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Pollination dynamics and bee foraging cycles in a tropical squash field

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Abstract

Temporal changes in the composition and abundance of floral visitors during floral anthesis influence the rate of deposition and germination of pollen in stigmas, as well as the rate of removal of pollen from anthers. In the tropics, the squash (*Cucurbita pepo*) is visited by a great diversity of bees, but little is known regarding the dynamics of its pollinators and pollination during its floral anthesis in tropical environments. We studied the foraging cycle of different species of bees in squash crops in Costa Rica and the relationship of this cycle with nectar production, pollen loads in anthers and bees, and number of pollen grains and pollen tubes in stigmas and styles. It was determined that despite the short duration of the floral anthesis of this crop, there are changes in the composition of visitors during anthesis from bees with twilight flight capacity that visit male flowers in the opening to primarily social bees at later hours. Loss of pollen from anthers and deposition of pollen on stigmas occurs rapidly in early hours. Pollen transport by bees may be facilitated by pollen accumulation at the bottom of the corolla of male flowers. After 6 a.m., little change occurs in crop pollination levels. The dominance of stingless bees in the flowers after 6:30 a.m. appears to displace other bees from these flowers, despite the permanence of nectar flow. The *Cucurbita* species may be an important crop for maintaining bee diversity and enhancing the pollination of other crops in agricultural ecosystems.

Keywords Squash · Pollination · Nectar foraging · Pollen dispersal · Pollen tubes

Introduction

Some plant species attract a range of species of floral visitors, among which there is variation in their pollination capacity. This variation is determined by the shape and size, behavior, pollen-carrying capacity, visiting hours, and local abundance of each floral visitor (Herrera 1987, 1989; Santiago-Hernández et al. 2019). In addition, there can be temporal variation in the composition of species that visit flowers during floral anthesis, a variation that depends on the daily activity patterns of floral visitors, as well as changes in the quantity and quality of resources offered by the flowers during anthesis (Willmer and Corbet 1981; Herrera 1990; Stone et al. 1996; Rader et al. 2013). Although turnover of pollinators during anthesis may be derived from differences

between pollinators in the optimal foraging environmental conditions (e.g., temperature, humidity, and insolation) (Herrera 1990), evolutionary processes can stabilize or increase the temporal separation of floral visitors during the anthesis of a plant species. For example, temporal separation can be enhanced by competition between pollinators (interference reduction) or by plant timing of floral rewards to match the activity peaks of the most efficient pollinators or to prevent hybridization between co-flowering plant species (Stebbins 1970; Willmer 1983; Herrera 1990; Stone et al. 1996; Ali et al. 2015).

The effects of diversity and temporal changes in pollinator species on the reproduction of plants are important factors to evaluate the effect of the loss of pollinator diversity and abundance in cultivable species and natural ecosystems (Winfree et al. 2018). Greater diversity of bees in agricultural crops has been associated with higher productivity of certain species of cultivated plants (Hoehn et al. 2008), and it has been suggested that changes in the composition of pollinators during floral anthesis promote complementarity in the pollination process, which increases the probability of deposition of pollen loads (Pisanty et al.

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2016) and can offer greater resilience to temporal or spatial changes in pollinator composition (Rader et al. 2013; Delgado-Carrillo et al. 2018; Winfree et al. 2018).

Cultivated species of the genus *Cucurbita* are monoecious plants, the transfer of pollen being obligatorily mediated by pollinating bees. Both staminate and pistillate flowers produce nectar secreted by nectaries located at the base of the reproductive structures (Nepi and Paccini 1993). In staminate flowers, the nectaries are inside a chamber or recess accessible through three nectary pores (Nepi and Pacini 1993). The anthesis of the showy yellow flowers occurs in the morning period. The floral visitors include several species of the subgenera *Eucera* (*Peponapis*) and *Eucera* (*Xenoglossa*), native to America, which are oligolectic bees specialized in collecting pollen exclusively from *Cucurbita* (Hurd et al. 1971). These bees have been identified as effective pollinators of the wild and cultivated species of *Cucurbita*, and their current distribution range has been attributed to the expansion of the crop (Hurd et al. 1971). However, both in temperate and tropical environments, a wide diversity of species of bees that visit these crops have been reported. In Neotropical regions, the most frequent species observed are *Apis mellifera*, several species of *Eucera* (*Peponapis*), and *Bombus*, as well as different species of the Meliponini tribe and the Halictidae family (Lobo and Bravo 2021). Although the composition of pollinating bees of these crops has been frequently studied, as well as the relative efficiency in the transfer of pollen of some bee species, little is known regarding the dynamics of their pollination during anthesis. In particular, it is interesting to determine if there is a daily temporal structuring of visits by different species of bees and the relationship of these visitation patterns with the dispersal and deposition of pollen in stigmas. These analyses are of particular interest in tropical environments, where there is less knowledge regarding the pollinating role played by the different bees that visit the crop (Canto-Aguilar and Parra-Tabla 2000; Delgado-Carrillo et al. 2018).

The objective of this study was to determine the temporal sequence of visitation by different species of bees to *C. pepo* flowers in a crop located in a tropical environment and its relationship with crop pollination. To this end, we will study the daily dynamics of pollen dispersal and its deposition in the stigmas, the production of nectar in male flowers (90% of the flowers available to bees) and how it relates to the foraging peaks of different species of bees. The dynamics of visitation by bees will also be studied in a locality in a high-altitude tropical region to determine how the temporal sequence of foraging by bees changes in an environment with differences in bee fauna. It is expected that this correlative analysis between pollination and bee foraging times will provide better information on the most important bee species for crop pollination.

Materials and methods

Study area

During the year 2019, sampling of bees, anthers and stigmas and video recordings were made in a small farm in the Central Valley of Costa Rica (Piedades, 9°56′–84°13′, altitude 800 m). Additionally, bees were collected, and video recordings were made in a small farm at a higher altitude (La Cima de Dota, 9°39′–83°54′, altitude 2150 m). Both crops were relatively large (> 50 open flowers at the time of study, 200–250 m^2 cultivation area). The Piedades farm is located in a semiurban region with a landscape made up of a mixture of peripheral neighborhoods, cultivation areas and patches of secondary vegetation. A gallery forest in a river canyon is located near the visited farm. The local temperature range is 18 °C min-28 °C max, and the annual rainfall is 1947 mm. La Cima de Dota has a landscape typical of cloud forest regions (temperature range of 10 °C min-21 °C max and annual rainfall of 2600 mm), where cultivated areas and grasslands are close to extensive areas of protected primary forest. In both places, squash crops are mixed with other crops, such as citrus and beans (Piedades) or vegetables and tubers (La Cima). Both locations have a rainy season from May to November and a dry season from December to April. In both locations, a squash crop is obtained at the beginning or in the middle of the rainy season (March–May in La Cima de Dota and July–August in Piedades) with a possible second harvest in the middle or end of the rainy season (November in Piedades).

This study was performed in the first squash crop during the rainy season (March in La Cima de Dota and August in Piedades). As observed in other studies (Vidal et al. 2006), the proportion of male to female flowers in the crop was very high. Censuses carried out at the time of the observations showed a ratio of approximately 15 male flowers to each female flower.

Observations of bee foraging

To obtain a more accurate and continuous record of the activity times of different groups of bees on male and female flowers, video recordings were made on flowers continuously from 5 a.m. to 8:30 a.m. The video recordings ended at that time because no changes in the composition of the floral visitors were observed after that time. Sony HDRCX700 Handycam video cameras (San Diego, CA, USA) were used for the recordings. After 10:30 a.m., many flowers showed signs of wilting.

In Piedades, three male and two female flowers per day were chosen for filming during four successive days,

totaling 70 h of video recordings. In La Cima de Dota, filming was performed with the same number of flowers per day during two succesive days, resulting in 35 h of video recordings. This comparison allowed us to visualize the differences in the daily visitation pattern between different locations in their floral visitors (see "Results" Section).

During video recordings, bees were collected periodically for identification and to quantify pollen loads. For its identification, published taxonomic keys and the collection of the Museum of Zoology of the University of Costa Rica were used. *Eucera* (*Peponapis*) specimens were sent to T. Griswold (Bee Biology and Systematics Laboratory, Utah) to be identified at the species level. In the recordings, it was possible to identify bees at the genus level in the cases of *Megalopta*, *Trigona*, *Caenaugochlora* and *Eucera* (*Peponapis*) and at the species level in the case of *Apis mellifera* and *Bombus ephippiatus*. Although *Eucera* (*Peponapis*) and *Megalopta* are genera with two or more species in Piedades (Lobo and Bravo 2021), their distinction was not possible in recordings. As bee collections showed that *Trigona* samples were *T. corvina*, bees of this genus observed in video recordings were classified as *T. corvina*.

To quantify bee visitation rates at different times, we divided the time between 5:00 a.m. to 8:30 a.m. in 10-min intervals. The total number of bees of each group that visited each flower gender (male and female) during each time interval was recorded. Next, the average number of visits of each bee group per flower gender for the 10-min time interval was calculated. Additional observations on the behavior of different groups of bees within the flowers were obtained from these recordings.

To quantify pollinator activity between 4:30 and 5:00 a.m., a period in which light conditions prevent recording, systematic counts of bees present in flowers were made, capturing all the bees found in open flowers during 10-min time intervals. The average number of visits per flower was obtained by counting the number of bees collected by the number of flowers sampled in each time interval.

Pollen grains and pollen tubes counting

To study the correlation between the activity of the different bee groups and the dynamics of pollination of *C*. *pepo* flowers, the amount of pollen in the anthers and stigmas, as well as the abundance of pollen tubes in styles, were analyzed during different time periods in the early morning hours. Female and male flowers were collected in Piedades for five contiguous days in during the same week of video recordings. The sampling of the two types of flowers was performed continuously from 4:30 to 8:00 a.m. for male flowers and from 5:00 a.m. to 9:00 a.m. for female flowers. Final samples sizes were 40 male and 38 female flowers.

Stamens were preserved in a fixed volume of 70% ethanol, from which 10 aliquots of 10 µl were taken to count the grains in a hematocytometer to infer the total number of grains in the stamen. As the presence of pollen in male flowers at the base of the corolla was observed during collection, samples of this pollen were obtained by washing the base of corollas and quantifying it with the same method used for the anthers. Five male flowers were bagged to obtain pollen counts on anthers and corollas in unvisited flowers.

Stigmas and styles were bagged for one day and subsequently fixed in 70% ethanol to allow the growth of the pollen tubes through the style (Quesada et al. 1993). The samples were softened in 8 N NaOH for 1 day and stained with aniline blue (Martin 1959). For pollen counting in stigmas, one of the three stigmas of each female flower was chosen. Several longitudinal sections of each stigmatic lobe were macerated on microscope slides to better visualize the adhered pollen grains. One of the three upper stylar columns (united to the same sampled stigma) was cut into several longitudinal sections using a razor blade and macerated on several microscope slides to visualize the pollen tubes using the same methods to stain stigmas. To count pollen tubes, digitized images of the style sections were taken, and the pollen tubes that crossed lines drawn transversely by the style were counted to avoid counting the same tube several times.

Measurements of standing nectar crop in male flowers

To determine the amount of nectar available in male flowers at different times during the morning hours, 2–3 male flowers were collected every 20–30 min for 2 days from 4:30 to 9:00 a.m (25–27 male flowers sampled per day). The volume of nectar accumulated in the nectary chamber was determined with 5 or 20 µl capillaries, depending on the volume. Five bagged flowers were collected at 8:30 a.m. to determine the volume of nectar accumulated. Some male flowers collected after 6:30 a.m. showed signs of Trigona bee bites in the nectary pores (Lobo and Bravo 2021). This condition was scored to determine the effect of this behavior on nectar volume.

Pollen loads in bees

To obtain estimates of the number of pollen grains in bees during the observation hours, 5–10 individuals per species were collected in male flowers during different times after anthesis. The bees were collected when they finished visiting the male flower during their departing flight. Each individual collected was bathed in 1 ml of 70% alcohol to remove all the grains, and 20 aliquots of 10 µl were

observed in a hemacytometer to estimate the total number of grains in the bee body.

Data analysis

To show the rates of visitation to the flowers of different species of bees throughout the morning, visitation rates were divided in four intervals (0–1.3, 1.4–2, 2–5, > 5 bees/flower/10 min). This intervals were chosen as they represent better the scale of the observed variation of this variable.

To better represent the relationship between the number of pollen grains or pollen tube loads and the time of day, both in floral parts and in bees, linear or exponential regressions were used between the time of day and the number of pollen grains or pollen tubes. The time of day was scaled to a continuous numerical scale (serial numbers), where 0 represents 4:30 a.m. and each unit of value represents 1 h. Decimal fractions represent time intervals within each hour. For example, 5:30 a.m. would have a value of 1.5 units on this scale.

Regressions were calculated using the lm function of R software. Significance of the regressions models were tested using the F test statistic (MS regression/MS residuals), where MS regression are the mean squares explained and MS residuals the mean squares not explained by regression (MS residuals) (Chambers 1991) The coefficient of determination (R^2), the quotient between the sum of squares and the total sum of squares, were calculated for each regression to present the fit of the regression to the observed data.

In the case of the analysis of the rate of change of the number of pollen grains on stigmas in relation to time, we observed a decrease in this rate after 5:30 a.m. Breakpoint regression, or segmented regression, was used to analyze these data. This analysis is used to estimate a linear relationship between two variables where the slope changes at a certain value of independent variable (breakpoint) (Muggeo 2008). The estimation of the parameters of this model (two slopes and the breakpoint) was carried out with the R segmented package (Muggeo 2008). The model that showed the maximum value of R^2 and the minimum AIC (Akaike Information Criteria) was chosen as the best to represent the data. The significance of these regression slopes were tested using the t-test statistic of the regression coefficient.

To test preferences in visitation frequency in relation to flower gender, the number of bee visits/flower/10 min intervals were compared between male and female flowers for each bee species. A Mann–Whitney non-parametric test was used to determine significant differences between visitation rate values.

Results

In the medium-elevation site of Piedades, a more diverse community of bees was observed than in the high-elevation site of La Cima. The most abundant groups in Piedades were *Megalopta (M. centralis* and *M. genalis), Caenaugochlora costaricensis, Apis mellifera, Eucera utahensis, Eucera limitaris* and *Trigona corvina.* Only two floral visitors were observed in La Cima: *Bombus ephippiatus,* the most abundant, and *Apis mellifera.* Although *Eucera (Peponapis)* and *Megalopta* are genera where two species can occur in the locality of Piedades (Lobo and Bravo 2021), identification of these bee genera at the species level was not possible in recordings. Although it is not possible to distinguish black *Trigona* species in recordings, there was only one species captured in Piedades, namely, *T. corvina.* Therefore, we defined that *Trigona* bees seen in recordings pertain to this species.

Visitation rates are represented for each bee species and flower gender for both localities (Piedades and La Cima de Dota) in Fig. 1. Observations at Piedades showed that bee activity starts at 4:30 a.m. with the opening of the male flowers, which begin their anthesis 30–40 min earlier than the female flowers (beginning of anthesis between 5:00–5:15 a.m., which is completed at 5:30 a.m.). The activity of the bees begins to decrease at 8:00 a.m. In the short interval of 4:30–8: 00 a.m., there is a sequence of foraging peaks by different bee species observed in both male and female flowers (Fig. 1). The first bees to arrive were *Megalopta* (first bees observed in the dark at 4:30 a.m.) on male flowers. These bees were the only ones observed before 5:00 a.m. From this time onward, the next groups that began to visit the male and female flowers were *Eucera* (*Peponapis*), *Apis mellifera* and *Caenaugochlora. Trigona corvina* was the most frequent visitor in all flowers from 5:45 a.m. *Eucera (Peponapis)* and *Apis mellifera* visitation rates were higher in female flowers than in male flowers (*Eucera U*-test = 115.5[*] $p < 0.05$, *Apis U*-test = 146.5[*] $p < 0.05$). The other bee species do not show significant differences in the visitation rates between male and female flowers. *Trigona* workers showed a different behavior from other groups: the individuals frequently inspected the nectaries and walked on the surface of the corolla with prolonged visit times per flower. Gradually, a circle of bees of this species formed around nectaries that remained there for the rest of the morning. Some individuals of *Apis* and *Eucera* (*Peponapis*) approached the flowers and desisted from the visit, apparently driven away by the accumulation of stingless bees. However, *A. mellifera* continued to enter the nectaries later in the morning. This late phase of bee activity did not change in the following hours until almost a complete decline in activity at approximately noon.
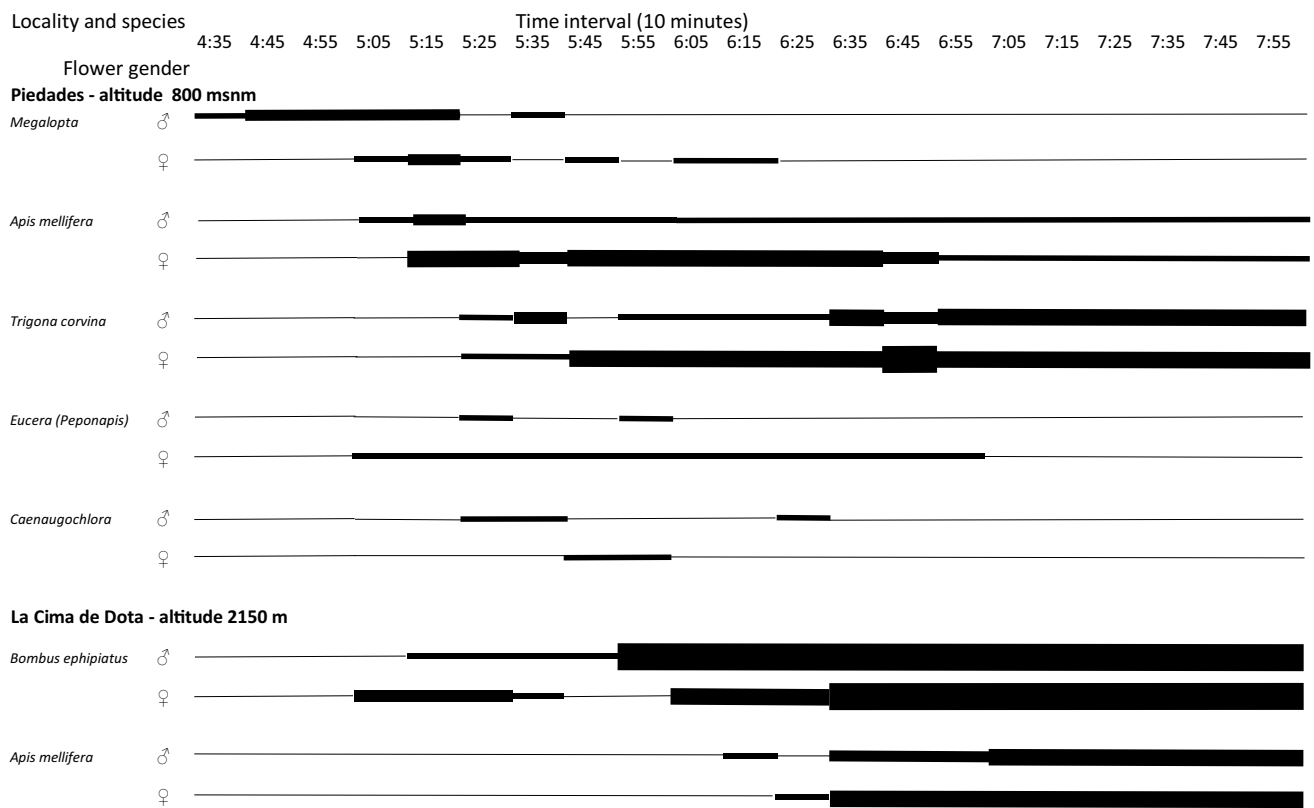
Fig. 1 Foraging cycle in *Cucurbita pepo* flowers of different bee genera in two locations in Costa Rica. Thickness of the line indicates the intensity of foraging, measured as the number of bees that reached male (♂) or female (♀) flowers in 10-min intervals from 4:30 a.m. until 8:00 a.m. (————— 0, ▬▬▬ 0–1.3, ▬▬▬ 1.4–2, ▬▬▬ 2–5, ▬▬▬ > 5 bees/10 min)

At La Cima de Dota, the activity began slightly later (5:05–5:15 am), and the bee that visited the crops earlier was *B. ephippiatus*, which was the predominant bee species that visited these crops. *Apis mellifera* appeared later (6:25–6:35 am) (Fig. 1). Note that the local temperature at 5:00 a.m. was 11 °C. Visitation rates per flower observed in this locality (up to 15 bees every 10 min) were higher than those observed in Piedades as a result of the high levels of activity of *B. ephippiatus*.

Figure 2 shows the dynamics of pollen release from anthers in Piedades. It was observed that the amount of pollen in the anthers decreased in a linear way from the opening of the male flowers (4:30 a.m.) until 8:00 a.m. [$b = -4411$ (578), $F_{(1,26)} = 58.24^{**}$ $p < 0.001$], when the anthers practically lost all the pollen. After 7:00 a.m., the amount of pollen available in the anthers was reduced by 75% (from 15,000 grains to approximately 5000). We observed that a considerable amount of pollen was present at the base of the corolla from very early hours (5:00 a.m.) until almost disappearing at 7:00 a.m. [$b = -2493$ (934), $F_{(1,15)} = 7.11^{*}$ $p < 0.05$]. Bagged flowers showed that pollen of the anther can fall spontaneously to the basal area of the corolla during that time interval, becoming more abundant than pollen in the anthers at 8:30 am. However, the reduction of pollen in anthers and corollas of open flowers shows that the activity of bees is responsible for the complete removal of pollen in male flowers.

Figure 3 shows how the volume of nectar available in male flowers increases during the morning [$b = 6.04$ (0.94), $F_{(1,26)} = 40.92^{**}$ $p < 0.001$]. It was found that when the first male flowers opened, the availability of nectar was very low. However, despite the intense foraging in the period between 5:00 a.m. and 7:00 a.m. (Fig. 1), the volume increased rapidly to a maximum volume of 15 μl, which was observed in bagged flowers. Flowers with bite marks in the nectary holes made by *Trigona* showed little volume of nectar in relation to the expected collection time.

Figure 4 shows the fast dynamics of pollen deposition in stigmas and the development of pollen tubes in styles. At 5:00 a.m., the recently opened female flowers were practically free of pollen, but by 6:00 a.m., many stigmas had more than 500 pollen grains. After that hour, the deposition

rate decreased drastically. The breakpoint regression analysis produced two regression lines, one with a slope $b = 1860$ (s.e. $= 886$, $t = 2.09^{*}$, $p < 0.05$) (rate of increase in the number of pollen grains per hour) for points between 5:00 a.m. and 5:46 a.m. and another with slope $b = 114$ (s.e. $= 89.4$, $t = 1.27$ n.s.) for observations after that time. The breakpoint model (AIC $= 982.8$, $R^2 = 0.23$) was superior to the simple linear model (AIC $= 986$, $R^2 = 0.13$). However, there is a wide variance in the number of pollen grains on stigmas after 6:00 a.m. and later. This variance may respond to differences between female flowers in the frequency of visitation by bees. However, almost all stigmas had more than 500 pollen grains at 9:00 a.m. The number of pollen tubes in the styles presents a different dynamic. This figure always stays below 500 pollen tubes per style and grows slowly throughout the morning with an average rate of change of 54 tubes per hour ($b = 54.1$, $t = 2.66^{*}$, $p < 0.05$). At 9:00 a.m., the average value is 250 tubes per style with a wide variance not explained by the regression ($R^2 = 0.19$). Note that the maximum pollination level (average of pollen tubes/style) of this population is achieved at approximately 6:15 a.m., which is 45 min after the full opening of the female flowers.

Figure 5 shows pollen grain counts in individual bees in the early hours of the morning. This variable also showed a rapid dynamic of change. Individuals with high pollen loads (> 3000 grains) were observed only between 5:00 a.m. and 6:00 a.m. After that time, bee pollen loads were reduced until almost no pollen grains remained in bees collected after 7:00 a.m. The highest number of pollen grains was found in *Megalopta* and *Eucera* (*Peponapis*) (7000–4000 pollen grains) in the first hour period. Pollen loads in the first collected *Caenaugochlora* and *A. mellifera* bees oscillated on a smaller scale (< 4000 grains).

Trigona corvina, which only appeared after 5:30 a.m., showed the lowest values (< 50 grains). At the high-altitude site (La Cima), although no direct pollen grain counts were made in bees, it was observed in the recordings and collections that the *B. ephippiatus* workers were saturated with pollen, especially those collected in the early hours of foraging. Due to the size and hairiness of the bees, they possibly exceeded the maximum number of grains recorded in Piedades bees. *A. mellifera* bees collected in La Cima carried considerably smaller pollen loads than *Bombus* bees.

It was observed in the recordings of male flowers that while *Eucera* (*Peponapis*) approached the male flower by landing on the stamen and then walking towards the nectaries, the other species of bees posed on the petals and then walked towards the base of the corolla to reach the male nectaries. In bees other than *Eucera*, pollen impregnation occurred by contact of the dorsal part of the bee with the anther while foraging for nectar at the base of the corolla. These bees also contacted pollen deposited at the base of the corolla, where it fell by gravity or by the activity of *Eucera*. However, note that no specific behavior of pollen gathering was observed in any of the visits recorded in male flowers. All bees walked directly to the nectaries at the base of the corolla and stayed in that area until they departed. Therefore, in all observed cases, pollen impregnation occurred because of nectar-collecting behaviors.

Fig. 2 Number of pollen grains per anther or corolla versus the morning time in a population of *Cucurbita pepo* in Piedades, Costa Rica. Black circles are the number of grains estimated per anther. White circles are the number of pollen grains in the region of the corolla near the base of the stamen. The observations at 8:30 a.m. represent the average values and standard errors of bagged flowers up to that time. The lines represent the regression lines for counts in anthers [solid line, $b = -4411$ (578), $R^2 = 0.62$] and corollas [dotted line, $b = -2493$ (934), $R^2 = 0.32$]
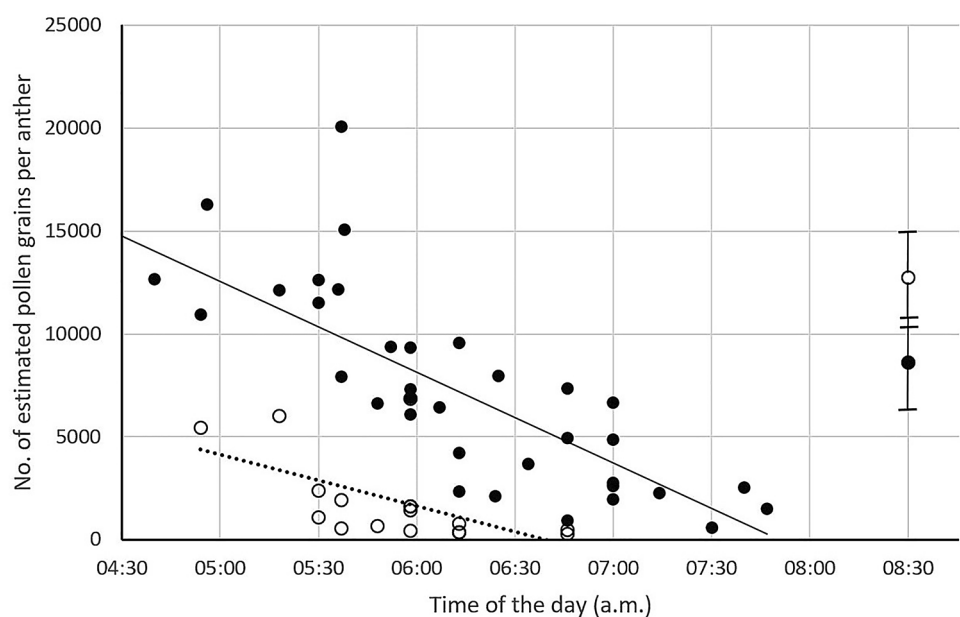
Fig. 3 Nectar volume (µl) in open male flowers collected at different early morning hours. The nectar volume of open undamaged flowers (black dots), open flowers with nectaries bitten by Trigona (X), and the average volume (and standard error) of bagged flowers collected at 8:30 a.m. (white circle) are shown. The line is the regression between time and nectar volume for undamaged open flowers [$b = 6.04$ (0.94), $R^2 = 0.61$]
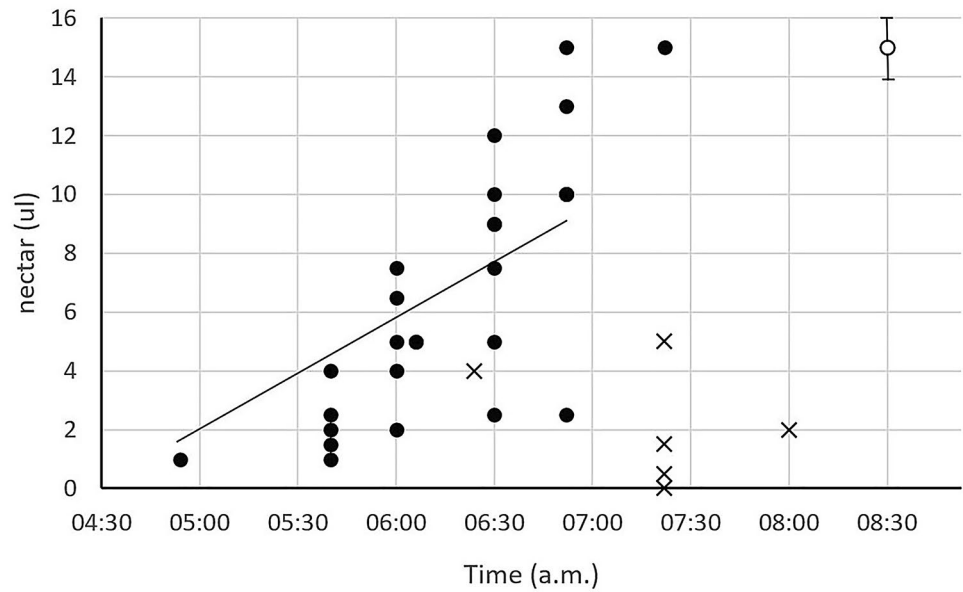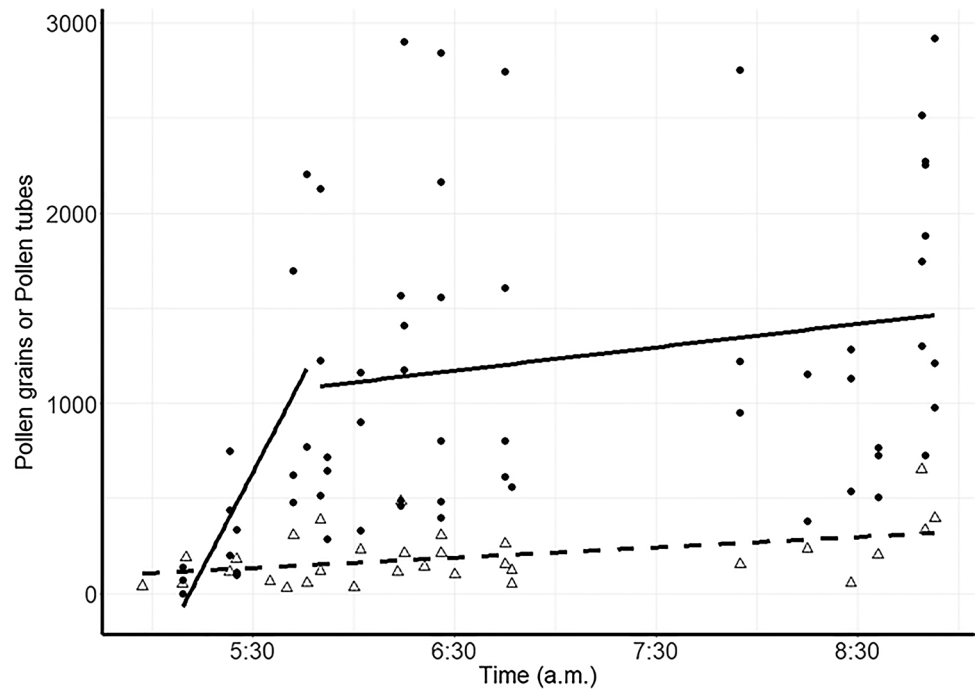
Fig. 4 Number of pollen grains on stigmatic lobes (points) and the number of pollen tubes in styles (triangles) of *C. pepo* flowers during the first hours after anthesis are shown. Lines represent the best fit breakpoint linear regression (continuous line: pollen grains on stigmas, $R^2 = 0.23$) and simple linear regression (dashed line: pollen tubes in styles, $R^2 = 0.19$)

Discussion

Although in our study sites, male and female flowers of *C. pepo* were visited by bees only during a short time period (5–6 h, from 4:30 am), it was possible to identify temporal changes in the foraging schedules of different bee species. At high altitudes, such as La Cima, the foraging onset of *Bombus* bees (5:00 am) precedes that of *A. mellifera* by over an hour, showing the early foraging capacities at low temperatures recognized for this genus (Willmer and Stone 2004). In Piedades, in a lower altitudinal belt, bees with a greater capacity for crepuscular foraging (*Megalopta*) initiate the use of male flowers but are quickly succeeded by *Apis*, *Eucera* (*Peponapis*) and *Caenaugochlora*, while from 6:00 am, *Trigona* workers occupy almost all the flowers with the intermittent presence of *A. mellifera* during the rest of the morning, especially in female flowers.

Different proposals have been made to explain the temporal structuring of visits by pollinators to a plant species, such
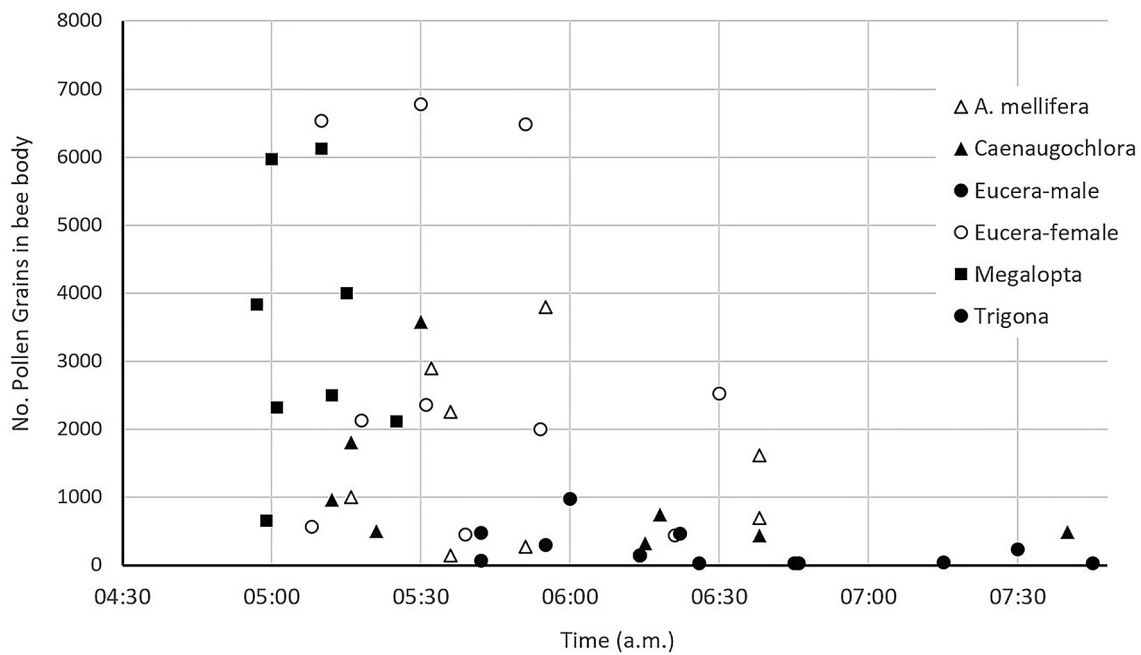
Fig. 5 Estimates of the number of pollen grains of *Cucurbita pepo* in the bodies of different species of bees visiting the crop in the early morning at the locality Piedades

as the temporal separation of foraging due to competition between species (Hubbell and Johnson 1978) or because of physiological differences between pollinators, which determines greater foraging capacity in different environmental conditions (Willmer 1983; Herrera 1990). It has also been proposed that plants respond with anthesis and the presentation of flower resources adjusted to the activity schedules of the most efficient pollinators (Herrera 1990; Stebbins 1970). Both proposals can be considered to explain the observations of this study. The rapid turnover of the foraging activity of different groups of bees in *C. pepo* is related to the different flight and orientation capacities in early day conditions of each group (such as the early foraging capacity in cold conditions of *Bombus* or *Megalopta's* crepuscular flight ability). Various studies have verified a size gradient, extending from large to small bees, during morning foraging cycles in plants visited by various pollinator species (Muniz et al. 2013). However, early foraging for nectar can also be a result of competition, especially in the presence of *Trigona* bees in later hours of the morning. The capacity of these bees for social foraging and recruitment, aggressiveness and other traits, such as biting nectary holes, allow *Trigona* bees to collect this resource later in the morning. It was found that male flowers damaged by *Trigona* bees present little nectar, and their numerous presence in flowers may keeps away other bees. However, observations on squash crop fields without *Trigona* bees are necessary to prove this detering mechanism. Notably, *Megalopta* starts foraging with little

supply of nectar from male flowers. Wcislo et al. (2004) suggested that escape from competitors may be one of the reasons for the evolution of crepuscular flight in *Megalopta*, where early foraging allows it to obtain flower resources before other groups of bees deplete them.

The maximum amount of pollen in the anthers was observed before 5:30 am (15,000–20,000 grains), similar to that observed in other studies (Nepi and Pacini 1993), although it was lower than other estimates in other varieties of squash (Vidal et al. 2006). Our observations suggest that pollen export from male flowers occurs in the first hour of morning light (5:00–6:00 a.m.). Part of the pollen that disappears from the anthers does not leave the flower but is deposited at the bottom of the corolla, where it can be removed by bees in their search for floral nectaries. Similar observations on the movement of bees and pollen within flowers were obtained by Nepi and Pacini (1993). This finding suggests a secondary form of pollen presentation, a phenomenon where pollen moves to regions of the flower other than the anther, where it can be exported more efficiently (Barman et al. 2020). In *C. pepo,* this movement of pollen can occur spontaneously due to the large size and weight of the pollen grains of this plant, as well as a result of the activity of bees in the anthers. The maximum pollen loads in the bees (4000–7000 grains) were obtained in the same hour as the maximum loads in the anthers. It is also the time of maximum activity of bee species that show the maximum pollen carrying capacity (*Eucera* males and females, Fig. 5).

As observed in the present study, Vidal et al. (2010) found maximum pollen loads of *C. pepo* in *Apis mellifera* in this range and a fast-decreasing rate of pollen loads between 7 a.m. and 10 a.m. Bees of the genera *Eucera* (*Peponapis*) and *Bombus* have been identified as the vectors with the highest pollen load in this crop (Artz and Nault 2011). Perhaps the genus *Megalopta* could be added as a bee with high pollen loads in this crop. This property could be explained by their early foraging behavior at the time of highest pollen availability in the anthers.

Therefore, the decline of pollen loads during the morning could be a result of two factors: the higher availability of pollen in anthers in early hours, as well as the early activity of bee species of high carrying capacity (*Eucera* and *Megalopta*). The importance of the amount of pollen in the anthers as determinant of the size of the pollen loads on bees can be better visualized in *Apis* and *Caenaugochlora*. Within these bee species, individuals collected early in the morning carry higher pollen loads than the ones collected after 6:00 a.m.

Differences in flower landing behavior can be one of the factors that explain higher pollen loads in *Eucera* (*Peponapis*) in our study: only these bees used the stamen as landing place to approach to the nectaries. *Bombus* also was observed with high pollen loads, although this bee species touch the stamen accidentally. The large size and hairiness of *Bombus* favors pollen adherence to its body as it forages for nectar in male flowers. It has been shown that bees like honey bees and bumble bees do not collect *Cucurbita* pollen (Brochu et al. 2020). This may explain the observed difference in the flower landing behavior between *Eucera* and the rest of the pollinators.

Pollen import into the stigmas appears to be completed at 6:30 a.m., half an hour after the proposed peak pollen export time. After that hour, the pollen deposition rate on the stigmas was reduced. At 9 am, 61% of the female flowers had more than 1000 grains per stigma, which could correspond to more than 1500–2500 grains in the three stigmas, a threshold proposed by various studies (McGrady et al. 2020), such as that necessary to achieve the maximum fruit set and vigor of the fruits in this crop. The low correlation between the number of pollen grains in the stigmas and the number of pollen tubes in the styles observed may have two explanations: (i) a proportion of pollen grains that do not germinate and (ii) a high proportion of pollen tubes that develop in the stigma but fall short of the style (Winsor and Stephenson 1995). Constriction in the upper style tube can be a limiting factor for the possible number of developing pollen tubes ("pollen tube attrition", Winsor and Stephenson 1995), resulting in many flowers with high levels of pollen deposition that are not reflected in an increased number of pollen tubes. Rapid deposition of pollen loads in stigmas was also observed by Winsor et al. (2000) in a wild *Cucurbita*

(*Cucurbita foetidissima*). The accumulation of pollen in early hours maximizes pollen tube competition, because in this case a high number of pollen tubes, frequently higher than the minimum required to fruit development, start growing simultaneously (Winsor et al. 2000).

The limiting factor for pollination time appears to be the pollen availability on male anthers, which disappears in the first hour and a half after flower anthesis. Pollen viability, stigma receptivity and nectar secretion in male and female flowers of this species remain high all morning after anthesis (Nepi and Pacini 1993; Nepi et al. 2001; Vidal et al. 2006). Therefore, these variables do not seem to limit the pollination time of this species to a few hours after anthesis. In our study, we verified that nectar is still available in open male flowers when the foraging activity of *Megalopta*, *Caenaugochlora* and *Eucera* (*Peponapis*) has ceased. Although flowers may remain attractive to pollinators, the depletion of pollen from anthers, as well as the decrease in activity of the most efficient bee species, would limit pollination to the earliest hours of the morning.

Pollination data show that bees that visit the male and female flowers of *C. pepo* after 6 a.m. contribute little to pollination. *Trigona corvina* practically monopolizes the space at the bottom of the corollas and around the nectaries after 6 a.m., a condition favored by social recruitment and long periods of exploratory behavior and foraging within the flowers. Similar observations have been reported for *Trigona spinipes* in Brazil by Serra and Campos (2010). Delgado-Carrillo et al. (2018) and Serra and Campos (2010) suggest that bees of the genus *Trigona* can be nectar thieves in *Cucurbita*. Perhaps these bees do not produce negative effects on male or female function in the population of *Cucurbita pepo* studied as they are active later in the morning and do not interfere with pollination.

It has been proposed that *Cucurbita* fruit production in the USA and Europe is not limited by pollinators (Pfister et al. 2017, McGrady et al. 2020). Our study shows that possibly not all the diversity of bees that visit the crop is required to achieve the observed levels of pollination. However, the diversity of pollinator bees observed in this study can ensure crop productivity in the face of environmental changes and fluctuations in pollinator populations throughout the year (Genung et al. 2017). For example, crops obtained at different times of the year may depend on different species of pollinators, where social bee species can provide more stable pollination services than those offered by solitary species with more restricted periods of activity (Delgado-Carrillo et al. 2018). In our main study location, Piedades, bee collections obtained at the end of the rainy season (November, second crop season) showed a greater preponderance of *Trigona bees* than that observed in this study (Lobo and Bravo 2021).

Finally, the crop plant species, not only pollinators, can be highlighted as conservation targets. Crops of *C. pepo*, especially on small farms in diverse agroecosystems, can be an important source of resources for the maintenance of local populations of social and solitary bees that pollinate other crops. In the agricultural areas studied, other crops, such as citrus fruits and beans, are part of the food production of the same farms where squash is grown. Stingless bees, which forage intensively on *C. pepo* without pollinating it, can obtain an important energy source from this crop in the form of nectar for their colonies, which facilitates a larger population of workers available for the pollination of other crops and wild plants of the agricultural ecosystem. Thus, the conservation of certain crops may be part of the strategy for the conservation of pollination services.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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