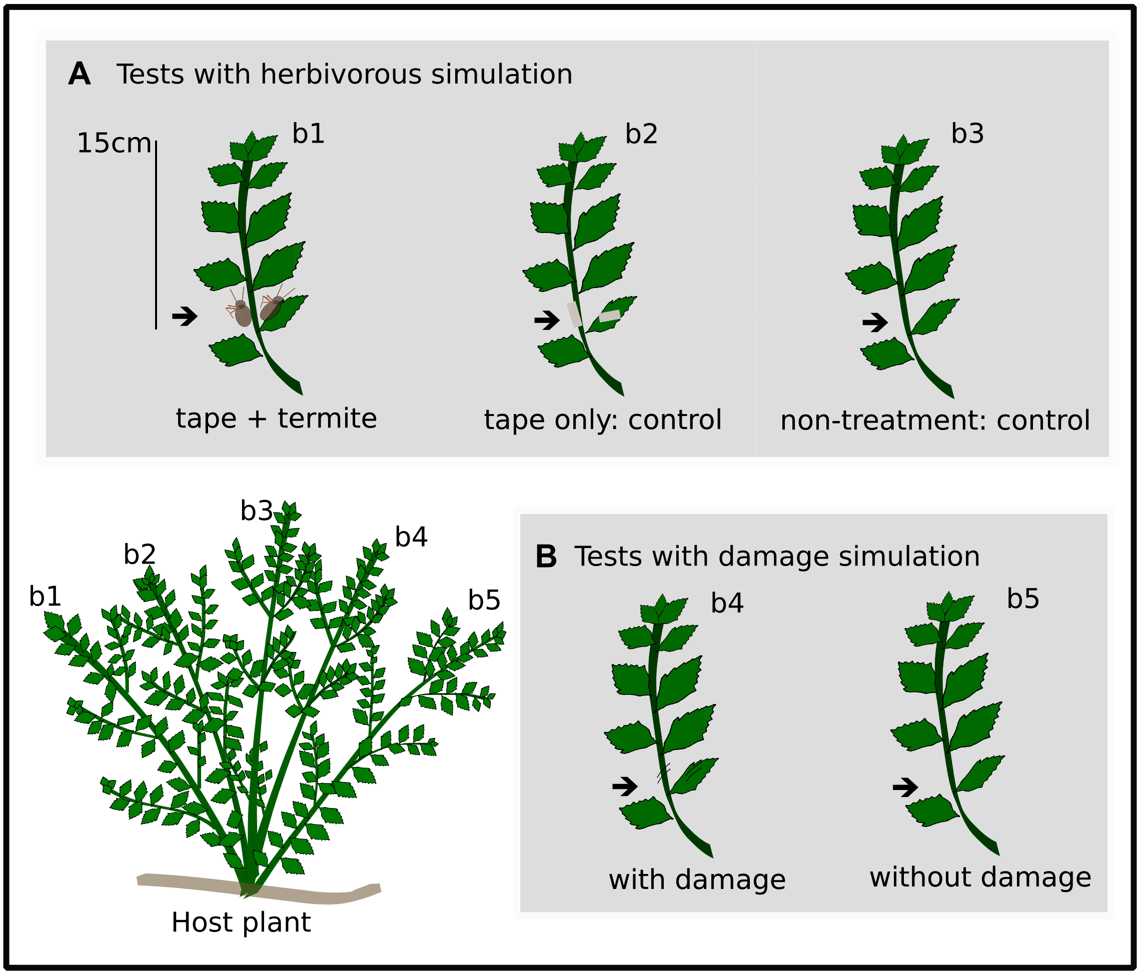

Fig. 1 Scheme of behavioral bioassays simulating the presence of herbivores and *Turnera subulata* structural damage. **a** Tests with herbivorous simulation: *b1* = branch containing treatments with tape + termite (taped termite "herbivore bait"); *b2* = branch with tape-only control [control (only tape)]; *b3* = branch with no-treatment controls [control (only stem or leaf)]. **b** Test with damage simulation: *b4* = branch with injury located on the stem or leaf; *b5* = no-damage branch (control: only stem or leaf). In all cases, ants were allowed to choose between treatments located on the stem or leaf. Each plant represented a true repetition of each treatment

arrival in each one of treatments and (ii) time spent for the first ant to attack the baits (in treatment with the presence of herbivore). At the end of each test, the tape and termites were carefully removed without causing damage or disruption to the plants.

Thirty minutes after the plant defense tests by ants, we evaluated ant responses to simulated plant herbivory cues. We selected randomly two branches that were different from those used in predation test, and inflicted the damage on the leaf and stem of each branch by cutting a 1-cm-long incision using a utility knife (probe). The treatments consisted of (i) mechanical injury and (ii) no-treatment controls (Fig. 1b). Leaves and stems on the same branch were always subjected to the same treatment. Observations were carried out in each one of the plants simultaneously for both treatments (mechanical injury and no-treatment controls) during 10 consecutive minutes. During the observation period, we measured the time spent for the first ant arrival in each one of treatments. For all bioassays, each plant was considered as a true repetition ($N = 60$), totalizing 1.200 min of observations.

Herbivory rates in *T. subulata*

After bioassays and quantification of the associated ant assembly, 50 plants were randomly selected to quantify herbivory rates (over the entire development of the plant until the sampling time). Shoots were removed and kept in a freezer to quantify the total number of leaves versus the number of leaves with injuries (punctures) caused by sucking insects. Subsequently, all leaves were removed from plants and photographed in order to estimate the total leaf area and the area lost to chewing insects. The images were processed using Image J (Wayne Rasband, National Institutes of Health, USA).

Statistical analyses

All analyses were performed using R software (R Development Core Team 2011) via generalized linear models (GLMs), followed by residual analysis to verify the suitability of distributions and the tested models.

The effect of variation across sampled sites and throughout the day, and the interaction between these factors on the composition of ants associated with *T. subulata* were tested by permutation multivariate analysis of variance (PERMANOVA). PERMANOVA was performed using the *Jaccard* dissimilarity index and multiple-paired comparisons with 999 permutations in the routine of the 'vegan' package. The terms "sampled sites" and "time periods" were included in the model as fixed explanatory factors, and the identity of each plant was included in the model as a random block effect. A similar model was used to evaluate whether there were differences in the assemblage of ant species that attacked the termite baits on plant stems and leaves.

The time spent for the visit and predation by ants in different treatments were analyzed using survival analysis with Weibull distribution ('survival' package). The censored values in the survival analysis were the time for ant arrival to treatments, or the time for the ant to attack termite baits. In all cases, each plant was considered a true repetition. Thus, the analysis provided the average time spent for 50% of the analyzed plants to be visited or for termite baits to be attacked by ants.

In order to check whether herbivory rates are correlated with the defense effectiveness of ants in the stem, the leaf, or both (stem + leaf), we conducted tests on independent models. The response variables were set as the proportion of area leaf consumed by chewing herbivores (leaf area consumed × 100/total leaf area); and the proportion of leaves with sucking insect damage (number of leaves with damage × 100/number of total leaves). The explanatory variables were the occurrence of predation on the stem, leaf, or both (stem + leaf; total predation) (ANODEV); and the total abundance and richness of associated ants (linear regression analysis). Data were analyzed under Negative Binomial.

Results

Change in species composition of associated ants

We collected 21 ant species associated with *T. subulata* belonging to 11 genera and four subfamilies (Table 1). The most frequent species and morphospecies considering all times of the day and all the sampled sites were *Solenopsis invicta* (occurring in 96.5% of plants), *Dorymyrmex* sp.1 (93%), and *Brachymyrmex* sp.1 (70%) (Table 1). All of the most common species considering occurrence on plants, time of day, and across sampled sites, belonged to the generalist guild (Table 1).

Ant assembly composition differed significantly across sampled sites and throughout the day (PERMANOVA, $P < 0.001$; Table SM01), and there were significant interactions between these factors (PERMANOVA, $P = 0.005$; Table SM01). The Monte Carlo test indicated that *Camponotus atriceps* and *Ca. melanoticus* were responsible for changes in the composition throughout the day (Table 2), while *Ca. leydigi*, *Cephalotes clypeatus*, *Ce. pusillus*, *Crematogaster obscurata*, *Paratrechina longicornis*, *Pseudomyrmex schuppi*, *Solenopsis invicta,* and *Wasmannia auropunctata* were responsible for changes in composition across sites (Table 2).

Ant responses to *T. subulata*-simulated herbivory and plant damage

Ants visited both stems and leaves on all plants. The proportion of visits by ants increased over the observation time (Fig. 2a–b). The percentage of visits by ants to stems were higher in the treatment containing the termite "herbivore bait" than for controls ($\chi^2 = 22.32$, $df = 180$, $P < 0.0001$; Fig. 2a). For leaves, a higher proportion of ants visited those with termite baits ($\chi^2 = 7.92$, $df = 180$, $P < 0.019$); however, there were no significant differences between the two control treatments ('tape only' and 'no-treatment') ($\chi^2 = 0.58$, $df = 179$, $P = 0.580$; Fig. 2b). In general, visits to stem baits were more frequent and faster than those for leaf baits ($\chi^2 = 44.93$, $df = 120$, $P < 0.0001$; Fig. 2c).

We observed ant attacks on a total of 59 termite baits, in which 74.6% of termite attacks by ants occurred on stems, and 25.4% occurred on leaves. The highest attack rates were observed in the 'generalist' ant guild (94.8%). *Solenopsis invicta* was the most common species, and also defended the plants most frequently. Termite baits were attacked by *S. invicta* in 35.6% of all studied plants (Table 1). Among plants with associated *S. invicta*, 70% had termite baits attacked. *Dorymyrmex* sp.1 attacked termite baits in 13.3% of the total plants and 50% of plants with which it was associated, while *Brachymyrmex* sp. did not attack termites. The proportion of attacked baits increased throughout the observation time. Ants attacked termite baits significantly more on stems than on leaves ($\chi^2 = 34.26$, $df = 118$, $P < 0.0001$; Fig. 3). The species composition of ants that attacked termite baits also differed significantly between the stems and the leaves of host plants (PERMANOVA, *pseudo* $F = 3.3227$; $P = 0.002$).

Ants also responded to signals from plant stems after mechanical injury (Fig. 4). The proportion of visits by ants to damaged plants increased over time, and it was

Table 1 Ant species and morphospecies, and their respective guilds in association with *Turnera subulata*, including occurrence throughout the day, occurrence (=number of plants found, $N = 60$) and the number of times that defense activity was observed in different host plant structures (for details see "Methods")

Species/morphospecies	Guild	Occurrence			Total occurrence	Number of defense activity		
		10–12 h	13–15 h	16–18 h		Leaf	Stem	Total
Dolichoderinae								
Dorymyrmex								
Dorymyrmex sp.1	Generalist	15	16	25	56	2	6	8
Ectatomminae								
Ectatomma								
Ectatomma brunneum	Predador and nectarivorous	3	2	2	7	0	2	2
Formicinae								
Brachymyrmex								
Brachymyrmex sp.1	Generalist	11	16	15	42	0	0	0
Camponotus								
Camponotus atriceps	Generalist	3	13	1	17	1	1	2
Camponotus blandus	Generalist	14	3	13	30	1	9	10
Camponotus crassus	Generalist	3	0	3	6	1	1	2
Camponotus leydigi	Generalist	4	2	3	9	0	1	1
Camponotus melanoticus	Generalist	1	8	0	9	0	0	0
Camponotus sp.1	Generalist	1	0	0	1	0	0	0
Paratrechina								
P. longicornis	Generalist	2	4	5	11	0	1	1
Myrmicinae								
Cardiocondyla								
Cardiocondyla emeryi	Predador	5	1	5	11	0	1	1
Cephalotes								
Cephalotes clypeatus	Pollen-feeding	1	0	1	2	0	0	0
Cephalotes pellans	Pollen-feeding	0	1	0	1	0	0	0
Cephalotes pusillus	Pollen-feeding	5	1	3	9	0	1	1
Crematogaster								
Crematogaster evallans	Generalist	1	1	1	3	0	0	0
Crematogaster obscurata	Generalist	7	8	6	21	1	5	6
Solenopsis								
Solenopsis invicta	Generalist	18	23	17	58	7	14	21
Pseudomyrmex								
Pseudomyrmex schuppi	Predador and nectarivorous	2	0	0	2	0	0	0
Pseudomyrmex simplex	Predador and nectarivorous	3	0	1	4	0	0	0
Pseudomyrmex termitarius	Predador and nectarivorous	0	0	1	1	0	0	0
Wasmannia								
Wasmannia auropunctata	Generalist	2	2	2	6	2	2	4
Total 21						15	45	60

São Cristóvão, Sergipe, Brazil. 2015

higher for stems than for leaves ($\chi^2 = 1974.59$, $df = 237$, $P = 0.001$). Damaged stems had more visits than stems without injuries ($\chi^2 = 6.98$, $df = 118$, $P = 0.008$). However, ant visited leaves with and without mechanical damage at a similar rate ($\chi^2 = 2.96$, $df = 118$, $P = 0.084$; Fig. 4).

Herbivory rates in *T. subulata* and ant defenses

The average percentage loss of leaf area was 3.37 ± 0.05% (mean ± SE), while the percentage of leaves damaged by sucking insect was on average 15.06 ± 2.61% (mean ± SE). No signs of stem herbivory were found on *T. subulata.*

The proportion of leaf area consumed and the ratio of leaves damaged by sucking insect did not correlate with ant protection of stems, leaves, or both (Table 3). The only exception was the proportion of leaf area consumed, which was significantly reduced on plants where ants defended the leaves (Table 3; Fig. 5).

Similarly, the proportion of leaf area consumed and the proportion of leaves damaged by sucking insects were not significantly correlated with the abundance and species richness of ants (Table 4).

Table 2 Ant species with a significant observed indicator value (IV), which is a measure of species occurrence (across sampled sites and throughout the day) from different samples according to the Monte Carlo test

Species	Indicator value (IV)	*P* value
Across sampled sites		
Camponotus leydigi	0.2029	0.002
Cephalotes clypeatus	0.1111	0.014
Cephalotes pusillus	0.1355	0.022
Crematogaster obscurata	0.1440	0.026
Paratrechina longicornis	0.1855	0.006
Pseudomyrme schuppi	0.1111	0.025
Solenopsis invicta	0.2093	0.008
Wasmannia auropunctata	0.1538	0.010
Throughout the day		
Camponotus atriceps	0.1657	0.002
Camponotus melanoticus	0.1185	0.005

Discussion

Our results showed that species composition of ants associated with *T. subulata* varied across sampled sites and throughout the day, and that visitation and attack rates depended on the plant structure (Figs. 2, 3, 4) and the species composition of ants associated with host plant. Herbivory rates did not correlate with timely defense by

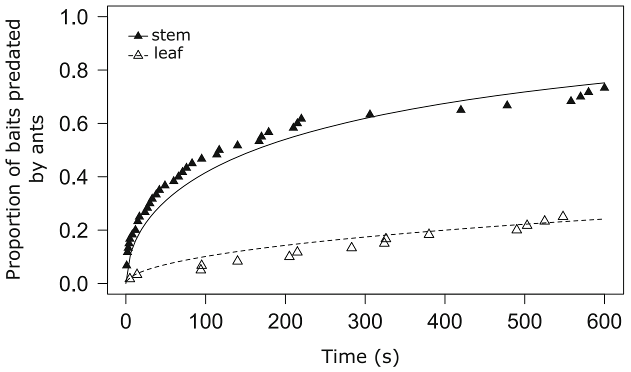

Fig. 3 Predation by ants throughout the day on *Turnera subulata* stems and leaves

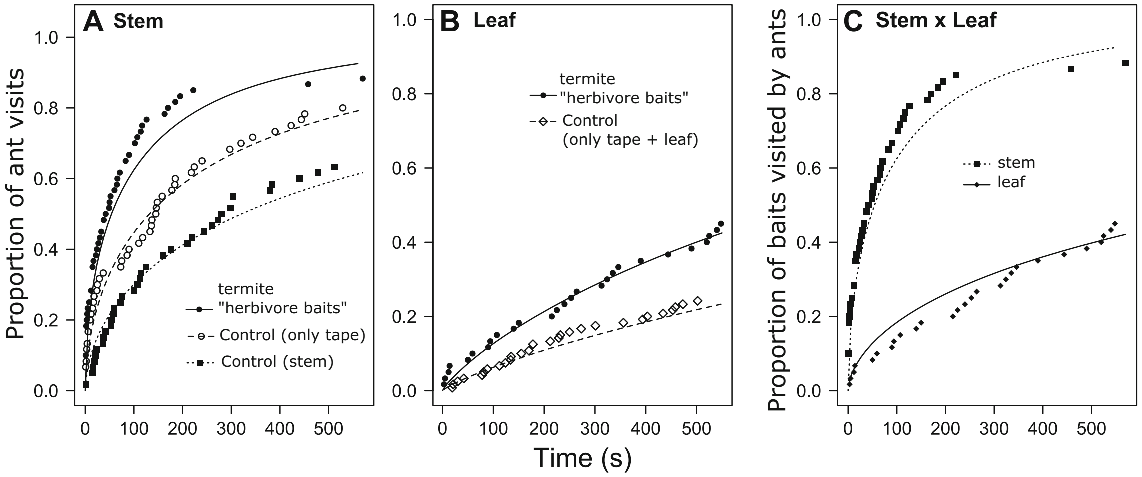

Fig. 2 Ant visits in *Turnera subulata* throughout the day. **A** Ratio of ant visits to the stem; and **B** ratio of ant visits to the leaves. Stems and leaves had the following treatments: taped termite "herbivore baits", control (only double-sided tape), and plant structure only (control: only stem or leaf). **C** Proportion of termite-baited stems and leaves visited by ants

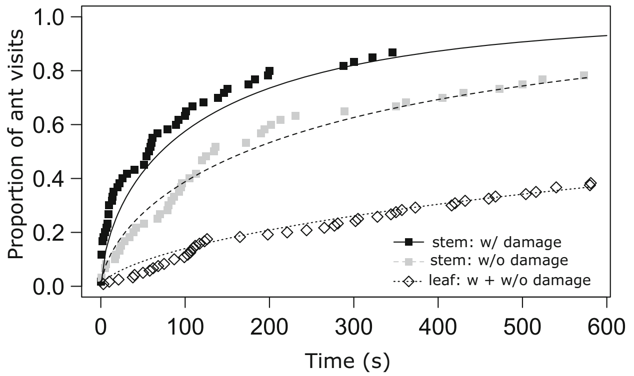

Fig. 4 Responses of ants over time to mechanical damage on *Turnera subulata steams and leaves*

ants (Table 3); however, a series of results observed here suggest that the presence of ants may have a positive effect on their host plants, since (i) most associated ants are considered potential predators; (ii) most ants carried out patrolling and defense (Table 1); and (iii) the proportion of leaf area consumed by chewing insects was lower on leaves defended by ants (Fig. 5).

In facultative mutualism interactions, the lack of shelter offered by host plants tends to produce rapid changes in the abundance and the composition of associated ants over the time (Heil and Mckey 2003). Such variation in ant assemblies have been reported to promote context dependency in these associations (Bronstein 1994; Di Gusto et al. 2001; Chamberland and Holland 2009). In the present study, the association of *T. subulata* with ants that depended on the resources offered by the plant was rare (13.6%) (e.g., predator and nectarivore guilds; Table 1), which suggests low fidelity of ant species to these plants. Our results seem to support this assumption, since the species compositions associated with the host plant varied across sampled sites and throughout the day (Table 2). Furthermore, not all species associated with *T. subulata* are efficient predators (Table 1). This apparent low associative fidelity may create temporal or spatial opportunities for attacking herbivores, resulting in an apparent lack of correlation between ant defense and herbivore damage noted here (Table 3).

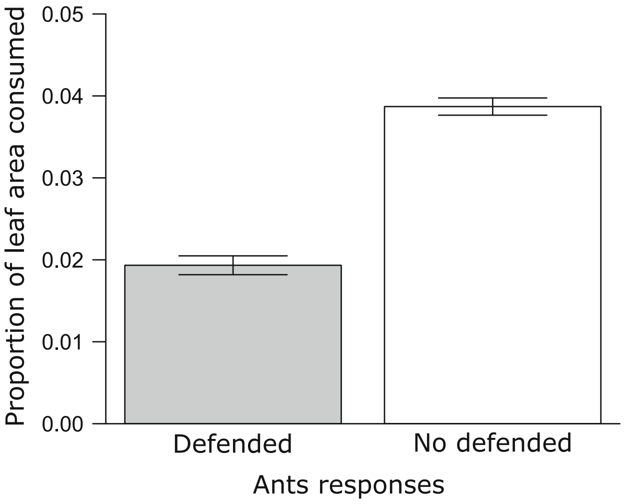

Fig. 5 Variation in proportion of leaf area consumed in *Turnera subulata* depending on ant responses

On the other hand, we must consider that associations with ants may provide other benefits to the plants in addition to reducing herbivory. Studies on *T. ulmifolia* demonstrated that its association with 25 different species of ants brings benefits to the host plant through seed

Table 3 Summary of the effects of ant defenses on stems, leaves, and both (stem + leaf; 'total predation') on herbivory rates, under the proportion of leaf area consumed and the proportion of leaves damaged by sucking insects

Term	*df*	Deviance	Resid. *df*	Resid. dev.	*F*	*P*
y = Percentage of leaf area consumed						
Null model			48	52.014		
Predation on stem	1	1.372	47	50.641	1.3724	0.241
Null model			48	55.021		
Predation on leaf	1	5.319	47	49.701	5.1399	0.021
Null model			48	605.37		
Total predation (stem + leaf)	1	10.496	47	594.88	0.8292	0.362
y = Percentage of leaves with sucking damage						
Null model			48	11.372		
Predation on stem	1	0.435	47	10.936	1.7767	0.186
Null model			48	11.372		
Predation on leaf	1	0.288	47	11.084	1.1578	0.284
Null model			48	1.6461		
Total predation (stem + leaf)	1	0.059	47	1.5863	1.7715	0.183

Each model was conducted separately

Table 4 Summary of the effects of the abundance and species richness of ants associated with *T. subulata* on herbivory rates, under the proportion of leaf area consumed and the proportion of leaves damaged by sucking insects

Term	*d.f.*	Deviance	Resid. *d.f.*	Resid. dev.	*F*	*P*
Percentage of leaf area consumed						
Null model			48	53.274		
Ants abundance	1	3.001	47	50.266	3.001	0.082
Null model			48	52.252		
Number of ants species	1	1.725	47	50.526	1.7257	0.189
Percentage of leaves with sucking damage						
Null model			48	28.204		
Ants abundance	1	0.183	47	28.021	0.1831	0.668
Null model			48	28.204		
Number of ants species	1	0.418	47	26.785	1.4189	0.233

dispersal (Cuautle et al. 2005). However, most ants associated with *T. subulata* (86.4%) belong to the potential predator guild, and two of the most common species were also more effective predators, highlighting the favorable role of ant defense for the host plant.

Ants associated with *T. subulata* visited faster and attacked intruders more on stems than those on leaves (Figs. 2, 3), which may explain the lack of herbivorous damage to the stems. Differences in ant activity between plant structures may be due to the location of EFNs; these structures are positioned on the leaf petiole base and to access them, it is not necessary for ants to walk on the leaf surfaces. In addition, ants seem to modulate their responses differently to the released signals (e.g., vibration, visual, and olfactory cues) between host plant structures. Ants responded differently to signals from the control treatments only in the stem (e.g., no-treatment and tape-only controls), while on the leaf, ants only perceived the presence of termite "herbivores baits." This suggests that vibration or kairomones released by herbivores—the only cues that were unique to the treatment-simulating herbivory—are the primary stimuli promoting leaf patrolling by ants. That is, although not actively patrolling the leaf, the ants are still able to perceive the presence of herbivores and initiate the defense. This assumption is supported by the significant reduction in the proportion of leaf area consumed by chewing insects on plant leaves defended by ants (Fig. 5). Indeed, it is widely recognized that the localization of prey by predators is facilitated by a number of cues, including those from chewing and moving herbivorous insects (*i.e.*, vibratory stimuli) (Pfannenstiel et al. 1995; Cocroft and Rodrigues 2005). This capability has been documented in *Azteca* ants, which increase patrolling with leaf vibration caused by insect intruders (Dejean et al. 2009).

Similarly, ants responded to damage signals only on the stem (Fig. 4). Several mechanisms acting alone or in combination could be responsible, including (i) the importance of the structure (stem) to the ants themselves (e.g., access to EFNs) or (ii) due to the differential responses of ant species regarding volatiles emitted by the plant. It is widely recognized that damaged plants can release volatiles as a means of indirect defense (Paré and Tumlinson 1997), as they can, for example, attract natural enemies of herbivores (Turlings et al. 1995; Kessler and Baldwin 2001). Despite the mechanisms involved, our results suggest that even though responses were stronger to stem cues, ants seem to be able to defend the leaf when herbivores are present (Fig. 2b). Future studies focusing on the mechanisms responsible for this difference in allocation of defense by plant structure may contribute to our understanding of the patterns observed here.

This is the first study describing the *T. subulata* associated ant fauna and their defensive roles for the host plant. Manipulative studies that control the presence of ants along host plant phenology may enhance our understanding of the interactions between these organisms. As mutualistic interactions can exert strong influence on communities (Rico-Gray and Oliveira 2007; Geange et al. 2011), such studies may elucidate evolutionary aspects and the communities structure under the influence of facultative ant–plant interactions.

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