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RESEARCH ARTICLE - BEES

Foraging ants on the extrafloral nectaries repel nectar thieves but not the effective pollinator of *Vigna luteola* (Fabaceae) in a Mexican coastal sand dune

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Abstract

While some studies have shown that ants that visit extrafloral nectaries may defend their host plants against potential herbivores, recent researches have shown that such ant-plant mutualism may be broken in some cases. For example, the presence of ants on plants could also drive away pollinators and seed dispersers. However, it is not yet known what mechanisms could favor that ant presence on plants does not affect other mutualistic interactions involving plants. In this work, we performed a series of field experiments to test the hypothesis that the presence of ants on EFNs located at the base of the inflorescences of *Vigna luteola* (Fabaceae) may have a negative effect on non-pollinators but not on pollinators in a Mexican coastal sand dune. In general, we found that the presence of ants on the plants decreased the rate of flower visitation. However, we observed that the time of visitation of the pollinator, the bee *Megachile* (*Pseudocentron*) sp. on the flowers was less compared to non-pollinators. This strategy may allow that ants cannot aggressively scare away the pollinator. In summary, we show that the pollinator of *V. luteola* present strategies that allow them to visit the flowers without being aggressively attacked by the ants that visit the extrafloral nectaries (EFNs). Therefore, the presence of ants on plants could have a dual function: protecting plants against potential herbivores as well as, filtering flowers against nectar thieves.

Introduction

Among the studies on ant-plant interactions, we can find those in which clearly there is a benefit for both interacting organisms through a mutual relationship (Koptur, 1984; Oliveira et al., 1999; Rico-Gray & Oliveira, 2007; Byk & Del-Claro, 2011). Plants provide rewards ranging from extrafloral nectar, elaisomes, food bodies (e.g., Beltian and Müllerian bodies in *Acacia* and *Cecropia* respectively) and nesting sites or refuges for ants (domatias) in genera such as *Cordia* (Koptur, 1992a; Rico-Gray & Oliveira, 2007; Pringle et al., 2012). In exchange for food and nesting resources, ants offer plants protection against herbivores, seed dispersion, and in some cases may serve as potential pollinators (Rico-Gray & Oliveira, 2007; De Vega et al., 2009; Dáttilo et al., 2015; Luna et al., 2018). However, these interactions between ants and plants are mediated by cost-benefit relationships in which plant resources are exchanged for services offered by animals, in this case by ants (Del Claro et al., 2016).

One of the main resources that plants offer ants is extrafloral nectar (Byk & Del-Claro, 2011), which is produced by glands called extrafloral nectaries (EFNs). The EFNs are located on almost any vegetative and reproductive structure, and are even common on inflorescences and on the external parts of flowers, but are not involved with pollination processes (Heil, 2011). The majority of studies of interactions between

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ants and plants with EFNs focus on the benefits conferred to the plant by the presence of ants attracted to its nectar-producing structures (Apple & Feener, 2001). This mechanism is beneficial for the plant because the ant aggressive behavior is likely to deter other insects from the plant structures (Altshuler, 1999), for example in *Chamaecrista debilis* (Fabaceae) it was found that the association with visiting ants significantly reduces herbivory compared to other plants from which ants were excluded (Nascimento & Del-Claro, 2010). However, this interaction may also have an impact on the plant reproductive potential as protection by ants can be beneficial when preventing herbivores from consuming developing fruits, but may be detrimental when potential pollinators and seed dispersers avoid flowers and fruits due to ant attendance (Altshuler, 1999). An example of this was found by Assunção et al. (2014) who confirmed that ants on the flowers of *Heteropterys pteropetala* (Malpighiaceae) were identified as a danger by bees that are responsible for pollination, but the avoidance response caused by ants in pollinators was not enough to negatively affect plant fitness. However, there are others studies that show that ants could have negative effects on the interaction with the plants, for example, the aggressiveness of arboreal ants on fruits can deter seed dispersers thus diminishing this ecological process (Falcão et al*.,* 2014). In the case of *Byrsonima intermedia* (Malpighiaceae), dried ants were pinned to inflorescences and the authors found that the presence of treehoppers increased with ant abundance (*Camponotus rufipes* Fabricius) on flowers, modifying the pollination process by oil-collecting bees (*Centris varia* Erichson), with negative consequences on the frequency and duration of floral visits and reducing fruit and seed set (Ibarra-Isassi & Oliveira, 2018). For *Banisteriopsis campestris* and *Banisteriopsis malifolia* (Malpighiaceae) it was found that the presence of ants on the flowers reduced the visitation rates of small bees (Tapinotaspidini, Tetrapediini, and Meliponini) rather than for large bees (Centridinii); also there was a variable effect of ant density on behaviour of bees and the consequences on the reproductive performance (Barônio & Del-Claro, 2018). However, there are other studies that showed no effect on floral visitors when ants are present. For example, in *Pasheolus lunatus* (Fabaceae) under different conditions of Jasmonic acid (which increases the nectar secretion by EFNs), the Jasmonic acid increases the ant abundance but does not have any effect on the number of flowers visitors found, however, there were effects when the ants were excluded on the number of flowers and seeds, this could have consequences on reproductive success in this plant, mediated by the ant-plant interactions (Hernández-Cumplido et al*.,* 2016).

Based on this background knowledge, we proposed to test if the presence of ants on EFNs located at the base of the inflorescences, could have a negative effect on the identity of floral visitors and the amount of time the visitors may expend foraging on the flowers of a species found in a Mexican coastal sand dune. Specifically our goals were to: 1) describe the arthropod fauna that visit the flowers and EFNs of *Vigna luteola* (Jacq.) Benth. (Fabaceae); 2) describe the morphology of extrafloral nectaries; 3) determine if the ant presence could negatively affect the identity and frequency of flower visitors and; 4) determine if the time expended by floral visitors and potential pollinators is modified by the presence of ants on the flowers.

Material and Methods

Study site

The study site is located on the Gulf coast in the southeastern portion of the state of Veracruz near the Los Tuxtlas Biological Field Station of the National Autonomous University of Mexico (UNAM – for further details of the study area see González-Soriano et al*.,*1997). During July and August of 2013 populations of *V. luteola* were located in the dunes along the sandy shores of a beach within the community of Balzapote (18°37’23.5” N; 95°04’26.4” W; 2m a.s.l.) where the vegetation is characterized by *Ipomoea pes-caprae*, *Ipomoea imperati* (Convolvulaceae), *Croton* sp. (Euphorbiaceae), *Passiflora* sp. (Passifloraceae), *Crotalaria incana* and *Chamaecrista nictitans* (Fabaceae). A previous study in the Los Tuxtlas region calculated that the flora with EFNs to be around 50 species of plants (16 families, 31 genera), representing 5% of the local flora, ranging from dune vegetation to cloud forest (1600 m a.s.l.). Plant families with the highest number of species with EFNs were Fabaceae (20 species) and Euphorbiaceae (6 species); and 52% of the species with EFNs in Los Tuxtlas region, while the other 48% were represented by 14 families, included Costaceae (2 species) and Malvaceae with three species (*Heliocarpus donell-smithii*, *Heliocarpus appendiculatus* and *Hibiscus tiliaceus*) (Aguirre et al*.,* 2013).

Study species

The genus *Vigna* has a pantropical distribution, and in America it is mainly found in the tropics and is comprised of over 90 species (Pasquet, 2004; Delgado-Salinas et al*.,* 2011). *Vigna* presents inflorescences with two or more flowers, in addition, extrafloral nectaries (EFNs) are found at the base of the inflorescences. These are important taxonomic traits at the genus level as they are used to differentiate between *Vigna* and *Macroptilium*, which are genera closely related to beans (*Phaseolus*) (McKey, 1989; Ojeda et al., 2014). *V. luteola* (Fabaceae) is a perennial vine that can be found up to 1450 m a.s.l., although it occurs frequently in coastal thickets, and is abundant in marshes along the coastal plains. The stems are glabrous or hirsute-pilose, have stipules of 5 mm of long or less; leaflets are ovate to lanceolate or elliptic from 3-9 cm in length. Flowers have yellow petals, the standard is about 1.5 cm long; and have several extrafloral nectaries at the base of inflorescences (Fig 1A, B). The corolla is zygomorphic; bee pollination and self-compatibility capacity are common in *Vigna* species, and high outcrossing levels are promoted by biotic pollinators in natural populations (Standley & Steyermark, 1946; De Sousa et al., 2017). In the study area is common to found associated to inflorescences of *V. luteola* ants under the flowers (Fig 1C).

Observations of floral and extrafloral nectary visitors

The observation of insect visitors on flowers and extrafloral nectaries in *V. luteola* were carried out every hour during daylight, over 4 hours on 5 consecutive days. Observations were carried out in intervals of 15 minutes of observation followed by 15 minutes of rest. These began at 10:00 and finished at 14:00h when the activity of visitors decreased strongly (in total we observed by 1200 minutes). For floral visitors, we recorded the visitation frequency while for extrafloral nectaries we considered the abundance of insects. Sample collections were taken for all insects and voucher specimens were deposited in the reference collection of insects of the Los Tuxtlas Tropical Biology Station.

Histology of EFNs

Samples of extrafloral nectaries were fixed in FAA (formalin – acetic acid – 96% ethanol, 5:5: 90 by volume) and dehydrated in a graded series of tertiary butyl alcohol (TBA) at 30, 50, 70, 96 and 100%. Finally, samples were embedded in paraffin of histological grade, mp 58 °C. Transverse and longitudinal sections were obtained from each sample at 12 µm of thickness, using a rotary microtome (Leica SM2010R) and stained with a combination of 0.1% aqueous safranin and 0.05% fast green dissolved in 96% ethanol, dehydrated in absolute ethanol, and cleared with xylene. Then, sections were mounted in Sigma™ synthetic resin, dissolved in xylene. Observations and images captured were made using a light microscope (Nikon Eclipse E600) with a digital camera (Nikon CoolPix 950).

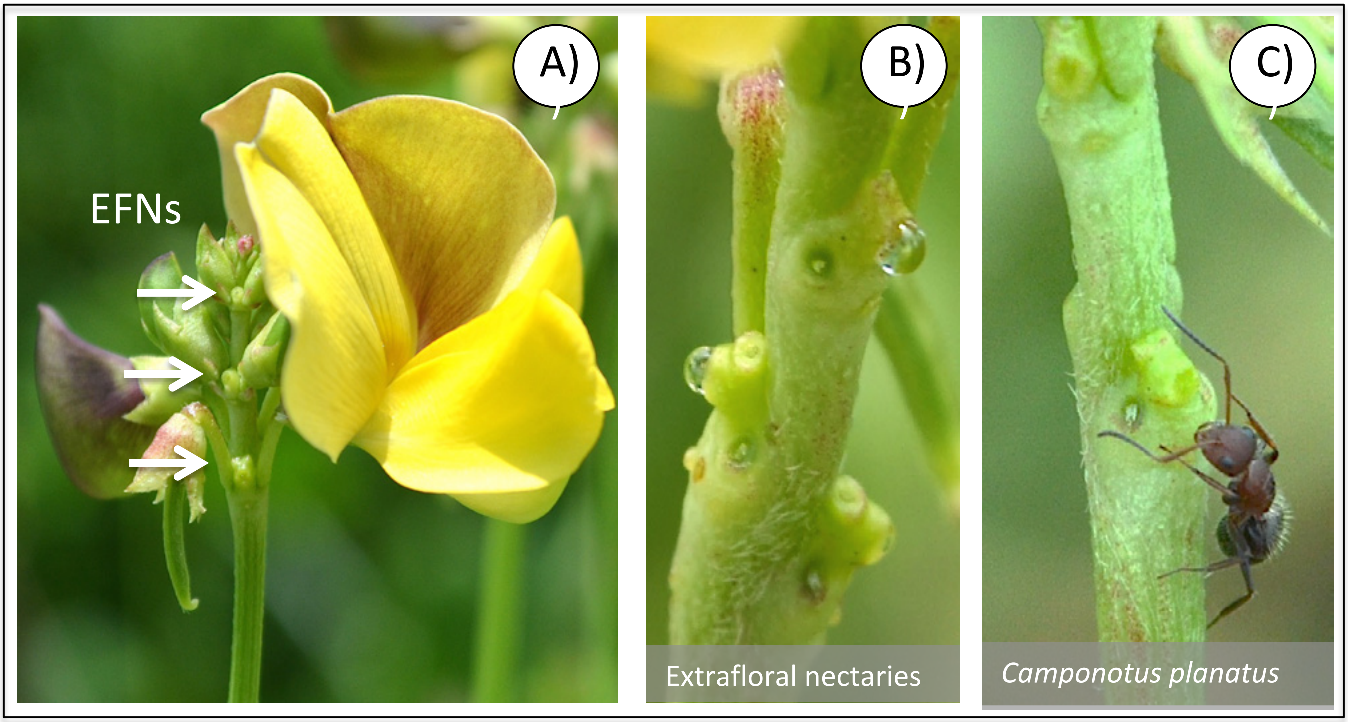

Fig 1. Inflorescence of *Vigna luteola* showing the extrafloral nectaries (white arrows) (A). Extrafloral nectaries located at the base of the inflorescence (B); and the principal visitor of the EFNs, the ant *Camponotus planatus* (C).

Effects of ants on non-pollinators and pollinators

To test the role of ants on the non-pollinators and pollinators, an experimental approach was carried out with 4 treatments in 100 plants (25 for each treatment) of *V. luteola*. The first treatment (NA) consisted of ant exclusion from plants by applying Tanglefoot™ at the base of plants which allowed the arrival of flying floral visitors. For the second treatment (WA) ants and the other visitors could roam freely over the entire plant; while in the third treatment (RA) the ants were excluded by adding Tanglefoot™ to the base of plants and ant dummies were placed on the main petal of the flower. Finally, in the fourth treatment (CG) the ants were excluded by adding Tanglefoot™, above every flower we put one dummy ant (1.5 cm length, US Toy Company) whitout antennas and legs. These were used to test if the ant shape could be identified by the floral visitors, or if they only detected some object on the petals. We observed 5 flowers per day during five days, and these were marked at a height of 30 cm above the ground. There were no neighboring plants around each *V. luteola* plant as to avoid the ants jumping from other plants. The artificial ants were fixed on the petals with glue, in total 50 artificial ants were utilized for this experiment.

Statistical analysis

All statistical analysis was performed in the R statistical software (R Development Core Team, 2016). Differences in percentage of flower visited were calculated by Chi-square test. Initially, we used a T-test to evalute if visitation time (in seconds) of pollinators differ from visitation time spent by non-pollinators on the flowers of *V. luteola*. Then, we used one-way ANOVAs to test if visitation time (in seconds) of pollinators and non-pollinators differ between treatments on the flowers. Post hoc Tukey test was used to determine significant differences between treatments. The normality of each variable was tested by using the Shapiro-Wilk test ($p < 0.05$).

Results

Taxonomic representativity of arthropods visiting flowers and EFNs

We quantified and measured EFNs present in 16 inflorescences (one per plant) of *V. luteola*, presenting 4.95 ± 1.06 (EFNs mean ± SD, N = 80) per inflorescence; the EFN size was 0.95 ± 0.18 mm (mean± SD, N = 80) diameter. On the flowers, we recorded a total of 2,017 visits, distributed in 41 morphospecies; while for the EFNs we recorded 1,486 visits of 19 morphospecies of arthropods (Fig 2).

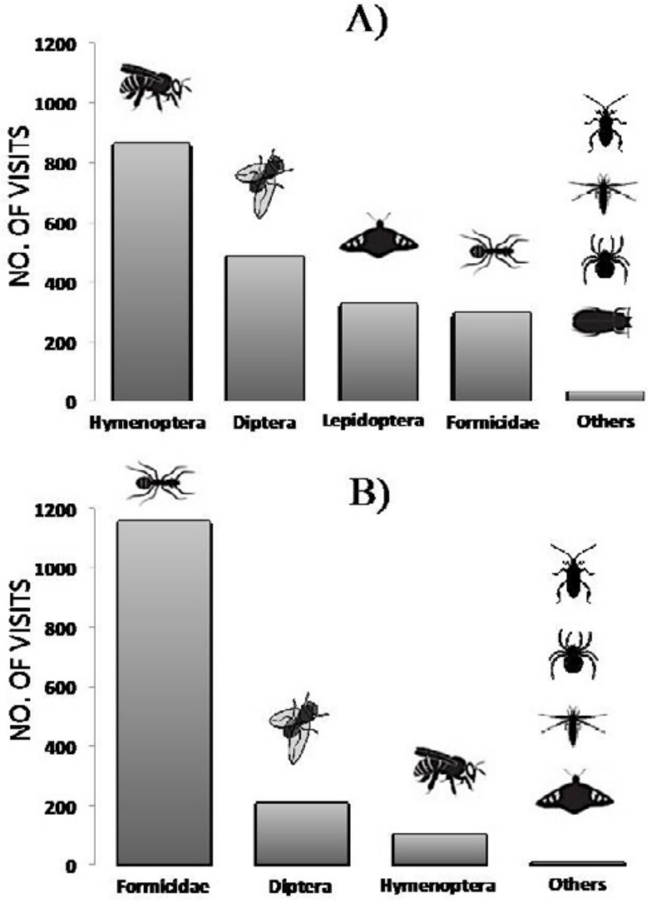

Fig 2. Number of visits of floral (A) and extrafloral visitors (B) in *Vigna luteola*. In flowers the most important visitors were bees and flies (A), while in extrafloral nectaries were ants and flies (B). We registered other non-pollinators like butterflies, thrips, spiders, beetles and bugs (Hemiptera).

For both flowers and EFNs, the order Hymenoptera showed the highest number of visits. In the case of flowers, arthropods visitors were by Apoidea and other Hymenoptera, which accounted 866 of visits, represented by 7 morphospecies of bees and 4 morphospecies of ants,

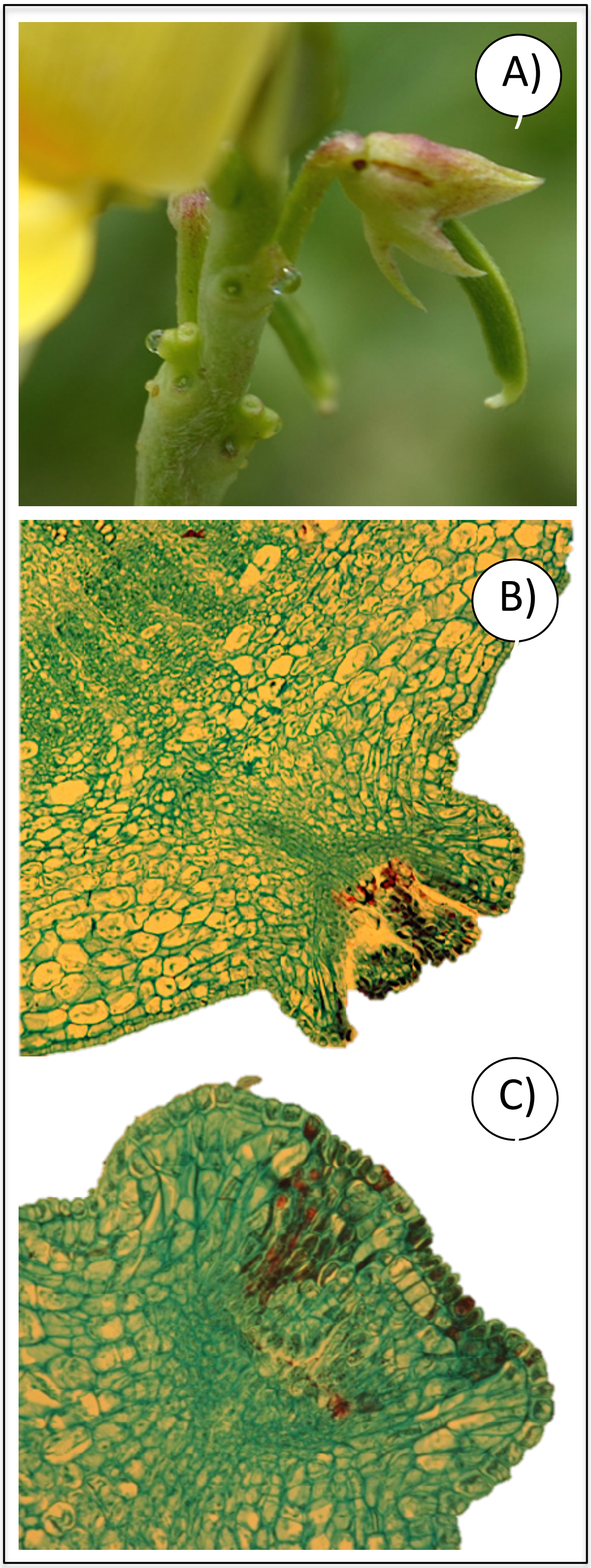

Fig 3. Extrafloral nectaries secreting nectar (A); longitudinal sections of EFNs showing the main structures, like central depression and the nectary parenchyma cells (B,C).

followed by Diptera with 489 visits (11 morphospecies), Lepidoptera with 329 visits (12 morphospecies) and the remaining orders accounted for 1.63% and 7 morphospecies in total, represented by Coleoptera with 3 (4 visits), Araneae with 2 (2 visits), Hemiptera with 1 (3 visits), Thysanoptera with 1(24 visits) (Fig 2A). For the EFNs, 84.2% of the visits were made by Hymenoptera, showing a strong predominance by the family Formicidae (77.2%), with a total of 1159 visits made by 4 morphospecies. On the other hand, Apoidea was only represented by one genus *Melipona*, which had 105 visits. The remaining visits corresponded to Diptera (with 9 morphospecies) (210 visits), Lepidoptera (2 morphospecies) (8 visits), Thysanoptera (2 morphospecies) (2 visits), Hemiptera (1 visit) and Araneae (1 visit) (each with one morphospecies) (Fig 2B).

The most frequent floral visitor was a bee of the genus *Megachile* (*Pseudocentron*) (Megachilidae), with a total of 600 visits, which represented 29.7% of the total visits. Regarding the EFNs, the most frequent visitor was the ant *Camponotus planatus* Roger with 953 visits (63.5% of the total visits).

Morphology and anatomy of EFNs

EFNs are located at the base of floral pedicels (Fig 3A), they are raised above the lamina with a central depression (Fig 3B) and they have no direct communication with the leaf vasculature. At the middle of the depression is located the nectary parenchyma cells (Fig 3B). The epidermis is formed by square cells, with a thin cuticle that becomes thicker from the center of the depression towards the margins of the EFN (Fig 3C).

Effect of the presence of ants on floral visitors

Our results showed that the percentage of flowers visited remained the same in the treatments without ants (NA), with ants (WA), and artificial ants without antennas and legs (CG), while in the treatment with only artificial ants (RA) the percentage of visits of both floral visitors and the potential pollinator (*Pseudocentron* sp.) was lower ($X^2 = 36.174$; df = 3; $p < 0.001$) (Fig 4A), reducing visits to almost 87.2% for the case of *Megachile* (*Pseudocentron*) sp. (Fig 4B) and to 65.2% for the remaining non-pollinators (Fig 4C).

Also when we compared the visitation times of *Megachile* (*Pseudocentron*) sp. Against the other non-pollinators we found that it was much less, 2.2 ± 0.11 (mean seconds ± SE; N = 70 visits); while other floral visitors spent more visitation time the flowers 9.79 ± 0.55 (N = 242 visits) (t = -7.61; p = 0.001). When we analyzed the visitation timeon flowers only by *Megachile* (*Pseudocentron*) sp. We found no difference between treatments (F = 0.73; df = 3; p = 0.53) (Fig 5A); the same pattern was observed when we analyzed the visitation time of the other floral visitors (F = 2.38; df = 3; p = 0.07) (Fig 5B).

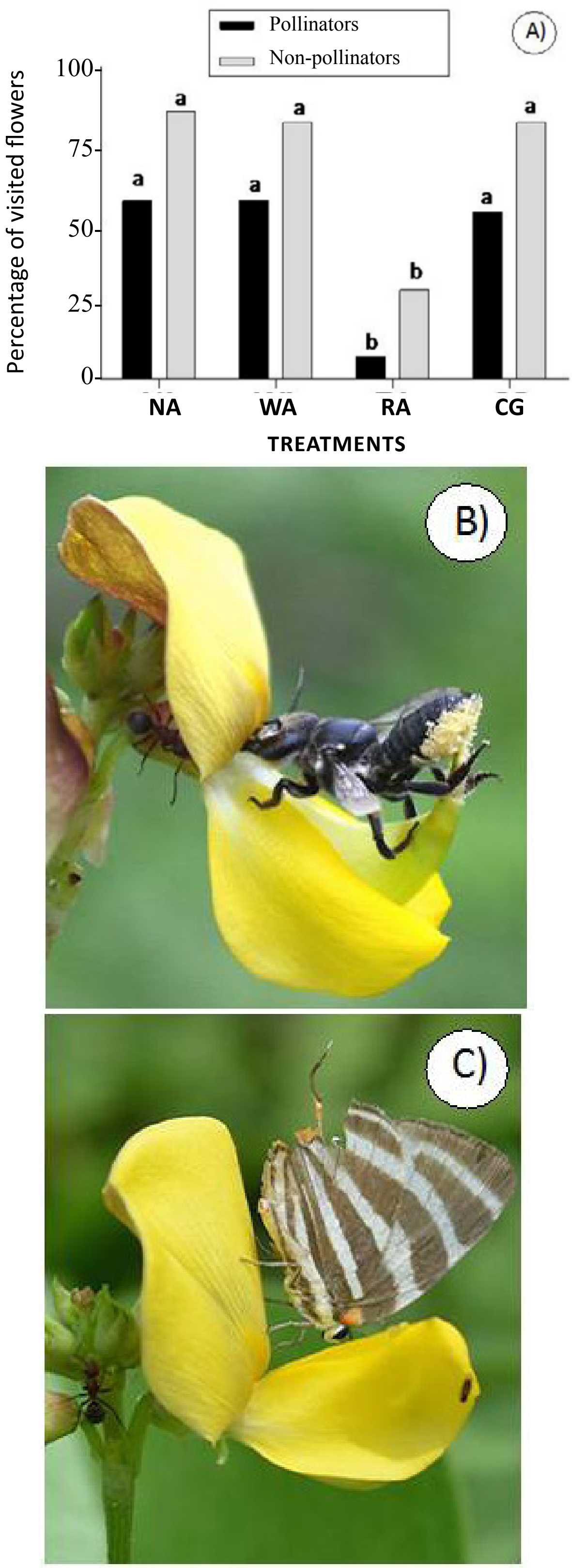

Fig 4. Percentage of flower visits in each treatment. There were less percentage of flower visited with the presence of dummy ants (RA), in the other treatments the visits were similars (A). NA = No ants (control); WA = with ants; RA = plastic ants on the flowers; CG = plastic ants without legs and anthenas. We show the pollinator *Megachile* (*Pseudocentron*) sp. of *Vigna luteola* (B), and behind the flower we appreciate to *Camponotus planatus* (B) and one of non-pollinators, a butterfly robbing nectar (C). Different letters show differences between treatments.

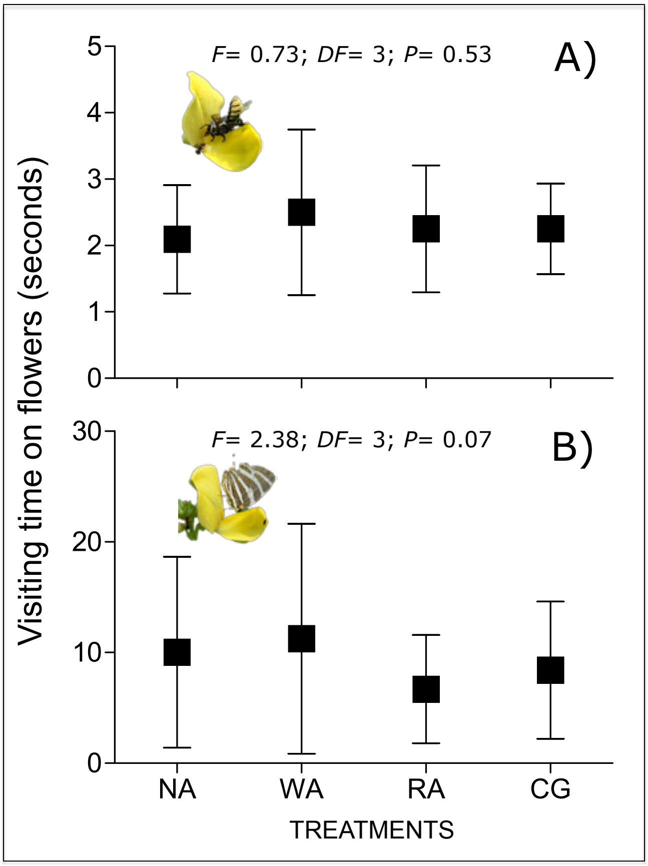

Fig 5. Time spend in the flowers in the four treatments by the pollinator *Megachile* (*Pseudocentron*) sp. (A), and by the non-pollinators (B); in both cases not found differences in the visiting time, however, the time on the flowers was three times less by the bee *Megachile* (*Pseudocentron* sp.). NA = No ants (control); WA = with ants; RA = plastic ants on the flowers; CG = plastic ants without legs and anthenas. Black boxes correspond to mean, and de lines to standar error.

Discusion

Although we found a moderate diversity of organisms associated with the flowers and the extrafloral nectaries of *V. luteola*, we detected a greater richness and frequency of visits associated with the flowers. Other studies in *H. pteropetala* (Malpighiaceae) have shown that the ant community dominates the visitors to both flowers and EFNs, with a total of 10 species of ants visiting both types of structures, while in the flowers only 5 species of bees have been reported. The most frequent ant visitors to the EFNs were two species of *Camponotus* and *Ectatomma tuberculatum* (Olivier) (Assunção et al*.*, 2014). In *Senna mexicana*, 9 species of ants were reported in the inflorescences, of which more than 80% of the visits corresponded to 4 species of *Camponotus* and to *Brachymyrmex obscurior* Forel with a total of 14 species of pollinators (Jones et al*.*, 2017). In other species of *Vigna* in Argentina, several groups of arthropod visitors have been reported to the EFNs, for example, ants, aphids, bruchids, beetles, Orthopterans, Heteropterans and leaf-cutting ants. For *V. luteola*, the presence of ants and aphids has been reported. The ants were identified as *Linepithema humile* (Mayr), *Solenopsis* sp., and *Acromyrmex lundi* (Guérin-Méneville) (Ojeda et al., 2013). While other studies report the presence of *Camponotus* with different intensity in mutualisms (Agulló et al*.*, 1993; Koptur, 1992b; Koptur et al*.*, 2010). In contrast, in our study, we found only one species of ant associated with the EFNs, *C. plantatus*, which could have up to two individuals per inflorescence and presented a very aggressive behavior.

The EFNs structure of *V. luteola* is very similar to most EFNs of Fabaceae species, which is considered of taxonomic value (Elias, 1983). In *Vigna candida* the EFNs arise from the aborted floral buds, but in *V. luteola* these originate at the node of inflorescence. The number and distribution of EFNs on *V. luteola* are comparable to those on *Vigna candida,* which have one to four EFNs in the nodes of the pedicels in the inflorescence (Gonzalez & Marazzi, 2018). But the EFN morphology of *V. candida* is volcano-shaped, in the central part of the EFNs there is an abscission region for nectar secretion and the EFNs may have two vascular bundles that reach the nectary parenchyma. Whereas in *V. luteola*, the cup-shape EFNs, the secretory structure in the central part and the absence of vascular bundles makes it anatomically different to those of *V. candida*. In Fabaceae, the elevated EFNs are typically located on the leaves (or petioles), but in *V. luteola* they are located on the inflorescence, which has a morphological evolutive implication because the EFNs can evolve independently from their bearing organ (Marazzi et al*.*, 2013). Elevated EFNs in Fabaceae has been described for the genus *Senna*, *Chamaecrista* (Pascal et al*.*, 2000), *Erythrophleum*, *Mimosa*, *Lysiloma*, *Acacia*, *Albizia*, *Inga* and *Leucaena* (Gonzalez & Marazzi, 2018). The typical organization of elevated EFNs involves the secretory epidermis and a nectary and subnectary parenchyma (Gonzalez & Marazzi, 2018).

It has been shown that EFN-bearing plants (or those that produce more nectar) have a large number of ants foraging on the foliage compared to plants without nectaries or with a low nectar production (Hernández-Cumplido et al*.*, 2016). Most of the studies have focused on the effect of this patrol of the ants on the plants to detect potential herbivores. However, little is known aboutthe other indirect interactions involving ants and EFN-bearing plants. Here, we present empirical evidence that objects resembling ant shapes on flowers were able to repel visually oriented floral visitors of *V. luteola*. These trait-mediated indirect interactions involving ants have been reported in the literature, as such: the presence of ants on plants can deter the oviposition of butterflies, pollination rate, seed dispersers, which could negatively (i.e., repelling mutualistc) or positively (i.e., repelling antagonists) affect the fitness of their host plants (Nascimento & Del Claro, 2010; Assunção et al*.*, 2014). However, we found that the pollinator of *V. luteola* (the bee *Megachile* (*Pseudocentron*) sp.) shows a strategy to visit the flowers without being aggressively attacked by the ants that visit the extrafloral nectaries. Specifically, we observed that the visitation time of the pollinator on the flowers is less than that compared to the non-pollinators. This behavior allows even though ants can sometimes scare away the pollinator, they have already performed their pollinating function (as shown in the video - Supplementary Material). Therefore, the presence of ants on individuals of *V. luteola* could have a dual function: protecting plants against potential herbivores and filtering flowers against potential nectar thieves, since non-pollinators spend a lot of time on the flowers, which allows the ants to scare away them. On the other hand, Barônio and Del-Claro (2018) observed in two species of Malpighiaceae that there was a decrease in the amount of time of visits by pollinators as ant density increased, negatively affecting the fruit set of these plants. In our study it was not possible to evaluate the effect on reproductive success, because the plants were consumed by free-ranging livestock.

However, one might assume that the presence of the ants could favor a greater cross-breeding of the plants, since the ants deter the bee *Megachile* (*Pseudocentron*) sp. which could probably be forcing this bee to visit more flowers in less time, and thus promote a higher flow of pollen. Finally, it is interesting how the use of artificial models can help to understand some biological processes involved with aspects of behavior and identification of floral visitors, or interactions such as predatory caterpillars or seed removal (Assunção et al*.,* 2014; Dáttilo et al*.,* 2016).

Conclusions

The presence of EFNs on plants may enhance the mutualistic interactions between potential pollinators and other organisms with some interactions being antagonistic. This study clearly shows that floral visitors are capable of detecting potential dangers such as aggressive ants and may avoid or spend less time visiting the inflorescences of plants and future evaluation of this aspect is important. The low number of ants and pollinators detected in our study as compared to other published studies may be due to the fact that the plants were located in a coastal dune where ant diversity may be reduced in this type of habitat. We also document the morphology and histology of EFNs of this species. More study is required to determine the effect that ants may have on the reproductive success of *V. luteola*.

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Supplementary Material

Megachile (*Pseudocentron*) sp. visiting *Vigna luteola* flower, in which we can appreciate that *Camponotus planatus* removes it and remains patrolling the flower surface.

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SUPPLEMENTARY MATERIAL

Video 1. *Megachile* (*Pseudocentron*) sp. visiting *Vigna luteola* flower, in which we can appreciate that *Camponotus planatus* removes it and remains patrolling the flower surface.