Oecologia (2018) 186:691–701
https://doi.org/10.1007/s00442-017-4045-7

PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Indirect effects of mutualism: ant–treehopper associations deter pollinators and reduce reproduction in a tropical shrub

Javier Ibarra-Isassi[1,2] · **Paulo S. Oliveira**[1]

Received: 22 September 2017 / Accepted: 10 December 2017 / Published online: 15 December 2017

Abstract

Animal-pollinated plants can be susceptible to changes in pollinator availability. Honeydew-producing treehoppers frequently occur on inflorescences, potentially enhancing ant-mediated negative effects on pollination services. However, the effect of ant-attended, honeydew-producing insects on plant reproduction remains uncertain. We recorded the abundance of treehoppers and ants on *Byrsonima intermedia* (Malpighiaceae), and monitored floral visitors in a Brazilian cerrado savanna. We manipulated the presence of ants and ant–treehopper associations on inflorescences to assess their effect on pollination and fruit formation. We used dried ants pinned to inflorescences to evaluate the effect of ant presence and ant identity on potential pollinators. Results show that the presence of treehoppers increases ant abundance on flowers and disrupts pollination by oil-collecting bees, decreasing the frequency and duration of floral visits and reducing fruit and seed set. Treehopper herbivory has no direct effect on fruit or seed production, which are independent of treehopper density. Pinned ants promote avoidance by floral visitors, reducing the number of visits. Ant identity mediates visitation decisions, with *Ectatomma brunneum* causing greater avoidance by floral visitors than *Camponotus rufipes*. Field videos show that pollinating bees are harassed by ants near flowers, prompting avoidance behavior by the bees. This is the first demonstration of indirect effects by honeydew-gathering ants, via disrupted pollination, on plant reproduction in tropical cerrado savanna. Our results highlight the importance of studying other interactions near flowers, in addition to just observing pollinators, for a proper understanding of plant reproduction.

Keywords Ant–plant–herbivore interaction · *Byrsonima* · Cerrado savanna · Oil-collecting bees · Flower avoidance behavior

Introduction

Arthropods and flowering plants are among the most abundant organisms in terrestrial landscapes and their life histories are bound to cross in many environments (Price et al. 2011). The relationships between plants and pollinators may be one of the most ecologically important types of animal–plant interactions, and about 87% of flowering plant species are animal pollinated (Ollerton et al. 2011). In addition to anthropogenic disturbance that can jeopardize pollination services, the presence of other organisms near flowers and their interactions with potential pollinators can also affect the final outcome of flower visitation (Romero et al. 2011). Thus, the study of ecological factors affecting animal pollination is becoming increasingly important.

Plant species have different degrees of flexibility regarding pollinators, being able to be pollinated by a single or by several species (Waser et al. 1996; Alarcón et al. 2008). In highly diverse communities, however, negative interactions between floral visitors may disrupt pollination services (Alarcón et al. 2008). The relationship between plants and low-efficiency pollinators can vary from commensalism to antagonism (Lau and Galloway 2004). In addition, these negative interactions involving floral visitors include

---

Communicated by Susan Whitehead.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00442-017-4045-7) contains supplementary material, which is available to authorized users.

✉ Paulo S. Oliveira
pso@unicamp.br

[1] Departamento de Biologia Animal, Universidade Estadual de Campinas, Campinas, SP 13083-862, Brazil

[2] Present Address: Department of Biology, Concordia University, Montreal, QC H4B-1R6, Canada

predation by arthropods such as wasps and spiders, which can affect pollination services (and in turn fruit formation) by harassing pollinators and prompting flower avoidance behavior (Romero et al. 2011). The interaction between mutualisms occurring near inflorescences (such as ants–treehoppers and pollinators–flowers) and their effect on plant reproduction remain understudied (Lach 2007; Styrsky and Eubanks 2010; LeVan and Holway 2015).

Although ants are often observed visiting flowers in a variety of environments, the outcomes of these interactions are frequently reported as negative for the flowers, rarely resulting in effective pollination (Beattie 2007; Rico-Gray and Oliveira 2007). Furthermore, floral visitation by ants can result in reduced pollen viability when pollen grains come into contact with antibiotic substances on ant integuments (Beattie et al. 1984; Dutton and Frederickson 2012). Ants may also cause damage to reproductive structures (Palmer et al. 2010), harassment of floral visitors (Tsuji et al. 2004; Ness 2006), avoidance or alteration of floral visitation behavior in response to olfactory (Li et al. 2014) and visual cues (Cembrowski et al. 2014), and depletion or modification of floral resources (Lach 2008; de Vega and Herrera 2013). However, floral visitation by ants may also result in positive outcomes for the plant if the ants can deter less effective pollinators (Gonzálvez et al. 2013), floral herbivores (Oliveira 1997; Bleil et al. 2011), or even pollinate the flowers (see review in de Vega and Gómez 2014). Although floral visitation by ants is pervasive in almost every terrestrial environment, few studies have assessed how ant–flower interactions can ultimately affect plant reproduction (Hanna et al. 2015; LeVan and Holway 2015). Moreover, the importance of the identity of the ant species as a factor affecting plant reproduction remains uncertain (see Ness 2006).

Given that the presence of honeydew-producing insects (i.e., trophobionts) is known to increase ant visitation to plants (Del-Claro and Oliveira 1996), floral visitation by ants can be enhanced by honeydew-producing insects that feed near flowers. Yet, the ecological consequences for the plant remain unclear (Styrsky and Eubanks 2007). Ants tending honeydew-producing insects often drive off other insects (predators, parasitoids, other herbivores, etc.), causing a positive effect on the trophobionts as well as an indirect positive effect on the plant by reducing herbivore damage (Oliveira and Del-Claro 2005; Pringle et al. 2011). Even though ants tending honeydew-producing insects may be distracted from foraging any floral resource (e.g., nectar), ants are commonly found on inflorescences (Lach 2007). Moreover, and despite the ubiquity of ant-mediated interspecific interactions near flowers in many terrestrial ecosystems, few studies have experimentally assessed the effects of ant–trophobiont associations on pollinators and their consequences for plant reproduction. Previous work focusing on ant–aphid associations demonstrated that the interaction between red fire ants (*Solenopsis invicta*) and cotton aphids (*Aphis gossypii*) may enhance cotton plant (*Gossypium hirsutum*) reproduction by reducing non-aphid herbivore damage (Styrsky and Eubanks 2010). In contrast, LeVan and Holway (2015) demonstrated that ant–aphid interactions negatively affect cotton plant reproduction, because the ants harass other floral visitors.

Here, we investigate if associations between ants and honeydew-producing treehoppers (Membracidae) on inflorescences of *Byrsonima intermedia* (Malpighiaceae) affect bee pollination (Fig. 1) and plant reproduction in a reserve of cerrado savanna in southeast Brazil. We manipulated the presence/absence of ants and treehoppers to determine their effect on the behavior of bee pollinators, as well as on the reproductive output of the plant. Specifically, our observations under natural conditions and experimental manipulations assessed (1) how the presence of treehoppers affects ant abundance near flowers of *B. intermedia*; (2) how ant presence near the flowers and ant species identity affect visitation behavior of potential pollinators (evaluated as duration of visits and number of approaches to flowers); and (3) how ant–treehopper associations affect fruit and seed set.

Materials and methods

Study area and system

Fieldwork was carried out at the cerrado reserve of the Estação Ecológica de Itirapina (22°14′43″S, 47°53′23″W), in the state of São Paulo, southeast Brazil. The vegetation physiognomy corresponds to a cerrado sensu stricto, characterized by a dense scrub of shrubs and trees in an herbaceous matrix (Oliveira-Filho and Ratter 2002). The climate of the

Fig. 1 a Oil-collecting bee, *Centris varia*, approaches an inflorescence of *Byrsonima intermedia* occupied by workers of *Camponotus rufipes*, which **b** attend nymphs of honeydew-producing *Amastris undulata* treehoppers. **c** Bees pollinate the flowers of *B. intermedia* while collecting oil at the calyx glands (arrow). Photo credits: J. Ibarra-Isassi (a, b); H. Soares Jr (c)

region is characterized by a rainy/warm season from November to March (mean temperature 24.8 °C and mean rainfall 1128 mm) and a dry/cold season from May to September (mean temperature 17.8 °C and mean rainfall 331 mm; Zanchetta et al. 2006).

In January 2015, we tagged 60 shrubs of *Byrsonima intermedia* (≥ 1.5 m from one another), where we had observed ants and/or treehoppers within 80 ha of cerrado containing nearly 200 shrubs of this species. For each plant, we recorded the phenological state, height, number of inflorescences, the presence of treehoppers, and the presence of ants (see below). *Byrsonima intermedia* is a shrub (0.4–1.8 m tall) with terminal, racemose inflorescences, and hermaphroditic, zygomorphic flowers with five pairs of elaiophores (oil-producing glands on the calyx; Fig. 1c). Flowers are diurnal, last one day and anthesis is variable: some flowers open around 9:00 h, while others may delay opening until around 15:00 h (Oliveira et al. 2007, Boas et al. 2013). Flowering lasts 9 months (August–April) with a peak in the beginning of the rainy season; fruiting lasts 8–9 months (Boas et al. 2013). The ovary is ovate, superior, tricarpellate, and trilocular, with one ovule per locule, which translates into three seeds per fruit formed (Souto and Oliveira 2005). *Byrsonima* shrubs are self-incompatible plants, although *B. intermedia* has been reported to have facultative self-compatibility (Boas et al. 2013), and even autocompatibility (Oliveira et al. 2007). Most *Byrsonima* species depend on pollinators, since no fruit formation occurs after self-pollination (Boas et al. 2013). Visitors to *Byrsonima* flowers collect pollen from anthers and/or oil produced by the elaiophores, but only a few visitor species exhibit a buzz-pollination behavior specialized for foraging on this type of flower (Fig. 1a; video Online Resource 1; Sigrist and Sazima 2004; Boas et al. 2013). Some bees (*Bombus* sp., *Centris varia*, *Epicaris flava,* and *Xylocopa ordinaria*) make quick visits (2–4 s) and collect pollen by vibration or buzz pollination (further details in Sigrist and Sazima 2004; Oliveira et al. 2007; Boas et al. 2013).

Effect of treehoppers on ant abundance

We carried out observations and manipulative experiments in the field to investigate the effect of the presence of honeydew-producing treehoppers on ant abundance, as well as the effect of ant–treehopper associations on flower visitors of *B. intermedia* shrubs (Fig. 1). In the cerrado, treehoppers are commonly tended by ants that harvest their carbohydrate-rich honeydew; in return, the ants may reduce treehopper mortality due to predators and parasitoids (Lopes 1995; Del-Claro and Oliveira 2000). If unattended by ants, treehopper aggregations may flick honeydew droplets to the ground to attract ants, which climb onto the plant and start tending activities (Del-Claro and Oliveira 1996). From January to April 2015, during sunny days, we monitored weekly treehopper and ant abundances on each of 30 individuals of *B. intermedia* (chosen randomly from the previously 60 tagged individuals), between 8:00 and 18:00 h, for 5 min per plant. The order and period in which the plant individuals were observed was randomized every observation session. We recorded the species and the abundance of ants and treehoppers (nymphs and adults) on each plant. As a measure of ant activity, we used time-averaged estimates equivalent to the mean abundance of ants per inflorescence per plant across weekly observation periods.

We used generalized linear models (GLM) to test the effect of the mean number of treehoppers on ant abundance, and the effect of ant–treehopper interactions on the number and duration of visits by floral visitors of *B. intermedia* (see below). We assumed a Poisson error distribution, since it is appropriate for count data showing a positive skew (Quinn and Keough 2002). In addition, we controlled for overdispersion and used log link function and Laplace approximation and maximum likelihood methods for parameter estimation. As a measure of model fit, we calculated marginal and conditional R^2 (Nakagawa and Schielzeth 2013). All statistical analyses were performed in R 3.0.2 (R Core Team 2013) using the packages 'lme4' (Bates et al. 2015) and 'MuMIn' (Barton 2016), respectively.

Effect of ant–treehopper associations on floral visitors

Ness (2006) suggests that frequency of floral visitation by bees varies depending on the ant species present on the inflorescence. Bees use several signal cues that influence their decision of visiting or avoiding the flower. When ants are present, their smell and chemical trails may affect bee decisions (Junker et al. 2007; Sidhu and Wilson-Rankin 2016). However, *Bombus* bees (Apidae and Bombini) can learn through experience that the smell of the ant can be ignored and that visual cues are more important (Ballantyne and Willmer 2012).

To record the assemblage of winged floral visitors to *B. intermedia*, 15 flowering individuals were observed (chosen randomly from the initial 60 marked plants). We counted the total number of floral visitors and the duration of visits for 30 min throughout the day. Plants were randomly chosen during each observation period. Daily field observations were carried out at 08:00, 10:00, 12:00, 14:00, 16:00, and 18:00 h (6 daily observation events for 5 days and totaling 900 min of observation). Any insect contacting the flower for at least 0.5 s was considered a floral visitor.

We used a GLMM (assuming a Poisson distribution, log link function, and maximum likelihood for parameter estimation) to analyze the relationship between ant abundance on the inflorescence and winged insect visitation frequency and duration while also considering the plant as a random effect. We considered each visit as a unit for analysis ($N = 51$). We did not include observations that had no visitation in our analysis. We calculated marginal and conditional R^2 (Nakagawa and Schielzeth 2013) as a measure of model fit. The statistical analyses were performed using the packages 'lme4' (Bates et al. 2015) and 'MuMIn' (Barton 2016), in R 3.0.2 (R Core Team 2013).

To estimate the relative effect of natural encounters between floral visitors and ant species commonly found on *B. intermedia*, we took an experimental approach. Ant occupation of inflorescences was simulated by pinning dead, recently-captured ants to inflorescences (following Sendoya et al. 2009; ants were killed by pinching and stabbing the thorax with pin). We pinned dead workers of *Camponotus rufipes* (~ 0.7 cm; Formicinae) and *Ectatomma brunneum* (~ 1.0 cm; Ectatomminae), because these two species are morphologically and behaviorally distinct from one another, and are commonly found foraging on the inflorescences of *B. intermedia* (see below). We selected ten plants with similar heights (~ 0.8–1.2 m) and numbers of inflorescences (12–15), and similar numbers of recently opened flowers (5–8); no treehoppers or other sap-sucking herbivores were present. We randomly selected four inflorescences on each plant and assigned each to one of the following treatments: (1) "No ants"; (2) "1 Pin" (by itself as a control); (3) "1 Pinned worker of *Camponotus rufipes*"; and (4) "1 Pinned worker of *Ectatomma brunneum*". All other inflorescences were clipped off to induce prospective visitation to experimental flowers. Ant access to all inflorescences was prevented by applying a sticky barrier of tanglefoot resin (Tanglefoot Co., Grand Rapids, Michigan) at the base of inflorescences, as well as by pruning plant bridges. We recorded visitation or avoidance behavior of winged visitors for each treatment block (individual plant) for 30 min between 8:00 and 18:00 h (40 observation events in 4 days and totaling 1200 min of observation), randomizing each observed plant throughout the day.

We constructed a generalized linear mixed model (GLMM) to explain the results of the "pinned insect" experiment. We used the treatment as a variable that may explain the number of approximations (visitation or avoidance) and individual plants were considered a random effect (blocks) assuming binomial error distribution (visitation/approaches ratio). We established a priori orthogonal contrasts for this analysis: No ants vs. *C. rufipes* and *E. brunneum*, No ants vs. *C. rufipes*, No ants vs. *E. brunneum*, No ants vs. Pin, and *C. rufipes* vs. *E. brunneum*. We estimated parameters using logit link function, Laplace approximation, and maximum likelihood. This analysis was performed using 'lme4' (Bates et al. 2015), the 'multcomp' (Hothorn et al. 2008) and the 'lattice' (Sarkar 2008) packages for R software version 3.0.2 (R Core Team 2013).

Effect of ant–treehopper associations on plant reproduction

The honeydew produced by treehoppers is very attractive to several species of ants in the cerrado (Lopes 1995), and the associated ants may prey on other insects (Del-Claro and Oliveira 2000). However, few studies have investigated the effect of ant–treehopper associations on fruit formation (Oliveira and Del-Claro 2005; for ant–aphid effects, see Styrsky and Eubanks 2010; LeVan and Holway 2015). We tested the effect of ant–treehopper associations (ATA) on the plant's reproductive output through exclusion experiments using *B. intermedia* inflorescences.

We selected another subset of 30 plants (taken from the original 60, but excluding the 15 used in the previous experiment) that were in similar phenological phase, had the same height (~ 0.7 m tall) and similar numbers of inflorescences (12–15), and were at least 3 m apart from one another. For each plant, we established the following treatments: (1) "No exclusion", inflorescence left untouched (winged floral visitors and ATA), and (2) "Exclusion", flying insects allowed, but treehoppers and ants excluded (tanglefoot resin applied to base of branch and selected inflorescences). Treatment (1) had a speck of resin applied on only one side of the branch, so that ants could still access the inflorescence. Inflorescences in Treatment (2) had no evidence of treehopper or ant presence. We used pairs of inflorescences on the same individual plant to control for potential among-plant variation (i.e., age and location) from confounding treatment effects (see Lach 2007). We made an initial count of treehoppers on each inflorescence of Treatment 1 (Treatment 2 inflorescences had no treehoppers, thus we did not perform a count, though we checked for the absence of treehoppers) and surrounding branches. Both treatments were checked every 3 days for 1 month to confirm the presence/absence of organisms on the experimental inflorescences. We recorded the number of flower buds of each experimental inflorescence. After 1 month, we recorded the number of treehoppers on each inflorescence, bagged all inflorescences using voile bags (which allow flowers to bloom, but exclude visitors), and applied a ring of tanglefoot resin to exclude all crawling visitors. Then, after 2 months, we counted the number fruits formed. Fruits were dried (48 h at 70 °C) and the number of seeds was counted for each fruit.

We used a Generalized Linear Mixed Model (GLMM), assuming Gaussian distribution, to test the effect of the presence (mean number) of treehoppers on fruit and seed set of *B. intermedia* while considering the individual plants as a random variable in our model (blocks). We then analyzed the fruit set in each experimental group of inflorescences using a GLMM using the treatments as the predictor variable and the number of fruits formed divided by the number of initial flowers (fruit/flower ratio) as the response variable (assuming a binomial error distribution). For this model, we also considered the individual plants as a random effect for our model. For this model, we used the Laplace approximation and maximum likelihood for parameter estimation. As we had many zeroes in our data (i.e., when no fruits were formed in either treatment), we excluded them from our analysis to avoid possible interpretation errors caused by zero-inflated models as a result of the treatments that had zero fruits formed. We used a similar approach to analyze seed formation, and considered the number of seeds formed per number of fruits (seed/fruit ratio) as the response variable. The analyses were carried out using the 'lme4' package (Bates et al. 2015) for R software version 3.0.2 (R Core Team 2013).

Results

Field surveys revealed that two species of treehoppers, *Enchenopa gracilis* and *Amastris undulata*, were associated with 84% of *Byrsonima intermedia* individuals ($N = 60$; Fig. 1b). In addition, 11 ant species were registered visiting 95% of the plants, the most frequent of which were *Camponotus rufipes* (66%), *C. crassus* (16%), and *Ectatomma brunneum* (9%). Plants hosting *E. gracilis* (45%) or *A. undulata* (39%) were pooled together in our analyses, since they did not differ in the average number of tending ants ($F_{1,59} = 3.141$; $P = 0.09$), and as such their potential effects on plant reproduction were considered the same.

Effect of treehoppers on ant abundance

We found that the number of treehoppers (nymphs) increased the abundance of ants on *B. intermedia* inflorescences (Fig. 2; $Z = 7.971$; $df = 29$; $P < 0.001$; $R^2_{(m)} = 0.21$; $R^2_{(c)} = 0.25$). When analyzed by treehopper species, we found similar trends for both species (Fig. 2; *A. undulata*: $Z = 5.211$; $df = 14$; $P < 0.001$; $R^2_{(m)} = 0.21$; $R^2_{(c)} = 0.25$; *E. gracilis*: $Z = 5.795$; $df = 14$; $P < 0.001$, $R^2_{(m)} = 0.22$; $R^2_{(c)} = 0.22$).

Effect of ant–treehopper associations on floral visitors

We recorded nine species of winged floral visitors belonging to Apidae (4 species), Halictidae (2 species), Syrphidae (1 species), and Muscidae (1 species) (Table 1). Oil-collecting *Centris varia* bees (Apidae) were by far the most frequent visitors to flowers of *Byrsonima intermedia* (≥ 5 visits per hour; Fig. 1a), whereas the other species were considered occasional visitors (≤ 1 visit per hour; Table 1). All visitors were active during the sampling period; visits by winged insects were more frequent early in the morning (08:00 h) and between 10:00 and 14:00 h, whereas visits by ants peaked at 10:00 and 16:00 h (Fig. 3a). The number of visits by winged insects declined with increasing number of ants (Fig. 3b; $Z = -2.799$; $df = 51$; $P = 0.005$, $R^2_{(m)} = 0.21$; $R^2_{(c)} = 0.30$). Likewise, the duration of floral visits by all insects declined with increasing number of ants on *B. intermedia* inflorescences (Fig. 3c; $Z = -2.604$; $df = 51$;

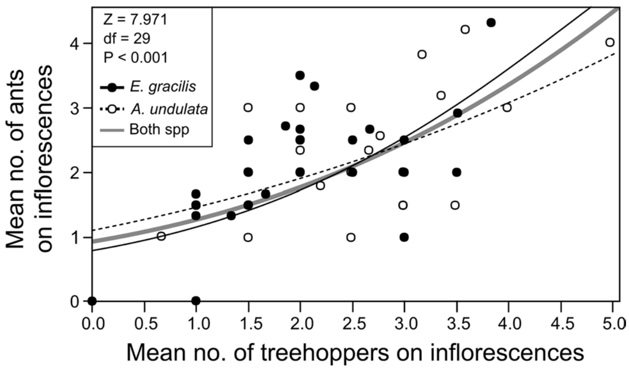


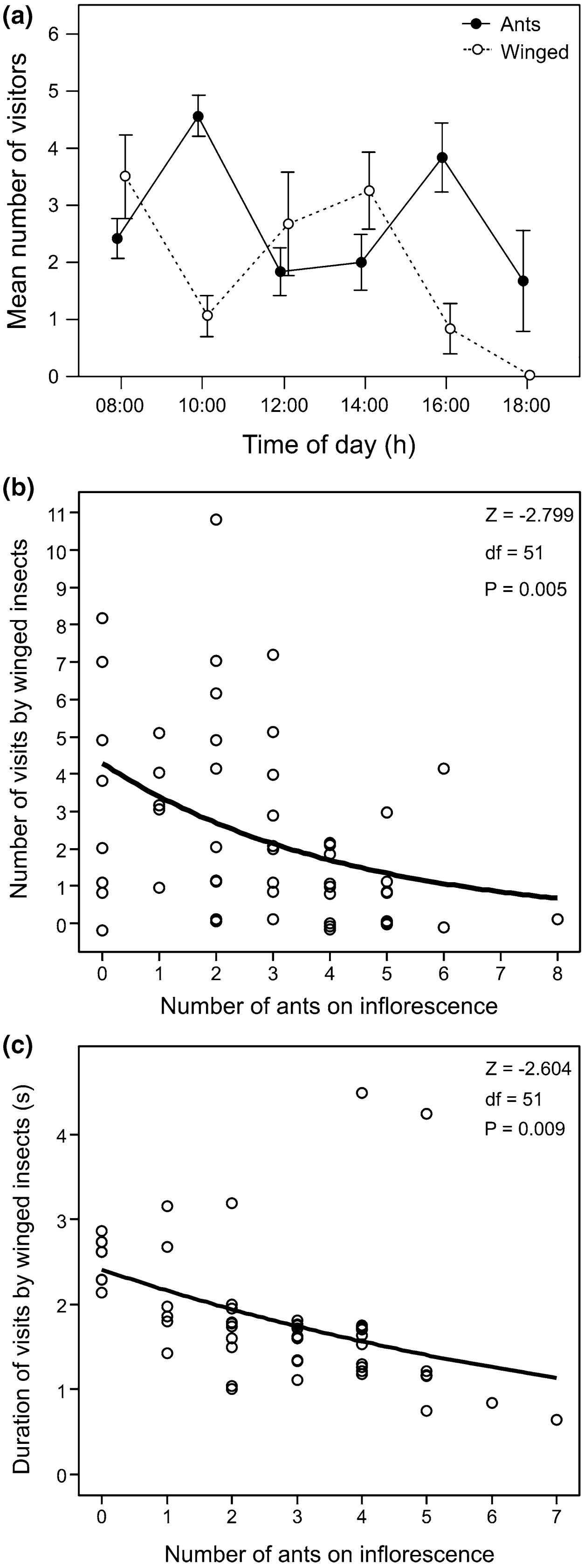
Fig. 2 Number of ants and treehoppers per shrub of *Byrsonima intermedia*. Each point represents the number of ants tending a treehopper aggregation. Black dots and black trend line represent *Enchenopa gracilis* treehoppers and white dots and dashed trend line represent *Amastris undulata* treehoppers. The test and P value correspond to a Poisson GLMM considering both species (gray trendline; $R^2_{(m)} = 0.21$; $R^2_{(c)} = 0.25$)

Table 1 Frequency of visitation (number of visits/total number of hours of observation) and floral resource collected by winged floral visitors of *Byrsonima intermedia* in a cerrado reserve in southeast Brazil

Floral visitor[a]	Visitation frequency (per hour)	Resource collected[a]
Apidae		
Apis mellifera	0.56	Pollen
Centris varia	5.44	Pollen, oil
Trigona spinipes	0.56	Pollen
Xylocopa ordinaria	0.16	Pollen
Halictidae		
Augochloropsis sp.	0.88	Pollen
Augochlora sp.	0.24	Pollen
Muscidae		
Muscidae sp.	0.08	Pollen
Syrphidae		
Toxomerus sp.	0.08	Pollen

[a] See also Boas et al. (2013) for additional data on visitors and floral resources of *Byrsonima* spp. in other cerrado areas

Fig. 3 **a** Visitation by ants and winged insects to *Byrsonima intermedia* throughout the day. Each point represents the mean number of ants (black) or winged insects (white) observed (± SE) visiting the inflorescences. **b** Number of visits by winged insects to flowers of *B. intermedia* as a function of the number of ants present on the inflorescence. Data points represent individual floral visits per observation period. Test and *P* values correspond to a Poisson GLMM ($R^2_{(m)} = 0.21$; $R^2_{(c)} = 0.30$). **c** Duration of visits by winged insects to flowers of *B. intermedia* as a function of the number of ants present on the inflorescence. Data points represent individual floral visits per observation period. Test and *P* values correspond to a Poisson GLMM ($R^2_{(m)} = 0.18$; $R^2_{(c)} = 0.31$) ▶



$P = 0.009$; $R^2_{(m)} = 0.18$; $R^2_{(c)} = 0.31$; see video Online Resource 1).

The experiment using pinned ants (*Camponotus rufipes* and *Ectatomma brunneum*) revealed that ant presence promotes avoidance behavior by floral visitors, reducing the total number of visits (Fig. 4; Binomial GLMM; $\chi^2 = 47.679$; $df = 3$; $P < 0.001$; see video Online Resource 1). Pins alone had no effect on floral visitation: bees and other insects visited equally inflorescences with or without pins ($Z = -1.041$; $df = 39$; $P = 0.6379$). Furthermore, we showed that ant identity is an important factor affecting the number of approaches by floral visitors (No ants vs. *C. rufipes*: $Z = -2.495$; $df = 39$; $P = 0.0433$; No ants vs. *E. brunneum*: $Z = -4.632$; $df = 39$; $P < 0.001$), with *E. brunneum* causing greater avoidance behavior by floral visitors than *C. rufipes* ($Z = -2.471$; $df = 39$; $P = 0.0462$; Fig. 4). Indeed, *E. brunneum* was extremely aggressive toward intruders around inflorescences (Online Resource 2).

Effect of ant–treehopper associations on plant reproduction

The reproductive output of *Byrsonima intermedia* increased when ant–treehopper associations were excluded from the inflorescences. Both the proportion of fruits (Binomial GLMM, $Z = 2.526$; $df = 17$; $P = 0.0115$) and seeds formed ($Z = 2.127$; $df = 17$; $P = 0.0334$) were significantly reduced by the presence of ant–treehopper associations on the inflorescences (Fig. 5; see Online Resources 3, 4). This result seems to be independent of the effect of treehopper herbivory, and indeed, our regression analyses show no significant relationship between the density of treehoppers and fruit and seed set (Fig. 6).

Discussion

The presence of honeydew-producing treehoppers increased ant abundance on inflorescences of *Byrsonima intermedia*, which disrupted pollination by flying insects and ultimately reduced plant reproduction. Ant presence near flowers

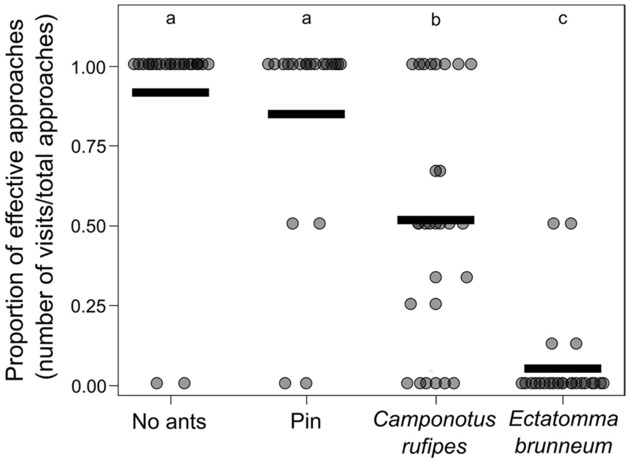


Fig. 4 Number of effective approaches to flowers of *Byrsonima intermedia* (visits/total approaches) by winged visitors per treatment ($N = 10$). Ant occupation on inflorescences was simulated by pinning dead, recently-captured ants to inflorescences. Treatment groups: (1) "No ants"; (2) "1 Pin"; (3) "1 Pinned worker of *Camponotus rufipes*"; and (4) "1 Pinned worker of *Ectatomma brunneum*". Different letters denote significant statistical difference ($P < 0.05$). Data points represent individual approach events; the points are jittered to show data aggregation (darker points show point overlap). Horizontal black lines represent the mean proportion of effective approaches to flowers

negatively affected both the frequency and duration of floral visitation by flying insects. Although a combination of different factors may affect *B. intermedia* fruit formation (Boas et al. 2013), our field experiments demonstrate a negative effect of the presence of ant–treehopper associations on the reproductive output of *B. intermedia* as expressed by decreased fruit and seed set.

Ant–treehopper associations have been thoroughly studied in cerrado plant species (Lopes 1995), and in general, ant attendants have a negative effect on damaging herbivores (Oliveira and Del-Claro 2005). Increased floral visitation by ants in the presence of honeydew-producing treehoppers has already been reported in other systems (e.g., Lach 2007), including associations occurring on inflorescences in the cerrado (Lopes 1995; Del-Claro and Oliveira 2000; Kaminski et al. 2010). In our study system, ants rarely visited *B. intermedia* inflorescences in the absence of honeydew-producing treehoppers, since flowers do not appear to offer any resource for the ants (e.g., pollen and oil). This indicates that treehoppers are the basis for the interaction between ants and floral visitors of this plant.

All winged insects visiting flowers of *B. intermedia* in our study area collect pollen from it, but only *Centris varia* bees collect oil from elaiophores at the base of flowers and exhibit buzz-pollination behavior (Sigrist and Sazima 2004; Boas et al. 2013). This information suggests that large oil-gathering *C. varia* may be the only effective pollinators of *B. intermedia* in our study area. Although floral visitation by

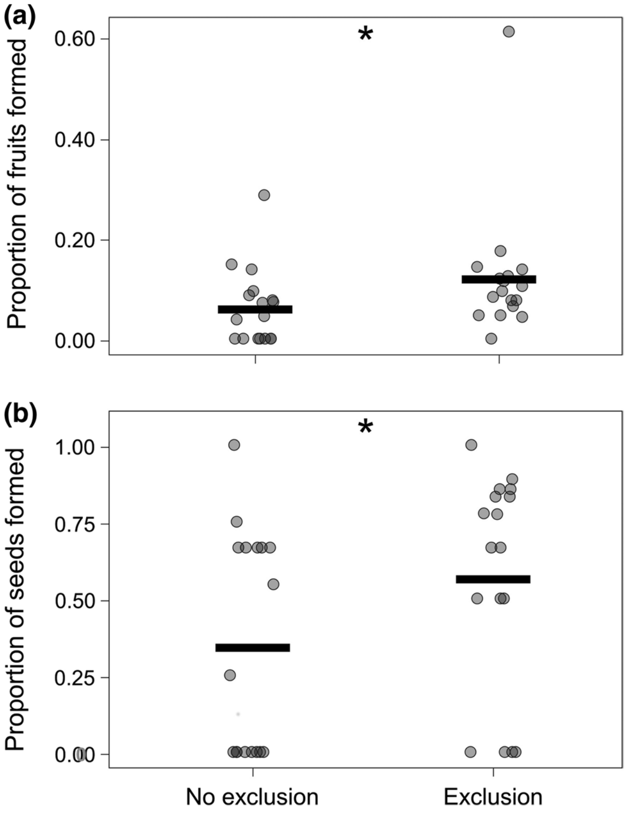


Fig. 5 Exclusion experiment examining the effect of ant–treehopper associations (ATA) on the reproductive output of *Byrsonima intermedia*. Treatment groups: (1) "No exclusion", inflorescence left untouched (winged floral visitors and ATA); (2) "Exclusion", flying insects allowed, but treehoppers and ants excluded (tanglefoot resin applied to base of branch). Data points represent individual inflorescences; the points are jittered to show data aggregation (darker points show point overlap). Asterisks (*) denote significant statistical difference ($P < 0.05$). **a** Proportion of fruits formed (number of fruits formed per number of flowers in inflorescence) in each plant. **b** Proportion of seeds formed (number of seeds formed per number of fruits) in each plant

C. varia was typically fast (normally 1–4 s per flower), buzzing behavior frequently triggered an aggressive response by nearby ants tending treehoppers. The alerted ants would then reduce the duration of the visits by buzz pollinators, likely reducing pollination efficiency (see video Online Resource 1; Boas et al. 2013). Similarly, our data on the daily activity pattern at *B. intermedia* by both winged visitors and ants show that flower visitation by winged insects peaks at periods of low ant activity, suggesting that visitors other than bees might also avoid encounters with ants (Tsuji et al. 2004; Sendoya et al. 2009; Li et al. 2014; Ibarra-Isassi and Sendoya 2016).

Our experimental results on pollination disruption by tending ants corroborate the study by LeVan and Holway (2015) with cotton (*Gossypium hirsutum*), which shows that increased numbers of aphid-tending Argentine ants

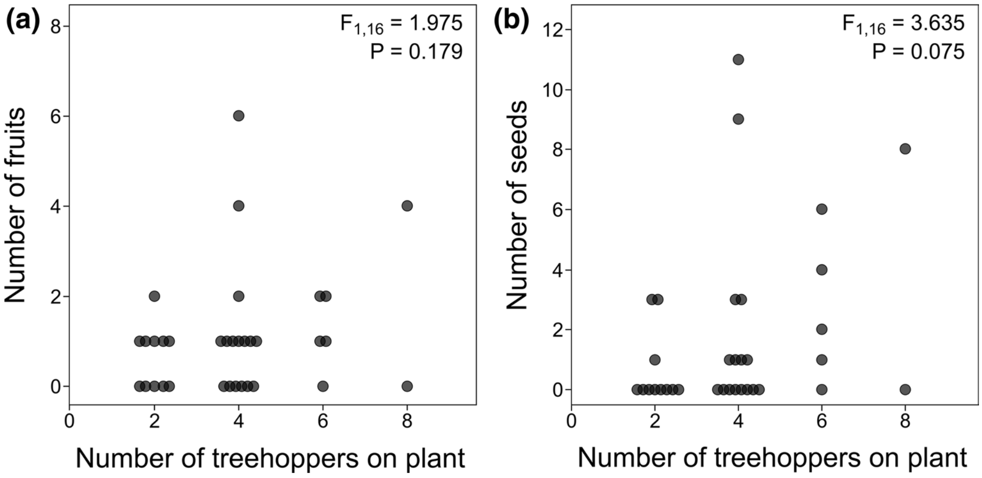


Fig. 6 Relationship between the number of treehoppers and **a** the number of fruits and **b** the number of seeds formed. Data points represent individual inflorescences; the points are jittered to show data aggregation (darker points show point overlap)

(*Linepithema humile*) on the plant decreases the duration of visits by honeybee pollinators. It is important to note, however, that shorter visits may benefit the plant through increased numbers of visited flowers per unit time, which might lead to an increased rate of pollen transfer (Altshuler 1999; Ness 2006). Thus, it is important to consider the time and number of flowers visited to fully assess the effect of ant presence on plant reproduction, especially in buzz-pollinated flowers, because a short visit might not be enough for the flower to be stimulated enough to release the pollen grains (Sigrist and Sazima 2004). Our experiment using pinned ants on inflorescences demonstrated that ant presence mediates flower visitation by winged insects and further detected a species-specific effect on flower visitors, which showed increased avoidance behavior towards *Ectatomma brunneum* compared to *Camponotus rufipes* ants. There is evidence suggesting that winged insects use visual or chemical recognition cues to avoid aggressive ants, altering egg-laying decisions and even discriminating different species of potential predatory ants by size and form (Sendoya et al. 2009). Although both ant species behaved aggressively on leaves, alertness by larger *E. brunneum* standing poised with mandibles open and ready to attack any intruder was remarkable (Online Resource 2). Since *C. rufipes* did not present this behavior, we believe that bees are able to recognize the "guarding" behavior presented by *E. brunneum* as a threat, and thus avoid visiting inflorescences occupied by this ant. This was evidenced in prior field observations, which in turn motivated the inclusion of *E. brunneum* in our pinning experiments. It was beyond the scope of this study to disentangle the mechanisms underlying the differential avoidance by floral visitors to these two species. Other studies have shown that the presence of ants on flowers (in general) affects the visitation or avoidance behavior of pollinators (Keeler 1977; Cembrowski et al. 2014; LeVan and Holway 2015). Our pinning experiment confirms that it is important to consider the identity of the ant species when studying any kind of ant–based interaction. Furthermore, Ness (2006) reported that floral visitation by bees varies in both duration and actual frequency depending on the species of ant present on the flowers of *Ferrocactus wislizeni* (Cactaceae). Recent theoretical models suggest that when the level of ant aggressiveness is above a certain threshold, it may drive pollination mutualisms to extinction (Wang and Wu 2015). Other models, however, show that when ant interference level is weak, coexistence between ants and pollinators may occur (Wang and Wang 2015). In our study system, ants commonly found foraging on the inflorescences of *B. intermedia* are considered very aggressive (Oliveira 1997; Sendoya et al. 2009; Alves-Silva 2011), and since there is a reduction in the reproductive output of the plant, our experimental evidence supports both the proposed theoretical models. Considering that effective pollinator diversity is low in our study area compared to other cerrado sites (Boas et al. 2013), further investigation should examine how ant-induced effects on the pollination ecology of *B. intermedia* vary through time and space in the cerrado landscape.

Our exclusion experiment suggests that the negative effect of ant–treehopper associations on plant reproduction probably results from the effect of tending ants on floral visitors rather than from a direct effect of treehoppers on the inflorescences. This is supported by our data showing that fruit and seed set do not vary with treehopper density on *B. intermedia* inflorescences (see Figs. 5, 6). In a similar study, LeVan and Holway (2015) also argue that pollination disruption by aphid-tending ants, rather than aphid herbivory, probably accounts for reduced seed set in cotton plants. Furthermore, other studies show that aphid herbivory alone has little effect on plant reproduction (Rosenheim et al. 1997; Styrsky and Eubanks 2010).

Although ant–trophobiont interactions may enhance host plant reproduction (Styrsky and Eubanks 2007), the evidence found in this and other recent studies (Wielgoss et al. 2014; LeVan and Holway 2015, Canedo-Júnior et al. 2017) indicates that the indirect effects are dependent on the identity, abundance, and behavior of the ant species involved. Positive effects on plant reproduction normally result from ant-induced herbivore deterrence (Messina 1981; Kaplan and Eubanks 2005; Pringle et al. 2011). Yet, with increased ant foraging on flowers due to the presence of ant-associated herbivores, plant reproduction may be decreased because of interactions between ants and other floral visitors (Horvitz and Schemske 1984; Rico-Gray and Thien 1989; Rico-Gray and Castro 1996; LeVan and Holway 2015). Therefore, the net effect of ant–hemipteran interactions on host plants depends on the balance between the benefit resulting from ant-induced herbivore deterrence and the cost of the negative effect resulting from pollinator disturbance (Ohm and Miller 2014). The outcome of such multispecies interaction systems can be influenced by the identity of participant ant species due to their foraging behavior and aggressiveness (Ness et al. 2009), as well as by the degree of pollination disruption caused by ant-induced disturbance of floral visitors (Lach 2003).

Our results add to the understanding of how host plants may be affected indirectly by the presence of mutualistic associations. We demonstrate that plant reproduction can be negatively affected by the presence of ant–treehopper associations near flowers. Our field observations confirmed our hypothesis that ant abundance follows increased abundance of treehoppers on *B. intermedia* inflorescences. Since ants were rarely seen visiting inflorescences when no treehoppers were present, the latter act as a bridge for the interaction between ants and floral visitors. Our study thus highlights the importance of including other interactions occurring near the flower, in addition to just pollinator observations, for a proper understanding of the negative effects of ants on plant reproduction. The detection of ants, or even of ant–trophobiont associations by floral visitors, may likely represent an important evolutionary step in the establishment or disappearance of particular pollination systems or mechanisms (such as buzz pollination). Finally, our study adds another dimension to ant-based mutualisms mediated by liquid rewards on cerrado foliage, and their indirect effects on associated species (Sendoya et al. 2009; Kaminski et al. 2010; Vidal et al. 2016). This is a first report of indirect effects by honeydew-gathering ants, via disrupted pollination, on a plant's reproductive output in ant-rich cerrado.

Acknowledgements We thank A. Freitas, S. Sendoya, R. Cogni, M. Vidal, A. Nogueira, C. Vieira, and M. Azevedo-Silva for reading early drafts of the manuscript. The final version was considerably improved by comments from S. Koptur, L. Lach, S. Murphy, and M. Pareja. Constructive criticisms from the handling editor, S. Whitehead, and two anonymous reviewers greatly enhanced the revised manuscript. A. Tacioli helped in the field, and S. Sendoya assisted with the statistical analyses. We also thank M. Azevedo-Silva for help with the figures, and H. Soares Jr for editing the videos and for the flower photo. JI-I was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and the São Paulo Research Foundation (2014/12486-8); PSO was supported by the São Paulo Research Foundation (2014/23141-1), and the Brazilian Research Council (306115/2013-1).

Author contribution statement JI-I and PSO conceived and designed the experiments. JI-I performed the experiments and analyzed the data. JI-I and PSO wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alarcón R, Waser NM, Ollerton J (2008) Year-to-year variation in the topology of a plant–pollinator interaction network. Oikos 117:1796–1807

Altshuler D (1999) Novel interactions of non-pollinating ants with pollinators and fruit consumers in a tropical forest. Oecologia 119:600–606

Alves-Silva E (2011) Post-fire resprouting of *Banisteriopsis malifolia* (Malpighiaceae) and the role of extrafloral nectaries on the associated ant fauna in a Brazilian savanna. Sociobiology 58:327–339

Ballantyne G, Willmer P (2012) Nectar theft and floral ant-repellence: a link between nectar volume and ant-repellent traits? PLoS ONE 7:e43869

Barton K (2016) MuMIn: Multi-model inference. R package version 1.15.6. https://CRAN.R-project.org/package=MuMIn. Accessed 21 Nov 2017

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48

Beattie AJ (2007) The evolution of ant pollination systems. Botanische Jahrbücher Systematik 127:43–55

Beattie AJ, Turnbull C, Knox RB, Williams EG (1984) Ant inhibition of pollen function: a possible reason why ant pollination is rare. Am J Bot 71:421–426

Bleil R, Blüthgen B, Junker RR (2011) Ant–plant mutualism in Hawai'i? Invasive ants reduce flower parasitism but also exploit floral nectar of the endemic shrub *Vaccinium reticulatum* (Ericaceae). Pac Sci 65:291–300

Boas JCV, Fava WS, Laroca S, Sigrist MR (2013) Two sympatric *Byrsonima* species (Malpighiaceae) differ in phenological and reproductive patterns. Flora 208:360–369

Canedo-Júnior EO, Santiago GS, Ribas CR, Zurlo LF, Cuissi RG, Souza B, Faria LD, Rabello AM, Braga DD, Silva E (2017) The effect size of aphid-tending ants in an agricultural tri-trophic system. J Appl Entomol 00:1–10. https://doi.org/10.1111/jen.12475

Cembrowski AR, Tan MG, Thomson JD, Frederickson ME (2014) Ants and ant scent reduce bumblebee pollination of artificial flowers. Am Nat 183:133–139

de Vega C, Gómez JM (2014) Polinización por hormigas: conceptos, evidencias y futuras direcciones. Ecosistemas 23:48–57

de Vega C, Herrera CM (2013) Microorganisms transported by ants in duce changes in floral nectar composition of an ant-pollinated plant. Am J Bot 100:792–800

Del-Claro K, Oliveira PS (1996) Honeydew flicking by treehoppers provides cues to potential tending ants. Anim Behav 51:1071–1075

Del-Claro K, Oliveira PS (2000) Conditional outcomes in a neotropical treehopper-ant association: temporal and species-specific variation in ant protection and homopteran fecundity. Oecologia 124:156–165

Dutton EM, Frederickson ME (2012) Why ant pollination is rare: new evidence and implications of the antibiotic hypothesis. Arthropod-Plant Interact 6:561–569

Gonzálvez FG, Santamaría L, Corlett RT, Rodríguez-Gironés MA (2013) Flowers attract weaver ants that deter less effective pollinators. J Ecol 101:78–85

Hanna C, Naughton I, Boser C, Alarcón R, Hung KLJ, Holway D (2015) Floral visitation by the Argentine ant reduces bee visitation and plant seed set. Ecology 96:222–230

Horvitz CC, Schemske DW (1984) Effects of ants and an ant-tended herbivore on seed production of a neotropical herb. Ecology 65:1369–1378

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biom J 50:346–363

Ibarra-Isassi J, Sendoya SF (2016) Ants as floral visitors of *Blutaparon portulacoides* (A. St-Hil.) Mears (Amaranthaceae): an ant pollination system in the Atlantic Rainforest. Arthropod-Plant Interact 10:221–227

Junker RR, Chung AYC, Blüthgen N (2007) Interaction between flowers, ants and pollinators: additional evidence for floral repellence against ants. Ecology 22:665–670

Kaminski LA, Freitas AVL, Oliveira PS (2010) Interaction between mutualisms: ant–tended butterflies exploit enemy-free space provided by ant–treehopper associations. Am Nat 176:322–334

Kaplan I, Eubanks MD (2005) Aphids alter the community-wide impact of fire ants. Ecology 86:1640–1649

Keeler KH (1977) The extrafloral nectaries of *Ipomoea carnea* (Convolvulaceae). Am J Bot 64:1182–1188

Lach L (2003) Invasive ants: unwanted partners in ant–plant interactions? Ann Mo Bot Gard 90:91–108

Lach L (2007) A mutualism with a native membracid facilitates pollinator displacement by argentine ants. Ecology 88:1994–2004

Lach L (2008) Floral visitation patterns of two invasive ant species and their effects on other hymenopteran visitors. Ecol Entomol 33:155–160

Lau JA, Galloway LF (2004) Effects of low-efficiency pollinators on plant fitness and floral trait evolution in *Campanula americana* (Campanulaceae). Oecologia 141:577–583

LeVan KE, Holway DA (2015) Ant–aphid interactions increase ant floral visitation and reduce plant reproduction via decreased pollinator visitation. Ecology 96:1620–1630

Li J, Wang Z, Tan K, Qu Y, Nieh JC (2014) Giant Asian honeybees use olfactory eavesdropping to detect and avoid ant predators. Anim Behav 97:69–76

Lopes BC (1995) Treehoppers (Homoptera, Membracidae) in southeastern Brazil: use of host plants. Revista Brasileira de Zoologia 12:595–608

Messina FJ (1981) Plant protection as a consequence of ant–membracid mutualism: interactions on Goldenrod (*Solidago* sp.). Ecology 62:1433–1440

Nakagawa S, Schielzeth H (2013) A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol Evol 4:133–142

Ness JH (2006) A mutualism's indirect costs: the most aggressive plant bodyguards also deter pollinators. Oikos 113:506–514

Ness JH, Morris WF, Bronstein JL (2009) For ant-protected plants, the best defense is a hungry offense. Ecology 90:2823–2831

Ohm JR, Miller TEX (2014) Balancing anti-herbivore benefits and anti-pollinator costs of defensive mutualists. Ecology 95:2924–2935

Oliveira PS (1997) The ecological function of extrafloral nectaries: herbivore deterrence by visiting ants and reproductive output in *Caryocar brasiliense* (Caryocaraceae). Funct Ecol 11:323–330

Oliveira PS, Del-Claro K (2005) Multitrophic interactions in a neotropical savanna: ant–hemipteran systems, associated insect herbivores and a host plant. In: Burslem DFRP, Pinard MA, Hartley SE (eds) Biotic interactions in the tropics: their role in the maintenance of species diversity. Cambridge University Press, Cambridge, pp 414–438

Oliveira MIB, Polido CA, Costa LC, Fava WS (2007) Sistema reprodutivo e polinização de *Byrsonima intermedia* A. Juss. (Malpighiaceae) em Mato Grosso do Sul, Brasil. Revista Brasileira de Biociências 5:756–758

Oliveira-Filho AT, Ratter JA (2002) Vegetation physiognomies and woody flora of the cerrado biome. In: Oliveira PS, Marquis RJ (eds) The cerrado of Brazil: ecology and natural history of a neotropical savanna. Columbia University Press, New York, pp 91–120

Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals? Oikos 120:321–326

Palmer TM, Doak DF, Stanton ML, Bronstein JL, Kiers ET, Young TPJ, Goheen R, Pringle RM (2010) Synergy of multiple partners, including freeloaders, increases host fitness in a multispecies mutualism. Proc Natl Acad Sci USA 107:17234–17239

Price PW, Denno RF, Eubanks MD, Finke DL, Kaplan I (2011) Insect ecology: behavior, populations and communities. Cambridge University Press, Cambridge

Pringle EG, Dirzo R, Gordon DM (2011) Indirect benefits of symbiotic coccoids for an ant-defended myrmecophytic tree. Ecology 92:37–46

Quinn GP, Keough MJ (2002) Experimental design and data analysis for biologists. Cambridge University Press, Cambridge

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rico-Gray V, Castro G (1996) Effect of an ant–aphid interaction on the reproductive fitness of *Paullinia fuscecens* (Sapindaceae). Southwest Nat 41:434–440

Rico-Gray V, Oliveira PS (2007) The ecology and evolution of ant–plant interactions. University of Chicago Press, Chicago

Rico-Gray V, Thien LB (1989) Ant–mealybug interaction decreases reproductive fitness of *Schomburgkia tibicinis* (Orchidaceae) in Mexico. J Trop Ecol 5:109–112

Romero GQ, Antiqueira PP, Koricheva J (2011) A meta-analysis of predation risk effects on pollinator behavior. PLoS ONE 6:e20689

Rosenheim JA, Wilhoit LR, Goodell PB, Grafton-Cardwell EE, Leigh TF (1997) Plant compensation, natural biological control, and herbivory by *Aphis gossypii* on pre-reproductive cotton: the anatomy of a non-pest. Entomol Exp Appl 85:45–63

Sarkar D (2008) Lattice: multivariate data visualization with R. Springer, New York

Sendoya SF, Freitas AVL, Oliveira PS (2009) Egg-laying butterflies distinguish predaceous ants by sight. Am Nat 174:134–140

Sidhu CS, Wilson-Rankin EE (2016) Honey bees avoiding ant harassment at flowers using scent cues. Environ Entomol 45:420–426

Sigrist MR, Sazima M (2004) Pollination and reproductive biology of twelve species of neotropical Malpighiaceae: stigma morphology and its implications for the breeding system. Ann Bot 94:33–41

Souto LS, Oliveira DMT (2005) Morphology, anatomy, and development of Byrsonima intermedia A. Juss. (Malpighiaceae) fruit and seed. Braz J Bot 28:697–712

Styrsky JD, Eubanks MD (2007) Ecological consequences of interactions between ants and honeydew-producing insects. Proc R Soc B 274:151–164

Styrsky JD, Eubanks MD (2010) A facultative mutualism between aphids and an invasive ant increases plant reproduction. Ecol Entomol 35:190–199

Tsuji K, Hasyim A, Nakamura K (2004) Asian weaver ants, *Oecophylla smaragdina*, and their repelling of pollinators. Ecol Res 19:669–673

Vidal MC, Sendoya SF, Oliveira PS (2016) Mutualism exploitation: predatory drosophilid larvae sugar-trap ants and jeopardize facultative ant–plant mutualism. Ecology 97:1650–1657

Wang Y, Wang S (2015) Persistence of pollination mutualisms in the presence of ants. Bull Math Biol 77:202–229

Wang Y, Wu H (2015) Stability of plant–pollinator–ant co-mutualism. Appl Math Comput 261:231–241

Waser NM, Chittka L, Price MV, Williams NM, Ollerton J (1996) Generalization in pollination systems, and why it matters. Ecology 77:1043–1060

Wielgoss A, Tscharntke T, Rumede A, Fiala B, Seidel H, Shahabuddin S, Clough Y (2014) Interaction complexity matters: disentangling services and disservices of ant communities driving yield in tropical agroecosystems. Proc R Soc B 281:20132144

Zanchetta D, Reis CM, Delgado JM, Silva CEF, de Luca EF, Fernandes FD, Lutgens HD, Tannus JLS, Pinheiro LD, Martins MRC, Sawaya R (2006) Plano de Manejo Integrado. Estações Ecológica e Experimental de Itirapina-SP. Primeira Revisão. Instituto Florestal, São Paulo