Differences among ant species in plant protection are related to production of extrafloral nectar and degree of leaf herbivory

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Many studies assume that all ant species collecting extrafloral nectar defend plants against herbivores, although ant–plant interactions are facultative, generalized and have variable outcomes. With over 280 h of observations in an area of Rupestrian Grasslands (Ouro Preto, Brazil), we described the network of interactions between 2313 ants of 23 species and 200 plants of 10 species. Ants from all species were further submitted to an identical task of finding and removing a standardized herbivore surrogate (termites) to quantify the relative protection effectiveness of different ant species. We then correlated ant protection to the volume and concentration of sugar in the nectar as conditional factors and the herbivory damage as interaction outcome. We found that 11 of the 23 species of ants attacked and removed 933 of 2000 termites. All plant species interacted with effective ant protectors, although the identity of the best protector varied among plants. The degree of ant protection was positively associated with sugar concentration of nectar, which further explained the variation in leaf damage among plant species. Our study provides evidence that ant protection varies among ant species and is enhanced by the plant investment in nectar reward, resulting in less herbivory for the partner plant. We also showed that the most protecting ant species are those that are most connected and thus spread the benefit throughout the network.


INTRODUCTION

Extrafloral nectaries (EFNs) are indirect plant defences consisting of structures that secrete nectar to attract ants which then repel or kill potential herbivores (Rosumek et al., 2009; Del-Claro et al., 2016), thereby reducing herbivory and increasing seed production (Oliveira, 1997; Nascimento & Del-Claro, 2010). However, ant–plant interactions based on extrafloral nectar are often generalized and facultative (Heil & McKey, 2003; Rico-Gray & Oliveira, 2007); thus, species of plants and ants can interact with several partners at different frequencies (Lange, Dattilo & Del-Claro, 2013; Belchior, Sendoya & Del-Claro, 2016; Fagundes et al., 2016), which often alternate in space and time (Dáttilo, Guimarães & Izzo, 2013; Lange et al., 2013; Chamberlain, Bronstein & Rudgers, 2014; Anjos et al., 2017), resulting in conditional mutual benefits (Bronstein, 1994; Rosumek et al., 2009; Trager et al.,...
At a multispecific level, the asymmetric variation in the frequency of interactions between ants and plants creates a network with a nested structure (Bascompte et al., 2003). In nested networks, few ant species recruit foragers to collect nectar from most plant species, while most ant species forage on only few plants (Dáttilo, Díaz-Castelazo & Rico-Gray, 2014; Fagundes et al., 2016). Because ant species vary in foraging behaviour, recruitment effort, patrolling ability and predatory activity, these differences in the frequency of interactions with ants can result in a gradient of benefits among plant species (Lange & Del-Claro, 2014; Del-Claro et al., 2016).

Interactions involving ants and EFN-bearing plants are particularly diffuse mutualisms because nectar is easily collected by almost all tree-dwelling ant species (Law & Koptur, 1986; Blüthgen & Fiedler, 2004; Blüthgen, Stork & Fiedler, 2004), although some species possess specific adaptations such as differentiation in the structure of the proventriculus to store and regurgitate nectar (Davidson, 1997, 1998; Davidson et al., 2003). In this facultative mutualism, some ant species can defend plants against herbivores (protectors), while others collect nectar, but do not protect their host plant (opportunists, exploiters or cheaters; Bronstein, 2001; Bronstein, 2015; Del-Claro et al., 2016). In exploitative interactions, one species does not fulfil its part of the bargain, as observed for pollination (Rodriguez-Rodriguez, Jordano & Valido, 2013) and seed dispersal systems (Cuautle, Rico-Gray & Díaz-Castelazo, 2005; Schupp, Jordano & Gómez, 2010). These interactions can lead to a neutral effect of the ant interaction if the costs of nectar production are minimal, defining a commensalism, or a negative effect if costs of producing nectar are high, defining an exploitative relation (Bronstein, 2015). Despite a well-known framework where mutualistic, commensalist and exploiter partners can generate ant–plant interactions (Jordano, Bascompte & Olesen, 2003; Rico-Gray & Oliveira, 2007; Genrich et al., 2017), the proportion of species in each of these functional groups and the role of protection effectiveness of ants as a mechanism structuring the ant–plant interactions at the community level remain unclear (Bronstein, 2001, 2015).

The ability of ants in defending plants varies in terms of taxonomic identity (Del-Claro & Marquis, 2015), frequency of nectar usage (Apple & Feener, 2001; Cuautle et al., 2005), recruitment of foraging workers (Agrawal, 1998), ability to capture herbivores (Fiala et al., 1989) and environmental conditions (Chamberlain & Holland, 2009). Therefore, an ant species would be an effective protector if it recruits many workers, presents aggressive behaviour and then quickly finds, attacks and removes a large proportion of herbivores (Bronstein, 2001; Rosumek et al., 2009). Recruitment and aggressiveness naturally vary between ant species, but can be enhanced by the presence of highly energetic, predictable resources (Blüthgen et al., 2004; Grover et al., 2007; Chamberlain & Holland, 2009). Therefore, plants providing better quality nectar could accumulate more interactions with aggressive and territorial ants, reducing the frequency of commensal and exploiter species (Blüthgen et al., 2004; Koptur, Jones & Pena, 2015). Consequently, the protection effectiveness of the partner ants might be related to the plant investment in nectar production, although the protection effectiveness of the ants might be dynamic and context-dependent (Holland et al., 2005; Chamberlain & Holland, 2009). This investment in attracting effective protectors can return to the plant as reduced herbivory damage and increased reproductive output.

Variation in the interaction outcome of ant–plant interactions is ecologically plausible and easily observed, although its driving mechanisms are poorly understood. Exploring this variation in ant defence ability could help to understand the structure of this important protective mutualism. In this study, we evaluated the differences among ant species in their ability to remove potential herbivores, according to the recruiting effort and the aggressiveness of the ants, and the effect of nectar production on ant protection ability and, consequently, on the degree of herbivory protection received by the plant species. For this, we tested four hypotheses: (1) ant species vary in their recruitment effort and aggressive behaviour against herbivores, creating a gradient of protection effectiveness; (2) ant species that collect nectar from more plants are those more effective protectors; (3) the volume and concentration of the nectar affects the protection effectiveness of ant species by attracting more protective ants; (4) the degree of herbivory suffered by plants is related to the protection ability of its partner ants.

MATERIAL AND METHODS

STUDY AREA

We conducted the fieldwork at Parque Estadual do Itacolomi (PET), near Ouro Preto and Mariana municipalities (MG/Brazil), which covers an area of approximately 7000 ha of Atlantic Forest and Rupestrian Grasslands, at an elevation of 700–1770 m a.s.l. The climate is subtropical humid with a mean annual temperature of 21 °C and mean annual precipitation of 2018 mm. We conducted the experiments in a 10-ha area covered by Rupestrian Grasslands, an ecosystem composed of quartzite or iron rock outcrops surrounded by continuous grassland with small shrubs, trees and woody vegetation inserted in rock crevices or grouped large patches (Fernandes, 2016).

EXPERIMENTAL DESIGN

We marked 70 shrubs of each of 10 plant species bearing only EFNs and providing only nectar as reward
for ants. We did not detect other physical or chemical defence as well developed as the EFNs, although specific studies are needed to fully investigate this issue. We randomly split the shrubs in four experimental groups: one to sample the interactions (N = 20 per plant species); one to assess the protection ability of ants (n = 20); one to assess nectar features (n = 20) and one to assess the degree of herbivory (n = 10). We used analysis of variance (ANOVA) to compare group response to the treatments and simple linear regression (SLR) model to test the relation between explanatory and response variables. In cases in which the residuals of the analysis did not fit normal distribution, we applied Friedman ANOVA (FANOVA) as an alternative to ANOVA and Spearman correlation as an alternative to SLR. All analysis was performed in Action v. 3.1.43 package (Estatcamp), a statistical program for platform R v. 3.0.2 (R Development Team).

**Sampling ant–plant interactions**

We performed direct censuses in 20 shrubs of each plant species (N = 10) to quantify the number of workers recruited by each ant species in the early rainy season (November–December 2013) (for details, see Supporting Information S1). We constructed a network of paired ant–plant interactions using the bipartite package in R software (R Development Core Team, 2011). We calculated the nestedness index of network and tested it against a neutral model using ANINHADO software. To evaluate differences among ant species in the number of interactions with plants, we compared the number of workers recruited and shrubs visited using one FANOVA followed by a Bonferroni test for both dependent variables.

**Ant defence assay**

We experimentally quantified the protection effectiveness of ant species on each plant species, using a standardized bioassay in the mid-rainy season (January–February 2014). We used 20 shrubs of each plant species, totalling 200 samples. We placed termites (Syntermes sp.) on plants to simulate the presence of an herbivore intruder (sensu Oliveira, 1997), then we evaluated the outcome of interactions between each ant species and the termites (for details, see Supporting Information S2). This procedure allowed a standardized assessment of the protection ability of several species of ants and provided a statistically reasonable number of samples, which would be extremely difficult using natural herbivores.

Firstly, we excluded ants from the whole shrubs by manual removal and blocked the plant for 48 h. We randomly selected five different apical branches of each shrub to introduce one termite in each, as far distant as possible. After that, we removed the blockage and observed one shrub of each plant species per day, totalling 10 shrubs per day. We repeated the procedure with five new termites per shrub. After that, we blocked 10 new shrubs, one for each plant species, to reapply the experiment 48 h later. This procedure continued until we had applied the experiment to all shrubs.

In total, we sampled 2000 paired encounters between ants and termites and approximately 100 h of observation distributed over a period of 20 days. In each encounter, we measured the time spent by each ant species to forage in the plant from the moment when it climbed into the plant until the moment it finds and captured a termite. We did not consider in the analysis ants that climbed the plants and did not find the termites. We considered the outcome of the interaction as a success if ants found, captured or repelled the termite and as a failure when the ants found, but ignored the termites. When termites fell out the plant without ant interference, we simply put them back in the same place they were before fall and continued the experiment (for details, see Supporting Information S2).

We compared the protection effectiveness among the ant species (fixed factor, N = 23), using the average number of termites found, the average number of termites captured and the average time spent to find termites in each plant species (random block, N = 10). For each dependent variable, we performed a FANOVA followed by a Bonferroni test with the plant species as a random block.

**Protection effectiveness**

To evaluate the protection effectiveness of each ant species in each plant species in a multispecific level, we combined three variables of protection capability into an index of ‘protection effectiveness’ (for details, see Supporting Information S3). We used the landscape analysis of effectiveness proposed by Schupp et al. (2010) and Rodriguez-Rodriguez et al. (2013). We estimated the protection effectiveness for each ant species using the formula: \( PE = QNC \times QLC \) (for details, see Supporting Information S2). The quantitative component (QNC) is based in the number of workers an ant species recruits to a plant species. The qualitative component (QLC) is based on two parameters: the discovery time (t) and the herbivores removal rate (a), which is the percentage of termites found by an ant worker that were removed. Ant species were classified according to the values of PE (see details in Supporting Information S3). To confirm this ranking, we compared the average values of PE calculated for each plant species (random block, N = 10) among ant species (fixed factors, N = 23). We performed a FANOVA followed by a Bonferroni test.
FACTORS INFLUENCING THE PROTECTION EFFECTIVENESS

We evaluated four factors that could affect protection effectiveness of ants: (1) the volume of nectar and (2) the solute concentration of nectar to characterize the quality of nectar reward, (3) the height of the shrub and (4) the number of branches per shrub to characterize the structure of the foraging area. We used 20 shrubs of every plant species to measure these factors. We measured nectar volume and sugar concentration in 10 EFNs per shrub using a light refractometer (See details in Supporting Information S3). We compared the four predictor factors among plant species using one-way ANOVA combined with the Tukey’s test. We tested the relationship between the predictor factors and the protection effectiveness of ants using one SLR model for each factor. We tested each of the four predictor factors as explanatory factors for the variation of protection effectiveness of interacting ants among plant species. We considered each plant species as an independent case ($N = 10$). We also tested if the protection gain of each shrub varies between plant species by using a one-way ANOVA combined with a Tukey’s test. To calculate the protection received by each shrub, we calculated the sum of the number of ants foraging, the average time of forage, the average rate of termite removal and the sum of $PE$s of all ant species interacting with the respective shrub.

EFFECTS OF ANT PROTECTION EFFECTIVENESS ON HERBIVORY DAMAGE

We evaluated the relationship between the average protection effectiveness of the interacting ants and the foliar damage suffered by each plant species. We measured the herbivory damaged accumulated along 28 days by 18 leaves previously marked in 10 shrubs of each plant species. The percentage of damaged leaves (more than 50% of herbivory) per shrub ($N = 100$) was compared among plant species using one-way ANOVA with Tukey’s test.

We tested the relationship between the effectiveness of the interacting ants and the rate of herbivory suffered by the plant species by using a SLR model with logarithmic function. The general protection effectiveness for each plant species was the sum of the specific protection effectiveness of all ant species that interacted with each plant species. Each plant species was considered an independent case ($N = 10$).

RESULTS

ANT–PLANT INTERACTIONS

We recorded 89 distinct pairwise interactions, involving 2313 ants from 23 species collecting nectar from 200 shrubs of 10 plant species (Fig. 1), composing a network of interactions with nested structure (NODF = 37.4, $P = 0.01$). Ant species varied broadly in the total number of plant species with which they interacted (Table 1) and differed in the mean number of shrubs visited per plant species (Friedman: $\chi^2 = 113.3$, $P < 0.001$, d.f. = 22) (Fig. 1). The most interactive ant species were Camponotus crassus (visited 127 shrubs of 10 plant species), Ca. novo–granadensis (94; 10), Pseudomyrmex gracilis (55; 9) and Cephalotes pusillus (42; 8), which differed among themselves and with all other species (Bonferroni: $P < 0.05$); therefore, these highly interactive species were considered as the network core (Fig. 1). Among the other ant species, 13 interacted with less than 20 shrubs of 7 plant species in total and were therefore considered as the lowly interactive species of the network periphery, which did not differ among themselves in the number of interactions (Bonferroni: $P > 0.05$) (Fig. 1). The last six ant species interacted with only one shrub of one plant species in total and were considered as rarely interactive species of the network, which did not differ among themselves (Bonferroni: $P > 0.05$) (Fig. 1).

Ant species also differed regarding the mean number of workers recruited to collect nectar (Friedman: $\chi^2 = 107.9$, $P < 0.001$, d.f. = 22). The species Ca. crassus recruited the largest number of ant workers (1067 ants in total; 47% of the observed ants), followed by Ca. novo–granadensis (403; 18%), P. gracilis (230; 11%) and Ce. pusillus (227; 11%), which differed among themselves and with all other species (Bonferroni: $P < 0.05$) (Table 2). Eight other ant species recruited less than 130 workers each to collect nectar (less than 5%) and another 11 ant species recruited less than 10 workers each (less than 0.5%), which did not differ among themselves (Bonferroni: $P > 0.05$) (Table 1).

PROTECTION EFFECTIVENESS EXPERIMENT

All 2000 termites were found in all 200 shrubs by 23 ant species, but 933 termites (47% overall efficiency) were successfully removed from 163 shrubs by only 11 ant species (Table 1; Fig. 2). Ant species differed in the number of termites found (Friedman: $\chi^2 = 119.6$, $P < 0.001$, d.f. = 22) and captured (Friedman: $\chi^2 = 40.6$, $P < 0.001$, d.f. = 22), and also in the time spent to remove termites (Friedman: $\chi^2 = 93.5$, $P < 0.001$, d.f. = 22) (Table 1; Fig. 2). Camponotus crassus was the most quick and effective species in capturing termites, spending 416 min (3 min/termite) to remove 660 out of 902 termites (68%) from 127 shrubs of all plant species. Camponotus novo–granadensis spent 381 min (5 min/termite) to remove 98 out of 358 termites (25%) found in 77 shrubs of all plant species. Pseudomyrmex gracilis spent 298 min (5 min/termite) to remove 67 out of 182 termites (37%) found in 53 shrubs of 8 plant species. Cephalotes pusillus spent 209 min (4 min/termite) to remove 37 out of 242 termites (15%) found in 46 shrubs of 8 plant species. Camponotus rufipes spent 381 min (6 min/termite) to
Figure 1. Ant–plant interaction network recorded in rupestrian fields (Brazil). Rectangles at left depict plant species and at right depict ant species. Rectangle width represents the relative number of interactions of each species. Line width represents the values of protection effectiveness of each ant species for each plant species. Asterisks (*) denote the ant species that were effective in plant protection. Note that the values of $PE$, representing the protection effectiveness index (see Material and Methods), are represented as $(PE \times 10) + 1$, because some ant species had values of $PE = 0$. 

remove 38 out of 79 termites (48%) found in 15 shrubs of 3 plant species. All other 18 ant species combined spent 407 min (12 min/termite) to find 70 termites in 69 shrubs of all 10 plant species, which did not differ among themselves (Bonferroni: $P > 0.05$), but only 7 of those ant species were capable of removing 49 termites (70%) from 17 shrubs of 6 plant species (Table 1; Fig. 2).

The ant species that recruited more foragers were those who visited more shrubs (Pearson: $r = 0.98$, $P < 0.001$; $N = 23$) of a larger number of plant species (Pearson: $r = 0.91$, $P < 0.001$, $N = 23$). Those highly interactive ant species were those that found (SLR: $r^2 = 0.83$, $F_{1,22} = 103$, $P < 0.001$) and captured most termites (SLR: $r^2 = 0.76$, $F_{1,22} = 65.9$, $P < 0.001$), but were no faster than species with few interactions (SLR: $r^2 = 0.10$, $F_{1,22} = 2.1$, $P = 0.16$). Combining the recruitment effort, the time spent to find a termite and the proportion of termites removed into a protection index, we observed that ant species varied in their effectiveness to protect plants (Friedman: $\chi^2 = 42.5$, $P < 0.01$, d.f. = 10) (Fig. 2). *Camponotus crassus* was the most effective ant species ($PE = 3.4$) by recruiting several workers, quickly finding and successfully capturing most termites in nine out of ten plant species, and thus considered as high-effective protector (Table 1; Fig. 2). The species *Ca. novogranadensis* ($PE = 0.52$) and *Ce. pusillus* (0.16) quickly found termites but captured few of them in few plant species, while *P. gracilis* (0.27) and *Ca. rufipes* (0.16)

Table 1. Parameters used to evaluate interaction frequency and to calculate the protection effectiveness index for each ant species interacting with 200 plant species bearing extrafloral nectaries in rupestrian fields, Ouro Preto (Brazil)

<table>
<thead>
<tr>
<th>Ant Species</th>
<th>Recruitment of workers per plant species</th>
<th>Shrubs visited per plant species</th>
<th>Plant species interacted</th>
<th>Foraging time (min)</th>
<th>Termites found</th>
<th>Termites removed</th>
<th>Protection effectiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Camponotus crassus</em></td>
<td>1067</td>
<td>127</td>
<td>10</td>
<td>3.2 ± 1.8</td>
<td>902</td>
<td>660</td>
<td>3.34</td>
</tr>
<tr>
<td><em>Camponotus novogranadensis</em></td>
<td>403</td>
<td>94</td>
<td>10</td>
<td>4.8 ± 3.4</td>
<td>358</td>
<td>98</td>
<td>0.52</td>
</tr>
<tr>
<td><em>Pseudomyrmex gracilis</em></td>
<td>231</td>
<td>55</td>
<td>9</td>
<td>5.1 ± 2.6</td>
<td>182</td>
<td>67</td>
<td>0.15</td>
</tr>
<tr>
<td><em>Cephalotes pusillus</em></td>
<td>227</td>
<td>42</td>
<td>8</td>
<td>4.1 ± 1.9</td>
<td>242</td>
<td>37</td>
<td>0.12</td>
</tr>
<tr>
<td><em>Camponotus rufipes</em></td>
<td>139</td>
<td>17</td>
<td>5</td>
<td>6.2 ± 3.1</td>
<td>79</td>
<td>38</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Camponotus melanoticus</em></td>
<td>59</td>
<td>14</td>
<td>3</td>
<td>4.4 ± 1.6</td>
<td>36</td>
<td>15</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Crematogaster</em> sp.3</td>
<td>16</td>
<td>5</td>
<td>5</td>
<td>3 ± 2</td>
<td>38</td>
<td>14</td>
<td>0.02</td>
</tr>
<tr>
<td><em>Pheidole</em> sp.2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2.8 ± 1.6</td>
<td>5</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td><em>Crematogaster</em> sp.1</td>
<td>14</td>
<td>1</td>
<td>4</td>
<td>3.6 ± 3.1</td>
<td>12</td>
<td>8</td>
<td>0.006</td>
</tr>
<tr>
<td><em>Pseudomyrmex</em> termitarius</td>
<td>21</td>
<td>10</td>
<td>6</td>
<td>4.4 ± 2.9</td>
<td>23</td>
<td>6</td>
<td>0.002</td>
</tr>
<tr>
<td><em>Pseudomyrmex pallidus</em></td>
<td>30</td>
<td>13</td>
<td>5</td>
<td>8.2 ± 3.8</td>
<td>22</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td><em>Camponotus senex</em></td>
<td>32</td>
<td>8</td>
<td>3</td>
<td>4.6 ± 5.1</td>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Linepithema</em> sp.1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>12.5 ± 1.7</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pheidole</em> sp.1</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>7.8 ± 3.6</td>
<td>14</td>
<td>0</td>
<td>0</td>
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<tr>
<td><em>Brachymyrmex heeri</em></td>
<td>31</td>
<td>7</td>
<td>5</td>
<td>7.4 ± 3.3</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Crematogaster</em> sp.2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>8.4 ± 0.9</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Dorymyrmex brunneus</em></td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>12.8 ± 1.2</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Wasmannia</em> sp.1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>5.4 ± 0.4</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Linepithema</em> micans</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>6.2 ± 0.8</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Camponotus fastigatus</em></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5.6 ± 1.3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Gnamptogenys sulcata</em></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>6 ± 0.9</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Brachymyrmex pictus</em></td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>2.3</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td><em>Camponotus atriceps</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>10.2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The first three parameters and the following four parameters were collected in different experiments (see Material and Methods). The protections effectiveness is as index combining the recruitment effort, the foraging time and the rate or termite removal (see Material and Methods).
forage slowly and found few termites but captured most of them in few plant species (Bonferroni: \( P < 0.05 \)). These four species were considered as low-effective protectors. The other six ant species that had a mean \( PE \) value lower than 0.02 forage very slowly and capturing few termites in few shrubs of less than two plant species (Table 1), and were considered as ineffective protectors. The other 12 ant species that showed a null \( PE \) value by foraging very slowly and ignoring all termites found were considered as non-protectors.

**Factors influencing the protection effectiveness of ant species**

In each shrub, ants spent 4.2 ± 2.1 min to capture an average of 46 ± 34% of the termites. The average time of forage (ANOVA: \( F_{9,190} = 7.7, P < 0.01 \)) and the average rate of capturing termites (ANOVA: \( F_{9,190} = 25.1, P < 0.01 \)) varied between ant assemblages depending on the plant species with which they interacted (Table 2). Combining the \( PE \) values of all ant species interacting with a plant species, we observed that ants exhibited a different protection effectiveness depending on the plant species with which they interacted (ANOVA: \( F_{9,190} = 4.2, P < 0.01 \); Table 2; Fig. 3). Ants presented the highest \( PE \) values in *Stachytarpheta glabra* and *Banisteriopsis campestris* and the lowest in *Chamaecrista desvauxii*, *Peixotoa tomentosa* and *Senna pendula*, which differed among themselves and with all other species (Tukey: \( P < 0.05 \)) (Table 2; Fig. 3). Considering the protection effectiveness presented by each ant species separately, we observed that *Ca. crassus*, *Ca. novogranadensis* and *P. gracilis* had the highest \( PE \) values among all ant species in almost all plant species (Fig. 3). However, *Ca. crassus* was the most effective protector of eight plant species, while *Ca. novogranadensis* was the most effective protector in the other two plant species, in one of them along with *P. gracilis* (Fig. 3).

Plant species differed in height (ANOVA: \( F_{9,190} = 13.3, P < 0.01 \)), number of branches (ANOVA: \( F_{9,190} = 9.5, P < 0.01 \)), volume of nectar produced (ANOVA: \( F_{9,190} = 20.6, P < 0.01 \)) and concentration of sugar in the nectar (ANOVA: \( F_{9,190} = 96.1, P < 0.01 \)) (Table 2). *Banisteriopsis malifolia* was the tallest and most branched species. *Crotalaria micans* produced the most voluminous nectar, but with low sugar concentration. The sugar concentration of the nectar (SLR: \( r^2 = 0.82; F_{1,8} = 17.7, P = 0.003 \)) explained the variation between plant species in the protection effectiveness of interacting ants (Fig. 4a). However, the volume of nectar (SLR: \( r^2 = 0.001; F_{1,8} = 0.04, P = 0.9 \)), plant height (SLR: \( r^2 = 0.01; F_{1,8} = 0.01, P = 0.94 \) and

### Table 2. Characteristics of plant morphology (height and branches) and resource quality (nectar volume and sugar concentration) as predictor factors of the protection effectiveness of the interacting ant species

<table>
<thead>
<tr>
<th>Plant species Codes</th>
<th>Plant volume (m³)</th>
<th>Branches per plant</th>
<th>Sugar concentration (mg/µL)</th>
<th>Nectar volume (µL/plant/24 h)</th>
<th>Protection effectiveness</th>
<th>Herbivory rate (% leaf damage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banisteriopsis campestris</td>
<td>BAC 0.23 ± 0.23</td>
<td>13.8 ± 8.2</td>
<td>0.43 ± 0.12</td>
<td>0.02 ± 0.01</td>
<td>6.21</td>
<td>2.8 ± 3.9</td>
</tr>
<tr>
<td>Banisteriopsis malifolia</td>
<td>BAM 0.19 ± 0.19</td>
<td>82.2 ± 67.26</td>
<td>0.08 ± 0.04</td>
<td>0.10 ± 0.06</td>
<td>3.88</td>
<td>7.2 ± 5.9</td>
</tr>
<tr>
<td>Chamaecrista desvauxii</td>
<td>CHD 0.10 ± 0.05</td>
<td>28.9 ± 17.78</td>
<td>0.12 ± 0.06</td>
<td>0.15 ± 0.1</td>
<td>0.38</td>
<td>22.7 ± 9.6</td>
</tr>
<tr>
<td>Chamaecrista mucronata</td>
<td>CHM 0.14 ± 0.09</td>
<td>73.15 ± 43.04</td>
<td>0.39 ± 0.2</td>
<td>0.08 ± 0.07</td>
<td>3.05</td>
<td>24.4 ± 21.1</td>
</tr>
<tr>
<td>Crotalaria micans</td>
<td>CRM 0.09 ± 0.07</td>
<td>42 ± 43.43</td>
<td>0.38 ± 0.1</td>
<td>0.77 ± 0.69</td>
<td>2.46</td>
<td>14.4 ± 12.1</td>
</tr>
<tr>
<td>Heteropterys umbelatta</td>
<td>HEU 0.14 ± 0.12</td>
<td>33.4 ± 16.37</td>
<td>0.63 ± 0.23</td>
<td>0.40 ± 0.42</td>
<td>4.93</td>
<td>8.3 ± 8.3</td>
</tr>
<tr>
<td>Peixotoa tomentosa</td>
<td>PET 0.54 ± 0.39</td>
<td>84.25 ± 58.87</td>
<td>0.12 ± 0.05</td>
<td>0.06 ± 0.02</td>
<td>0.77</td>
<td>16.7 ± 12.8</td>
</tr>
<tr>
<td>Senna pendula</td>
<td>SEP 0.10 ± 0.08</td>
<td>56.3 ± 38.35</td>
<td>0.14 ± 0.08</td>
<td>0.06 ± 0.03</td>
<td>0.12</td>
<td>22.2 ± 25.6</td>
</tr>
<tr>
<td>Senna reniformis</td>
<td>SER 0.52 ± 0.34</td>
<td>41.2 ± 30.61</td>
<td>0.24 ± 0.09</td>
<td>0.08 ± 0.03</td>
<td>2.41</td>
<td>4.4 ± 8.1</td>
</tr>
<tr>
<td>Stachytarpheta glabra</td>
<td>STG 0.07 ± 0.07</td>
<td>57 ± 67.56</td>
<td>1.03 ± 0.05</td>
<td>0.29 ± 0.19</td>
<td>9.20</td>
<td>12.8 ± 17.2</td>
</tr>
</tbody>
</table>

Values are mean and SD. Protection effectiveness is the sum of the protection index of all ant species interacted with the plant species. Herbivory is the percentage of leaf damaged per shrub.
the number of branches (SLR: $r^2 = 0.03; F_{1,8} = 0.22, P = 0.64$) had no effect on protection effectiveness (Fig. 4b–d).

**Effects of Ant Protection on Herbivory Damage**

Plant species had an average $14 \pm 12\%$ of leaves damaged by herbivory ($N = 100$ shrubs; 1800 leaves). The rate of foliar damage differed between plants species (ANOVA: $F_{9,90} = 3.1, P = 0.003$) (Table 2; Fig. 5a). The leaves of *Ch. desvauxii* and *Ch. mucronata* were the most damaged, while the leaves of *S. reniformis* and *B. campestris* were the least damaged (Table 2; Fig. 5a). Mostly important, the rate of foliar damage suffered by a plant species responded negatively to the increase in protection effectiveness of the ant partners (SLR: $r^2 = 0.43; t_{1,8} = -2.5, P = 0.04$) (Fig. 5b).

**Discussion**

In this study, we observed that ant species differed in the frequency of interactions with plants and, more importantly, in their protection ability against plant intruders of small size, soft tegument and passive behaviour (simulated by the termites). In total, 11 out of the 23 ant species (40%) showed some potential in protecting plants against intruder herbivores. *Camponotus crassus*, *Ca. novogranadensis* and *P. gracilis* were the most protective ant species. These species are commonly observed interacting with EFN-bearing plants in Rupestrian Grasslands (Viana-Silva & Jacobi, 2012; Fagundes et al., 2016; Fernandes, 2016), Cerrado (Lange & Del-Claro, 2014; Belchior et al., 2016), Caatinga (Neves et al., 2010; Leal, Andersen & Leal, 2015) and Mangrove (Cogni & Freitas, 2002), suggesting that similar interactions occur in other systems. Of these species, *C. crassus* was the most important.
effective protector and six times more effective than the other 10 protector species. Twelve ant species ignored all encountered termites and did not offer any protection for plants. The low proportion of protector ant species (48%) and the low overall effectiveness of protection (47% of herbivore removed), presented by ants collecting nectar from plants, reflect the facultative nature of these mutualism, which, in some paired interactions, can be classified as mutualism, commensalism or exploitation (Bronstein, 2015).

Ant species removed the intruders by different mechanisms, suggesting four functional groups. Some species presented high recruitment and quickly found and removed most of the intruders of almost all plant species, being classified as high-effective protectors. Some species foraged intensively, quickly found and removed intruders, but only in some of the plants, which classifies them as low-effective protectors. Other ant species recruited few workers, found few intruders, removed or ignored them, and were therefore classified as ineffective protectors. The last group included ant species that did not find intruders and were classified as non-protectors. Broad variation in partner protection behaviour and effectiveness is common in generalized mutualisms including pollination, seed dispersal and plant root microbiomes (Schupp et al., 2010; Orona-Tamayo & Heil, 2013; Rodríguez-Rodríguez et al., 2013; Genrich et al., 2017), and can affect the outcomes of the interactions (Ruggera et al., 2016), as shown by our results. We admit that some specific protective behaviours were underestimated by our experiment, and some ant species might be highly effective against true herbivores (Alves-Silva et al., 2014; Alves-Silva & Del-Claro, 2014, 2015). We encourage studies with other types of simulators, or even true herbivores, to comprehensively assess the defence capabilities of these ants.

We observed a relation between the network structure and the protection effectiveness of the ants. The most interactive species of the network core were the best protectors and could extend the protection for all plant species; they are therefore key elements in the structure and stability of the network (Mello et al., 2015). In ant–plant networks, those species are...
generalist partners and perform facultative and weak interactions (Blüthgen et al., 2004; Belchior et al., 2016; Fagundes et al., 2016). However, interactions with various ant species in a generalized way may have four main advantages. First, different ant species that handle herbivores can increase defence success (Alves-Silva et al., 2014; Del-Claro & Marquis, 2015). For example, Ca. crassus defended plants with bites and spraying acid, while P. gracilis hunted and stung preys. Second, many herbivores are specialists in avoiding ant predation and only ant species with specific foraging behaviour are effective defenders against those herbivores (Trager et al., 2010; Pereira & Trigo, 2013). For example, P. pallidus and P. termittarius captured intruders hidden in flowers and leaf sprouts. Third, some ‘backup species’ (or redundant species, sensu Lockwood & Pimm, 1994) can replace absent protector species under different contexts (Mello et al., 2015), which could also be observed in this study. In plants where Ca. crassus showed low protection effectiveness, Ca. novograndensis was the main protector. And fourth, complementary species can interact to enhance overall protection in a synergic way (Zamora, 2000). In most plants studied here, at least three species were necessary to remove much of the intruders. Nevertheless, rather than discriminating mutualists, commensalists or exploiter species, our results reinforce the importance of the whole interacting ant assemblage in guarantying the mutualistic positive outcomes of the interactions as an emergent property of the multispecific network (Del-Claro & Marquis, 2015).

The novelty of our study is that we used a standardized experiment to show, at a multispecific level, that interaction effectiveness varied between partner species when considering a similar task. Additionally, this experiment revealed that the number of plants visited (interaction frequency), the effort in forager recruitment (patrolling intensity) and the rate of intruder removal (aggressiveness), but not the time spent foraging in the plant (foraging time), are important components of ant foraging behaviour and regulate protection effectiveness of ant species. Recruitment of several workers to most plants increases the probability of finding herbivores and aggressive, predatory behaviour increases the success of herbivore removal (Rosumek et al., 2009; Trager et al., 2010; Del-Claro & Marquis, 2015). In fact, the protection of plants is generally higher when a dominant, highly interactive ant species is involved (Del-Claro & Marquis, 2015). In our study, Ca. crassus was the most interactive and protective species; it is also considered as dominant species in the Rupestrian Grasslands (Costa et al., 2015; Fagundes et al., 2016) and Cerrado (Lange & Del-Claro, 2014; Belchior et al., 2016; Del-Caro & Marquis, 2015). Therefore, plant species interacting with numerical and behavioural dominant ant species may be key to the success of this mutualism.

Our results indicate that sugar-rich nectar attracts more aggressive ant species, as well as more workers of aggressive species were recruited, and increases aggressiveness. Aggressive species varied in aggressiveness between plant species and showed higher aggressiveness in plants secreting sugar-rich nectar. These results support the relation between the quality of the reward and the effectiveness of the mutualist partner (Koptur, 2005). Intense recruitment and predation rate, the two most important components of
ant protection effectiveness, are intrinsic characteristics of ant species, but can also be enhanced when an ant species dominates and exploits a large amount of energetic resources, such as nectar (Davidson, 1998; Davidson et al., 2003; Koptur, 2005). Plant species can increase ant patrolling by producing more EFNS (Fagundes et al., 2016) and by enhancing the quantity and quality of nectar (Davidson et al., 2003; Blüthgen & Fiedler, 2004; Chamberlain et al., 2010). The relationship between sugar concentration/composition and ant protection also exists for ant-tended aphids (e.g. Fischer, Voelkl & Hoffmann, 2005). However, future studies should investigate the effects of variation in the quantity and quality of nectar for the effective protection of ants, since, in addition to differences between species of plants, nectar may vary with plant growth, time of the day, seasonality and degree of herbivory (Heil et al., 2000; Heil & McKey, 2003).

Plants that interacted with highly effective ants were those which suffered lower rates of herbivory, indicating that plants benefit from the attraction of highly protective ant species. Our results are in line with recent reviews which have shown that the number of species protecting the plants negatively affects the amount of herbivory suffered by plants, while ant aggressiveness positively affects plant reproductive success (Rosumek et al., 2009; Trager et al., 2010; Del-Claro & Marquis, 2015). The benefit received by the plant in reduced herbivory is often related to the benefit in reproduction (Schmitz, Hambäck & Beckerman, 2000; Trager et al., 2010), so there might be an adaptive advantage in producing nectar more concentrated in sugar and in attracting more effective protector ants. However, high recruitment and aggressiveness against intruders can also reduce the reproductive success of plants when ants attack or prey on pollinators and other predators of herbivores. Termite was used as surrogate for herbivores, but it can simulate any intruder, including pollinators and herbivore predators like wasps and spiders (Gaume, Zacharias & Borges, 2005). It is also important to investigate if plants producing low attractive nectar may also rely in other defence mechanisms. We did not notice any prominent defence structure but EFNs in the studied plants, but chemical substances could be involved and future studies should address this issue, since trade-off between defence types are common (Koricheva, Nykanen & Gianoli, 2004).

In conclusion, this study empirically demonstrates, through a standardized field experiment, the variable degree of plant protection provided by different ant species at a multispecific level. Our results provide evidence that studies based solely on observation of ants collecting nectar without evaluating the protection ability of the ants could be overestimating positive interactions. We also underline the necessity of species-level, or even individual-level, approaches (Tur, Olesen & Traveset, 2015; Genrich et al., 2017) to better understand the variation in the outcomes of ant–plant interactions. Our results also stimulate studies to consider the variation in partner attributes, such as ant behaviour and plant investment in nectar. Therefore, considering the whole ant–plant network as mutualistic, or even classifying each paired interaction as positive, negative or neutral, might be overly simplifying the dynamical and conditional nature of these facultative interactions. In summary, our findings indicate three important factors regulating the protection effectiveness of ant species: (1) the quantity of ant workers and species interacting with plants, (2) the capability of ant species to remove intruders and (3) the quality of the nectar reward offered by the plant. We suggest that future studies investigate the correspondence of our findings with cases involving actual herbivores, especially those highly abundant or specialized, which cause serious plant damage. Finally, we reaffirm the importance of empirical studies on the natural history of interacting species to better understand the complexity of ant–plant interaction networks.

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REFERENCES


**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

S1. Detailing on sampling ant–plant interactions.

S2. Detailing on ant defence assay.

S3. Detailing on nectar sampling.