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ORIGINAL RESEARCH ARTICLE

Non-pollinating floral visitors of the *Cucurbita* genus plants and their relationship with the presence of pollinating bees

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ABSTRACT

Flower-visiting insects may be pollinators or, conversely, unrelated to the reproductive process of plants. Interactions between pollinating and non-pollinating flower visitors can negatively influence pollen transfer. Little is known about the effects of bee visits on pollination of squash *(Cucurbita* spp.) flowers and their interactions with the presence of other floral visitors. The study was conducted at the Facultad de Ciencias Agrarias (Universidad Nacional de Rosario) in the south of Santa Fe (Argentina) and evaluated the effect of the presence of non-pollinating floral visitors on bee foraging in the flowers of two cultivated squash species. Flower sex and squash species *C. maxima* and *C. moschata* were included as variables. A total of 937 visitors were recorded in 403 flowers. Bees of the tribes Eucerini and Apini were the most abundant pollinators with an average of 2.3 individuals per flower during 10 minutes of observation. Diptera, flower sex and squash species did not influence the number of bee visits, whereas the prolonged stay of coleoptera and formicids negatively affected the presence of bees on both squash species. The presence of coleoptera reduced bee visits by 38%, while in the presence of ants, bees did not visit the flowers. The theft of nectar and pollen by non-pollinating floral visitors could have a negative effect on the reproductive success of squash.

Keywords: Coleoptera; Diptera; Formicidae; Interaction; Squash

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1. Introduction

Biological pollination by native insects is a common good provided by the ecosystem for agricultural producers and therefore this ecological process is considered an ecosystem service. Pollination contributes to human well-being by maintaining and enhancing fruit and seed production in natural ecosystem species and crops within agricultural ecosystems. Ecosystem services depend on biodiversity and vice versa, because they involve a complex set of direct and indirect interactions between multiple functional groups of organisms such as herbivores, pathogens, predators and pollinators[1]. Floral visitors can play different roles in the flower, such as pollination in the case of pollinating bees, or be unrelated to the reproductive process, for example, herbivorous arthropods, although the latter consume nectar or pollen[2] or yeasts that decrease sugar concentration[3]. Some visitors may prey on floral structures such as petals, sepals and stigmas; for example, adult beetles of *Diabrotica speciosa* are known and very damaging predators of squash flowers[4].

Flower herbivory has indirect negative effects on pollinator visitation frequency due to reduced corolla size, flower number, flower lifespan, quantity and quality of available pollen, and destruction of entire sex organs[5–10]. Nectar consumption by non-pollinator visitors can produce changes in the behavior of legitimate pollinators by altering flight distances, the number of flowers visited, and the time spent at each flower[11], thus contributing to an antagonistic relationship between pollinator and non-pollinator visitors.

On the other hand, interactions between different visitors can influence the role of the main pollinator; for example, aggressive visitors that displace other floral visitors[12]. Ants are considered aggressive visitors because of their role in defending plants against herbivorous arthropods[13]. Several studies show an antagonistic relationship between different ant species with pollinators such as bees[14,15]. In fact, some plants have developed systems to protect their flowers by producing extrafloral nectar collected by ants and differentiated from floral nectar used by pollinators[16,17]. An example of these plants with extrafloral nectaries are several species of the genus *Cucúrbita*, since in addition to having nectar glands in the flowers that are related to crop pollination, they also have this type of glands in vegetative parts related to ant attraction[18]. Other plants have structures that hinder access to nectar by non-pollinating insects, such as trichomes on the nectaries or smooth, slippery and adhesive surfaces on the petals[19].

Coleoptera or Diptera can negatively influence the frequency of visits of main pollinators to flowers by consuming nectar and/or pollen[2,20,21]. *Diabrotica speciosa*, for example, is a very abundant coleopteran in squash crops, which in addition to feeding on leaves and petals, are consumers of pollen and nectar[22].

Fruit production in squash crop is pollinator dependent by having separate male and female flowers (dichlino-monoecious plants)[23]. Plants initially produce only male flowers and continue to produce them usually in a higher proportion to female flowers[24]; of the total female flowers produced by a plant, only 20–50% reach harvest as fruits. Flowers of both sexes of *C. maxima* and *C. moschata* produce nectar, although female flowers produce greater quantities than male flowers[24,25]. Added to this, size and floral morphology play an important role in the frequency of visits received by each flower type and among different *Cucurbita* species. For example, the male flowers of *C. moschata* possibly receive more pollinators than the female ones, because the corolla allows a faster and easier access of the pollinator to the nectary, which is larger and has a wider opening; at the same time, the flowers of *C. maxima* are smaller than those of *C. moschata* and the female ones have the style and gynoecium closer to the petals[24].

In squash, pollen grains are heavy, sticky and have pollen cement[26], and pollination has been described as entomophilous[27]. In South America, different species of bees have been found pollinating species of the genus *Cucurbita*. Among them, the most abundant are *Apis mellifera* (Tribe: Apini)[23,26,28–30,26] and *Trígona spinipes* (Tribe: Meliponini)[29]. In Central America the most abundant bees are *A. mellifera*[31,32], *Augochlora nigrocyanea* (Tribe: Augochlorini)[32,33], *Xenoglossa gabbi* (Tribe: Eucerini)[34] and species of the genus *Peponapis* (Tribe: Eucerini)[31,33,35].

It was hypothesized in this study that bee visits to squash flowers are negatively affected by the presence of non-pollinating floral visitors. Specific predictions were as follows: (1) *Cucurbita* flowers with non-pollinating floral visitors receive fewer visits from pollinating bees than flowers without; (2) the magnitude of the reduction in the number of bee visits in flowers depends on the identity of the non-pollinating floral visitor; and (3) *Curcurbita* species and flower sex differentially affect the number of bee visits per flower.

2. Materials and methods

The research was conducted in Santa Fe, Pampas region of Argentina, during the December to February 2019, which corresponds to the squash flowering season. Previously, this region was an extensive grassland that has become one of the largest agricultural regions in the world (~5 Mha)[36]. Herbicide-tolerant soybean and corn are the predominant summer crops, while fallow fields abound in winter[37]. Horticultural crops are restricted to small orchards near cities.

Three orchards in the south of the province of Santa Fe were selected for this study: one in the town of Funes (32°53′33.10″ S 60°49′50.16″ W) and two in the town of Zavalla (30°01′52.43″ S 60°53′50.02″ W and 33°01′49.32″ S 60°53′03.48″ W). The predominant soils in the area are Mollisols and the climate is temperate. Samples and observations were taken in the warm season with an average of 24 °C per day and an average rainfall of 110 mm per month. In the research orchards, *C. maxima* Duch. and *C. moschata* Duch. were planted simultaneously in plots of approximately 100 m^2, at a distance of more than 1,000 m from each other and with controlled agroecological management.

On sunny days with low wind speed, during the anthesis hours (5:30–13:30), random sampling stations corresponding to different plants were established in each orchard. The data recorded the results of evaluations on 403 flowers, 337 male and 66 female, belonging to 226 of *C. maxima* and 177 of *C. moschata*, during a total of 67 hours distributed in 16 days.

At each sampling station, a squash flower was observed for 10 min and records were taken on the type of floral visitors present on the corolla, squash species and sex of the flower. Floral visitors were classified as: (1) visitors of floral Pollinating (VFP) are those that remained for 10 sec or more in contact with the reproductive organs of the flower in search of nectar or pollen; in addition, they presented morphological characteristics adapted for the collection and dispersion of pollen, such as the presence of combs or corbiculae on the hind legs, presence of hairs on the abdomen, and remained in the flower for a period of time less than the complete observation period, that is, they have a high degree of displacement between flowers; (2) non-pollinating visitors (NPV), those that did not present the morphological characteristics mentioned above and that also remained in the same flower for a time greater than 10 min, with little displacement between flowers. In each observed flower, the number of bees or VFPs was counted to generate the variable number of bee visits, also taking into account the identity of each VFNP observed in order to evaluate the possible differential effect of each of these on pollinators. The importance of each VFP was rated according to the number of visits recorded; those whose appearance was less than 6% of the flowers evaluated were not considered in the comparative analysis. Each floral visitor was recorded in a photo and once captured, it was preserved in an insectary. Subsequently, each specimen was observed under a 40x magnifying glass and with the help of bibliographic records[38–40] were taxonomically classified to the lowest possible level; in the case of not being able to reach species, the concept of morphological species was used.

A binary data matrix included squash species, flower sex, VFNP and presence/absence of bees (VFP). This matrix was used to determine the ‘probability of bee presence’, that is, the probability of a flower occupied by VFNP for more than 10 min of being visited by bees (VFP). For this variable, the Bernoulli probability distribution was considered, due to the nature of the data (non-clustered binary). The matrix for the variable Number of Bee Visits contained the same variables mentioned above, but the response variable reached values between 1 and 15. The error distribution was determined through the Fitdist function of the Fitdistrplus package[41]. The probability of presence and number of bee visits were analyzed by Generalized Linear Models (GLMs) using the GLM function of the lme4 statistical package[42]. All data analysis and graphs were performed by means of the statistical software R Project[43].

The categorical explanatory variables of the corresponding models were in order of importance: presence of VFNP, *Curcurbita* species and flower sex. The most ‘parsimonious’ model was chosen by the maximum likelihood method through the Anova function. The plots were constructed with the Visreg function of the same package and are of contrast type[44]. To assess the quality of the fitted models of the binary response variable Probability of Presence, the normality of the residuals was observed using the DHARMa library[45] with 500 simulations. ROC (Receiver Operating Characteristic) and AUC (Area Under the Curve) curves corresponding to each

Table 1. Floral visitors recorded on squash flowers

Order	Superfamily	Family	Subfamily	Tribe	Genre	Species
Visitors of Pollinating floral (VFP)						
Hymenoptera	Apoidea	Apidae	Apinae	Apini	*Apis*	*A. mellifera*
			Apinae	Eucerini	*Peponapis*	*P. fervens*
			Apinae	Eucerini	*Thygater*	*T. analis*
			Apinae	Eucerini	*Melissoptila*	-
Low-abundance VFP						
Hymenoptera	Apoidea	Apidae	Apinae	Bombini	*Bombus*	-
		Halictidae	Halictinae	Caenonalictinis	-	-
Lepidoptera	Pyraloidea	Crambidae	Spilomelinae	Margaroniini	Diaphania	*D. hyalinata*
Non-pollinating floral visitors (NPV)						
Diptera	Ephydroidea	Drosophilidae	-	-	-	-
Coleoptera	Chrysomeloidea	Chrysomelidae	Galerucinae	Luperini	*Diabrotica*	*D. speciosa*
Hymenoptera	Vespoidea	Formicidae	Formicinae	Plagiolepidini	*Plagiolepis*	*P. alluaudi*

Low abundant VFP: pollinating floral visitors that appeared in less than 6% of the flowers evaluated. Halictidae were recorded in 21 of the 403 total flowers and bumblebees and lepidoptera were censused outside the sampling stations (flowers).
Source: Faculty of Agricultural Sciences (National University of Rosario), Santa Fe, Argentina.

model were calculated by means of the ROCR library[46]. To assess the quality of the models for the Poisson response variable "number of visits", the Graphics library[43] was used as supplementary material.

3. Results

The most abundant VFP were bees of the morphological Tribe Eucerini (3) and Apini: *A. mellifera* (1) (**Table 1**). A total of 937 individuals and an average of 2.3 visits per flower during 10 min were recorded. In some flowers no bees were observed pollinating, while in others a maximum of 15 individuals were recorded. The bees visited a total of 271 flowers of which 192 did not record VFNP. In those flowers the average was 2.7 visits per flower for 10 min. VFNP individuals were present in 132 flowers including: 76 small Diptera (<5 mm) of the family Drosophilidae, 28 Coleoptera of the species *D. speciosa* and 28 Formitidae of the species *Plagiolepis alluaudi* (**Table 1**; **Figure 1**). Bee visits in flowers occupied by VFNP were, on average, 2.2, 1.5 and 0 every 10 min, in flowers with presence of Diptera, Coleoptera and ants, respectively.

The Probability of Bee Presence was significant ($P < 0.001$) only for the variable Presence of VFNP, excluding the variables *Cucurbita* Species and Flower Sex (**Table 2**). No bees were observed on flowers with *P. alluaudi* present (**Figure 2**); consequently, for the second model with the response variable number of bee visits the *P. alluaudi* category was not incorporated. The Poisson probability distribution was the best fit for the response variable number of bee visits. The most parsimonious and explanatory model was the one that included the variable presence of VFNP ($P < 0.05$) and excluded the other variables (**Table 2**). Multiple comparisons between VFNP and flowers without VFNP presence showed that there were differences ($P < 0.05$) in the number of bee visits between *D. speciosa* categories and empty flowers; on the other hand, pollinator bee visits decreased 38% in flowers with coleopteran presence (**Figure 3**). On the other hand, the presence of Drosophilidae had no significant effect (**Table 2**).

Figure 1. Non-pollinating floral visitors (NPVs) found on squash flowers.

Table 2. Models constructed for each response variable and the significances of the explanatory variables included

Model	AIC	Gl	VFNP	Species of cucurbita	Sex of the flower
Probability of bee presence (error distribution: Bernoulli)					
VFNP + Cucurbita+ species sex of the flower	427.8174	6	P < 0.001	0.95506	0.07681
VFNP+Species	428.9485	5	P < 0.001	0.95506	-
VFNP	426.9517	4	P < 0.001	-	-
Number of bee visits (error distribution: Poisson)					
VFNP + cucurbita+ species sex of the flower	1017.041	6	P < 0.05	0.78106	0.20514
VFNP + species	1016.646	5	P < 0.05	0.78106	
VFNP	**1014.724**	4	**P < 0.05**	-	-

Source: Faculty of Agricultural Sciences (National University of Rosario), Santa Fe, Argentina.
AIC: Akaike information criterion, VFNP: non-pollinating floral visitors, Gl: degrees of freedom.

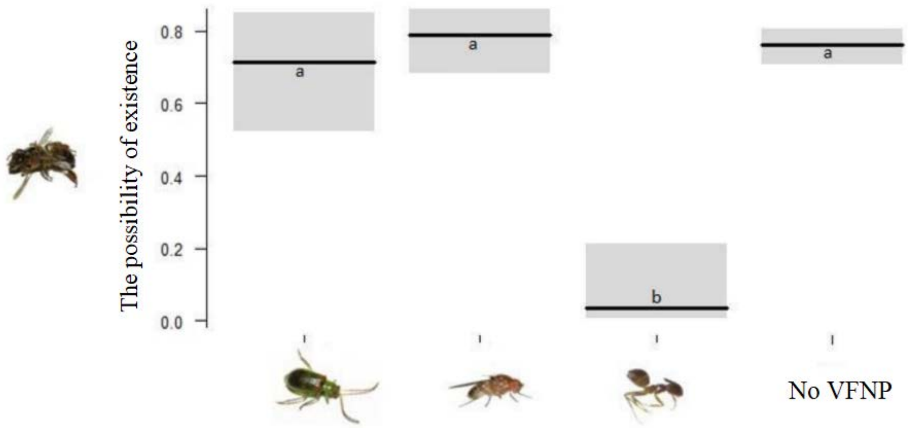

Figure 2. Probability of presence of pollinating bees in *Cucurbit* flowers with *Diabrotica speciosa,* Drosophilidae, and *Plagiolepis alluaudi,* and in flowers without VFNP (in order from left to right).
Note: The probability of pollinator bee presence decreased with the presence of *P. alluaudi* (P < 0.001).

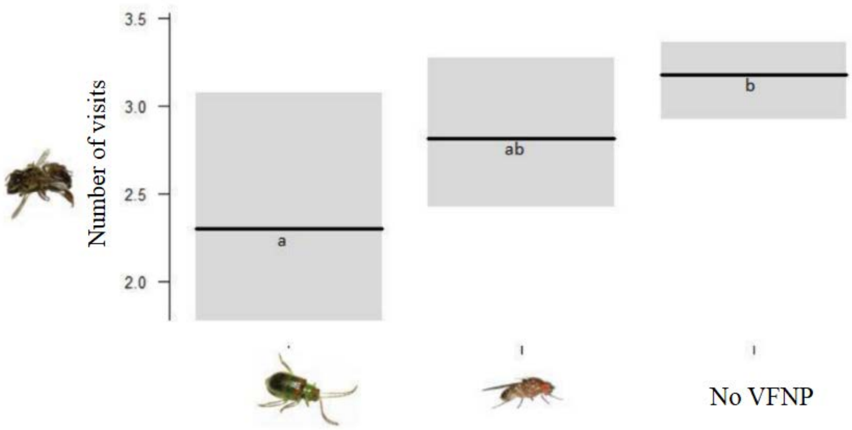

Figure 3. Average number of bee visits every ten minutes in flowers with *Diabrotica speciosa* and Drosophilidae, and without non-pollinating floral visitors (NPV). (in order from left to right).
Note: Visitation values are expressed as the result of the link function used by the Logit model. The horizontal lines on the x-axis represent the values for each category of the explanatory variable VFNP. Flowers with *D. speciosa* present had 38% fewer bee visits than empty flowers (P < 0.05).

From top to bottom: Female flower of *Cucúrbita moschata* with Drosophilidae and a bee feeding on nectar obtained from the floral nectaries. Male flower of *C. maxima* with *Diabrotica speciosa.* Male flower of *C. maxima* with *Plagiolepis alluaudi.*

4. Discussion

In several studies, representatives of the tribe Eucerini were found in different cultivated cucurbitas, including: *C.ficifolia, C. maxima, C. mixta, C. moschata, C. pepo* and in wild cucurbitas[23,27,31,33–35,47,48], while *A. melífera* was recorded only in some cultivated species such as *C. maxima, C. moschata and C. pepo*[24,26,29,30,47,49,50]. Although in this study it was observed that many squash flowers were not visited by bees due to the interference of VFNPs, visits were recorded in flowers without VFNP being, on average, 15.6 visits/flower and per hour, with a maximum of 90 visits; these values are high compared to other studies[30,49]. It is possible that at the time of the observations the recorded wild pollinator community was actively foraging the crop flowers without VFNP during anthesis being, on average, 93.6 the number of bee visits/flower during the whole floral opening.

Generally, squash flowers host Coleoptera **(***Diabrotica* sp., *Acalymma vittatum* and *Cyclocephala borealis)* and small Diptera (Drosophilidae) that stay up to 30 min in the flowers[24,26,47,50]. In this work, some of these groups were found to interfere with pollinator visits, so their presence would have impacts on the pollen transfer process in this crop.

No conclusive results were found on the possible influence of floral morphology and nectar and pollen supply on bee visits between floral sexes and *Cucurbita* species. Although some authors in Nigeria, Peru, and Italy did observe a higher number of bee visits on female flowers than on male flowers of *C. moschata* and *C. pepo* [24,51,52], although these were mainly by *Apis.* In contrast, the observations in the present study found members of the tribe Eucerini, whose genera are considered specialized pollinators for the genus *Cucúrbita*[35,48]. Both these bee genera and the genus *Cucúrbita* are native to the Americas[27,53,54] as evidenced by old records of some bees for Argentina[55-57]. It is possible that the foraging of eucerine bees is not differential between flower sexes due to the specialization of the genus mentioned above.

Ant behavior on flowers varies by species, some are aggressive, negatively affecting bee and bumblebee visits[14,15,58,59], while other species are not and their presence has shown no effect on pollinator activity in Boraginaceae and Orchidaceae[60,61]. In other cases, ants favored the presence of some large pollinators such as bumblebees of the genus *Xylocopa* in flowers of *Melastoma malabathricum***,** by deterring smaller and less efficient pollinators such as Halictidae of the genus *Nomia*[62].

Acuña-Perandrés[63] considers *Pheidole pallidula* as important in the pollination of *C. pepo*; although he considers that ants experience a great loss of pollen load that must be compensated with a high frequency of visits. In general, the role of ants as pollinators was discarded in this study, because the antecedents indicate that ants have little specificity in flower selection, do not have specific structures for pollen transport, and some species inhibit pollen grain germination by secretions from metapleural glands[19]. In addition, pollen grains in squash are comparatively large (0.1–0.2 mm in diameter)[24,26] to be transported by small insects, flowers remain open for few hours and the mobility of ants between flowers is very low probably as a consequence of the high nectar source in the flower. In addition, they tend to be pollinators of plants with dense inflorescences because of the type of movements they make, which is mainly within and between nearby inflorescences[64,65].

Some works show that by simultaneously increasing pollination and pest control, reproductive yields are exceeded compared to the sum of each activity separately[26,58,66]; a possible explanation could be the response of bees in the presence of coleoptera, as confirmed in this study. Bees tend to avoid C. *maxima* flowers when *Acalymma xantographa* and other coleopterans are present[26]. However, no aggressive behavior of coleopterans towards bees was recorded in this work, so the reduction in the number of visits in flowers with *D. speciosa* could be due to the decrease in space or resources available in the flower, since this coleopteran species consumes nectar and pollen. To confirm this inference, it would be interesting to evaluate during anthesis the amounts of nectar present in flowers with or without *D. speciosa***.** Also, in addition to this direct interaction between coleopterans and pollinators, a possible indirect interaction with an important role on bee foraging behavior caused by *D. speciosa* damage to flower structures should be considered. *speciosa* to flower structures (petals, nectaries, and buds) that impact bee attractiveness and reduce visits[6,7,29,67,68]. In addition, nectar and pollen theft by VFNP such as ants and coleoptera may have an overall detrimental effect on pollen deposition and fruit production in flowers with VFNP[69] so it would be interesting to evaluate this fact in future research.

5. Conclusions

The prolonged stay (>10 min) of ants and coleoptera on the flowers of both squash species negatively affected bee visits. The presence of *Plagiolepis alluaudi* is associated with a very low probability of bee presence, while the number of visits decreases with the presence of *Diabrotica speciosa*. *Drosophilidae* sp., flower sex and *Cucurbita* species had no direct influence on bee visits to squash flowers.

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Conflict of interest

The authors declared no conflict of interest.

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