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Cucurbit specialist, *Peponapis pruinosa*, is a more effective pollinator than generalist, *Apis mellifera* for cultivated squash

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by Maria Izabel Martinez

Committee in charge:

Professor David Holway, Chair Professor Elsa Cleland Professor Joshua Kohn

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The Thesis of Maria Izabel Martinez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair Chair

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ABSTRACT OF THE THESIS

Cucurbit specialist, *Peponapis pruinosa*, is a more effective pollinator than generalist, *Apis mellifera* for cultivated squash

by

Maria Izabel Martinez

Master of Science in Biology

University of California San Diego, 2020

Professor David Holway, Chair

The western honey bee (*Apis mellifera*) is a super-generalist in terms of floral visitation, but its effectiveness as a pollinator varies greatly. This variation has called attention to the importance of native insects as pollinators in agricultural systems and has raised concerns that floral visitation by honey bees can adversely impact some plant species. To clarify what factors underlie this variation, we use multiple approaches to compare the effectiveness of specialist (*Peponapis pruinosa*) and generalist (honey bees) pollinators of cultivated squash (*Cucurbita pepo*). Previous studies report conflicting results on the effectiveness of *P. pruinosa* and *A.*

mellifera as pollinators of *C. pepo* but have not typically considered how specific foraging behaviors (e.g., degree of contact with the stigma or anthers) influence their effectiveness. To resolve this uncertainty, I conducted single and multiple visit trials on *C. pepo* plants at the UC San Diego Biology Field Station to examine how visitation and behavior of these two bee species affect pollinator effectiveness. Visitation by female squash bees was found to have a greater positive effect on pollen removal, pollen deposition, fruit set, and seed set (i.e. seed number) compared to honey bee visitation. These findings were consistent with the behavior of the pollinator species. Cumulative duration of stigmatic and anther contact by female squash bees had a greater effect on pollen deposition and pollen removal compared to the same behaviors in honey bees. Previous studies on cultivated squash conducted in agricultural settings have found that the effectiveness of honey bees and squash bees appears more similar than what we report here. These contrasting results could be explained by differences in resource availability in large scale agricultural versus small scale plots. At our study sites, for example, honey bees primarily collected nectar from cucurbits and foraged for pollen on other plant species in the immediate environment.

Introduction

Pollination by insects and other animals represents an important ecosystem service in both natural and agricultural systems (Losey and Vaughan 2006, Ollerton et al. 2011). For instance, 70% of global food production results from pollination by animals (Klein et al. 2007), and the economic benefits provided by pollinators have been estimated to be \$235 - \$577 billion annually (Lautenbach et al. 2012, Potts et al. 2016). For these reasons, it has become increasingly important to learn more how pollination services are affected by different aspects of environmental change. Non-native pollinator species, for example, may differ in their effectiveness compared to native pollinators and could affect pollination services if they became abundant. Effective pollinators collect and transfer the most pollen, relative to other pollinators, thereby increasing reproductive success of a plant (i.e. fruit set and seed set) (Herrera 1987, Ne'eman et al. 2010, Williams and Thomson 2003). The western honey bee (*Apis mellifera*) is a globally distributed super-generalist pollinator that collects pollen and nectar from a wide variety of plant species and exhibits substantial variation in its effectiveness as a pollinator (Hung et al. 2018). To clarify what factors underlie this variation, we compare the effectiveness of specialist (native squash bees) and generalist (non-native honey bees) pollinators of cultivated squash (*Cucurbita pepo*).

We focus on *Cucurbita pepo* for the following reasons. This species is monoecious with separate female (pistillate) and male (staminate) flowers. Cultivated squash does not selfpollinate and therefore requires pollinators to transfer pollen from staminate flowers to pistillate flowers (Hurd et al. 1971). Squash flowers are large and short-lived, opening before sunrise and then wilting and becoming unreceptive by midday (Hurd et al. 1971, Tepedino 1981, Willis and

Kevan 1995). Important pollinators of cultivated squash include the western honey bee (*Apis mellifera*) and the eastern cucurbit bee (*Peponapis pruinosa*) (Hurd et al. 1974). As a specialist pollinator, *P. pruinosa* (hereafter referred to as the squash bee) requires cucurbit pollen to reproduce but can visit other plants to obtain nectar. Behavioral and physiological differences between honey bees and squash bees may also influence their effectiveness as pollinators. Honey bee workers forage throughout the day for pollen and nectar, whereas squash bees mainly forage during the morning and decrease their activity later in the day (Hurd et al. 1974). Squash bees also can collect resources with little to no light which makes foraging before sunrise a possibility (Hurd et al. 1974). Moreover, this observed activity coincides with early flowering times of squash plants. To further emphasize the specialist relationship between squash bees and squash plants, Hurd et al. (1974) demonstrated that annual nest emergence and construction occurs during the flowering period of squash plants. Squash bees have a physiological advantage in terms of collecting and transferring pollen because squash bees fly faster and begin to forage much earlier compared to honey bees. Additionally, female squash bees are larger and have hairier bodies than honey bees which results in pollen being collected passively. A caveat, however, is that squash bees have an advantage only when their population is well-established. This brings up the question of how effective squash bees are as pollinators if they are not as abundant.

 Tepedino (1981) further explained how effective honey bees and squash bees were at pollinating by looking at how single and double visits from either honey bees or squash bees compared regarding fruit set and pollen deposition. He found that there were no significant differences between fruit set or pollen deposition by squash bees and honey bees during single and double flower visits from each species. Therefore, both pollinators were equally effective

which further supported Hurd's (1974) claim that in areas with well-established squash bee populations, honey bees are not necessary (i.e., to provide pollination services in agriculture). However, Tepedino's result diverges from the findings of recent studies showing that squash bees deposit significantly more pollen during a single visit (Canto-Aguilar et al. 2000), and that honey bees do not remove pollen readily during a single visit (Williams and Thomson 2003). Therefore, it is still unclear if squash bees are more efficient than honey bees or if honey bees are equally as effective during a single visit to a squash flower.

 The results of studies that rely on single-visit trials to compare the effectiveness of honey bees and squash bees as pollinators may be influenced by environmental context and do not consider differential visitation(Ne'eman et al. 2010). Controlled multiple visitation experiments can be used to better understand how increasing visits from different pollinators affects a plant's reproductive success. Vidal et al. (2010), for example, showed that pistillate squash flowers required twelve honey bee visits to be successfully pollinated; after twelve visits there was no longer an increase in reproductive success. Therefore, this study suggests that a pollinators effectiveness when collecting and transferring pollen may differ with the number of visits (visitation) a flower receives and thereby affect reproductive success of squash plants differently.

In this study, I aim to determine the pollinator effectiveness of honey bees and squash bees (female and male) on cultivated squash. To do this I looked at (i) how increasing visitation (number of visits) from these bee species affected the amount of pollen deposited on stigmas of pistillate squash flowers and on removal of pollen from staminate squash flowers, (ii) how visitation affected squash reproductive success, and (iii) how behavioral differences between these species influenced their effectiveness as pollinators. Measuring the effects of multiple visitation builds on the results of studies that consider smaller numbers of visits (Artz and Nault

(2011) and Tepedino (1981)). Furthermore, behavioral differences, which have scarcely been quantitatively compared, can reveal the mechanisms underlying different degrees of pollinator effectiveness by honey bees and squash bees.

Methods

Study site

I grew *Cucurbita pepo* at the UC San Diego Biology Field Station (32°53'08.8"N, $117^{\circ}13'47.2''W$) from June through early September 2019. Individual plants (n = 142) were germinated and grown in a 19 x 13 m plot with each plant occupying its own 1.2 x 1.2 m section. Seeds were planted in mid-June, and individual plot sections were reseeded if there was no sign of germination five days after the initial seeding. After germination, plants were monitored daily by recording the emergence of cotyledons, number of true leaves, approximate amount of herbivory (if any), and presence of fungal pathogens (if any). A dripline system was used to irrigate plants daily. Individual 1.2 x 1.2 m sections were each traversed by two 0.64 cm drip lines in which equal-sized and evenly spaced holes were punctured such that all plants received similar amounts of water during scheduled hourly morning irrigation. To ensure that all plants received comparable levels of irrigation, a Field Scout TDR 100 soil moisture meter was used to measure the volumetric water content (VWC%) of the soil underneath each plant. After germination, soil moisture was measured one hour after watering in the morning, three times per week; the mean soil moisture throughout the summer was 33.4% VWC (SD = 5.9, n = 4503) measurements) (Appendix 1).

Pollinator visitation

Each plant was randomly assigned to an experimental pollinator visitation category that set the number of pollinator visits that every flower (of either sex) on that plant would receive. These categories included: 0 visits, 1 visit, 3 - 6 visits, 9 - 12 visits, or open pollinated. To determine which flower buds would open in a given morning, daily surveys were conducted on the previous afternoon in which Seedburo S27 wax treated pollination bags were placed on each of the flower buds that appeared ready to open the next day. Pollination bags placed over floral buds prevented insect visitors from entering flowers. For these flowers, visitation trials began at sunrise on the following day (at which time pollination bags were removed); trials lasted approximately two hours. Before beginning visitation trials, pollination bags were removed from a portion of staminate flowers from the plot (mean = 0.41 , SD = 0.09 , n = 43 days) (Appendix 2) in order to supply enough pollen for deposition on female stigmas. For staminate flowers in the 0-visit experimental group, flowers were removed from the plant but left inside the pollination bag to be measured later that day. For pistillate flowers in the 0-visit experimental group, each pollination bag was replaced with a Paper Mart standard gold organza 15 x 23 cm mesh bag, which excluded visitors while simultaneously permitting similar environmental conditions as open flowers (Kearns and Inouye 1999). Mesh bags were used to replace pollination bags since placing female squash flowers back into pollination bags may result in damaging the stigma or fruit bulb of the flower. Also, mesh bags contained draw strings which allowed for the bag to be secured onto the flower.

For flowers visited by pollinators, each open flower was observed until the designated number of visits (or range of visits) for that plant had occurred. The duration of an individual visit was defined as the total time spent inside the cup of the flower (from entry to exit). For pistillate flowers, once the designated number of visits had occurred, mesh bags were used to prevent additional visitors from entering the flower. After the designated number of visits had occurred for staminate flowers, these flowers were removed from the plant and placed in pollination bags so that remaining pollen could be weighed. On some trial days, the abundance of honey bees made it difficult to obtain an even balance of squash bees versus honey bees. For

this reason, an aspirator was used to blow puffs of air at arriving honey bees (i.e., prior to them entering the flower) to prevent individuals of this species from monopolizing visitation on plants in the 3 - 6 and 9 - 12 visit groups. Application of this method discouraged honey bees from landing in the flowers and did not seem to affect other visitors (Nabors et al. 2018).

 In addition to visual observation of flowers, all trials were videotaped except those in the 0-visit group. A Victure Action 4K Wi-Fi video camera was positioned on a tripod approximately 20 - 40 cm away from each focal flower. Camera distance was close enough to each flower so that the entire corolla was within the frame of the video but far enough away such that bee visitation did not seem to be affected. Videos began after pollination bags were removed and were directly compared to visual observations of visitation for each flower. In all videos, the number of visits by honey bees and by male and female squash bees was recorded. For each bee visit to pistillate flowers, the time spent drinking nectar and contacting the stigma were estimated. For each bee visit to staminate flowers, the time spent drinking nectar, the time spent contacting the anthers, and the time spent collecting pollen were estimated (Appendix 3). Flowers in the open pollinator group were videotaped but received no observation. Cameras set up for open-pollinated flowers began recording after pollination bags were removed and were left running for two hours.

Pollen deposition and pollen removal

Approximately 24 hours after the conclusion of visitation trials, a single-edged razor blade was used to separate stigmas from the fruit bulb of each pistillate flower. This span of time allowed for pollen grains to germinate and fertilize ovules in the pistillate flower without affecting fruit or seed set (*personal observation*). Each stigma was placed in a 50 mL centrifuge

tube filled with 100% ethanol. To estimate pollen deposition on each stigma, I added 4 drops of basic fuchsin dye solution to each tube to make pollen grains visible. Pollen deposition was counted at least one week after the addition of basic fuchsin dye. Pollen deposition on each stigma (including pollen grains in the ethanol dye solution) were counted using a dissecting microscope at 4x magnification.

To estimate how much pollen was removed from staminate flowers by bees, I conducted the following measurements. Staminate flowers that were not visited by any insect were collected from each plant. Using a small spatula $(0.64 \text{ cm wide} \times 19 \text{ cm long})$ pollen was removed from the anthers and weighed on a scale. The mass of the pollen was then averaged across unvisited staminate flowers within each plant. The average pollen mass was used as a proxy measurement of an individual plant's per flower pollen production. To estimate the amount of pollen removed from staminate flowers that received visits, the amount of pollen remaining after the visits occurred was first weighed. The remaining pollen mass after bee visitation was then subtracted from the average pollen mass for a given plant.

Fruit and seed set

Fruit bulbs of pollinated pistillate flowers were checked daily for evidence of fruit set. Of those that did set fruit, I let each fruit grow for 50 days following pollination and at that time separated them from the plant by cutting the stem 3 cm above where it met the top portion of the fruit. After harvesting, fruit volume was measured by submerging each fruit in a 2000 mL graduated pitcher filled with 1000 mL of water and measuring the displaced volume of water. Seeds were removed after the volumetric measurements of each fruit, counted, and placed in

mesh bags to dry for 14 days in the laboratory. Dried seeds were then weighed collectively for each fruit.

Statistical analysis

I used R (R Core Team, 2019) to analyze all data, and I prepared all figures using the package ggplot2 (Wickham, 2009). To determine how visitation (number of visits) from different bee species affected the reproductive success of squash plants, I used (i) multiple logistic regression with fruit set as a response variable, and (ii) multiple regression with mean seed mass (per fruit) and seed number (per fruit) as response variables. Seed number was log transformed to meet model assumptions. Predictor variables for the logistic regression analyses included visitation by honey bees, female squash bees, and male squash bees as well as pollination date (i.e. day of year). Predictor variables for the multiple regression analyses included pollination date, linear and quadratic visitation terms (for honey bees, female squash bees, and male squash bees), and interaction terms. The simplest best fit model was derived by first creating a full model that included all linear and quadratic terms as well as interaction terms. I performed model selection by successively removing non-significant terms from the full model and using Akaike Information Criterion (AIC) to identify the best supported model among all possible models (Crawley 2013). I report the full model and the simplest model with the lowest AIC value (see *Results*). This model selection approach was also used in analyses with pollen deposition (on the stigmas of pistillate flowers) and pollen removal (from the anthers of staminate flowers) as response variables. Pollen deposition was log-transformed to improve normality. Furthermore, to determine if partial regression coefficients were significantly different (within an individual analysis), I used a Wald-test based comparison.

To determine how the behavior of different bee species influenced plant reproductive performance, I again used multiple regression analyses with the cumulative time spent on floral stigmas by each bee species as the predictor variables and the following response variables: fruit set (logistic regression), seed set (multiple regression), and pollen deposition (multiple regression). Number of seeds was log-transformed to better meet regression model assumptions. I also used multiple regression to test the relationship between the cumulative time spent on floral anthers by each bee species and the amount of pollen removed. Time spent on anthers was log transformed to better meet regression model assumptions. For all regression analyses, I tested the assumptions for multiple regression models by plotting residuals to test for linearity and equal variances and inspecting q-q plots to determine if normality assumptions were met. Note that for each analysis I only used data from one flower per plant.

To determine if individual bee visits influenced subsequent visits, I used two-sample *t*tests to compare the duration of stigmatic contact of the second visitor (honey bee or female squash bee) to a pistillate flower when the first visitor was either a honey bee or a female squash bee. Similarly, I compared the duration of anther contact of the second visitor to a staminate flower, when the first visitor was either a honey bee or when the first visitor was a female squash bee. For these analyses, the duration of stigmatic contact and anther contact were logtransformed to improve normality.

Results

Fruit set, pollen deposition, and seed set

The likelihood of fruit set increased with visitation and the cumulative duration of stigmatic contact for both female squash bees and honey bees (Tables 1-2, Figure 1A). However, the effect of female squash bee visitation on fruit set was significantly greater than the effect of honey bee visitation (Figure 1A; $\chi^2 = 4.65$, df = 1, p = 0.031). Fruit set was independent of visitation by male squash bees. In an analysis that was restricted to flowers visited only by honey bees, the likelihood of fruit set increased with visitation (Table 3) but not with the cumulative duration of stigmatic contact (Table 4).

 For flowers that set fruit, pollen deposition by female squash bees linearly increased both with visitation (Table 1, Figure 1B) and with the cumulative duration of stigmatic contact (Table 2). Pollen deposition by honey bees quadratically increased with visitation (Table 1), but the parameter coefficient for this relationship was small, and the significance of the relationship between pollen deposition and honey bee visitation disappeared in an analysis that was restricted to flowers visited only by honey bees (Table 3). Moreover, the amount of pollen deposited by honey bees was independent of the cumulative duration of stigmatic contact (Table 2).

The number of seeds produced by squash fruit linearly increased with visitation by female squash bees, and linearly decreased with visitation by male squash bees (Table 1). Honey bee visitation affected seed number only in terms of its interaction with male squash bee visitation (Table 1), and the relationship between honey bee visitation and seed number were no longer significant in an analysis that was restricted to flowers visited only by honey bees (Table 3). The cumulative duration of stigmatic contact did not affect seed number for any type of

visitor (Table 2). In contrast to seed number, mean seed mass was independent of visitation by both squash bees and honey bees (Table 1).

Pollen removal

The amount of pollen removed from staminate flowers increased with visitation by both female squash bees and honey bees (Table 5). For each female squash bee visit, however, sevenfold more pollen was removed compared to the amount removed by a honey bee visit (Fig 1C; F $= 18.08$, df $= 1$, p < 0.001). The significant and negative quadratic term for female squash bee visitation (Table 5) indicates that pollen removal did not continue to increase with increasing visitation (i.e., likely because of pollen depletion). Pollen removal by honey bees also depended on visitation by male squash bees (Table 5) with the latter tending to depress pollen removal by the former. In an analysis that was restricted to flowers visited only by honey bees, visitation increased pollen removal by this species alone (Table 3). Pollen removal was positively related to the cumulative duration of anther contact for both female squash bees and honey bees (Table 4 and 5).

First visit effect on second visit

The stigmatic time of the second visitor, honey bee, was not significantly different when the first visit was either a female squash bee or honey bee (Fig 2A). Similarly, the stigmatic time of female squash bees did not significantly differ when the first visitor was either a female squash bee or a honey bee (Fig 2A). Furthermore, in staminate flowers duration of anther contact of the second visitor (honey bee or female squash bees) was not significantly different when the first visitor was either a female squash bee or a honey bee (Fig 2B).

Discussion

Effective pollinators collect and transfer pollen more proficiently relative to other pollinators, thereby positively affecting the reproductive success of the plants that they pollinate. Comparisons of different aspects of pollinator effectiveness in the present study revealed that female squash bees are more effective compared to honey bees in terms of the likelihood of fruit set, pollen deposition, seed set (seed number), and pollen removal. Behavioral differences when foraging help to explain differences in pollinator effectiveness. The time that female squash bees spent on the anthers of staminate flowers increased the amount of pollen removed, and the time spent on the stigma of pistillate flowers increased the amount of pollen deposited. These relationships held for honey bees, but the relationships were weaker compared to those of female squash bees. Male squash bees, however, did not appear to be as effective as female squash bees or honey bees. Additionally, it did not appear that the first visit to a squash flower affected the behavior of the second visitor; this indicates that visitation by squash bees is not influenced by visitation honey bees and vice versa.

 Previous studies have shown that specialist pollinators can be more effective pollinators compared to generalist pollinators (Cane et al. 2011, Hurd et al. 1974, Larsson 2005, Tepedino et al. 2016). Specialists collect and transfer pollen more successfully than do generalists (Larsson 2005, Tepedino et al. 2016). As with Canto-Aguilar et al. (2000), I found that squash bees deposit more pollen during a single visit than do honey bees, and honey bee visitation did not significantly affect pollen deposition for flowers visited only by honey bees. In this system, I typically observed that honey bees landed on the edge of a flower's corolla and then crawled to the base of the flower to collect nectar (Artz and Nault 2011). Therefore, during a visit it was possible that honey bees would not make any contact with the stigma. In contrast, female squash

bees tended to land on the stigma and remain on the stigma while drinking nectar. The constant contact during a female squash bee visit may explain the significant positive effect female squash bee cumulative stigmatic contact had on the amount of pollen deposited. It remains unclear whether these behavioral differences apply to other systems. Artz and Nault (2011), for example, found that squash bees and honey bees do not differ from one another with respect to single-visit, stigmatic pollen deposition. However, Artz and Nault (2011) pooled male and female squash bees in their analyses, and the disparity in the results of their study and the results of my study may stem from this difference in how squash bees were treated, perhaps especially if the number of male squash bees visits was higher than that of female squash bee visits within a treatment group (Cane et al. 2011).

Previous studies have reported no difference between honey bees and squash bees in terms of their effect on fruit set (Tepedino 1981, Artz and Nault 2011). My results, however, suggest that visitation by female squash bees causes a higher frequency of fruit set than visitation by honey bees than does honey bee visitation. The disparity in results of this and previous studies again may result in site-to-site variation in the extent to which honey bees collect squash pollen, and in the case of Artz and Nault (2011) the pooling together of male and female squash bees in their analyses. Although I did not see a significant effect of male squash bee visitation on fruit set as demonstrated by Cane et al. (2011), this result may be due to relatively low male squash bee visitation to pistillate flowers in my study. Cane et al. (2011) found that five or more visits by male squash bees resulted in a successfully pollinated flower with seed set comparable to that of an open pollinated flower. In my study, most of the flowers received one to three visits by male squash bees. Furthermore, male squash bees are typically active earlier in the season (mid-

June to early July) while female squash bees tend to be more active near the end of August (Tepedino 1981, this study).

Visitation by female squash bees positively affected the number of seeds produced by individual fruits, whereas honey bee visitation did not show this effect. Similarly, in flowers that only received honey bee visits, visitation did not affect the number of seeds. This finding contrasts Artz and Nault' s (2011) findings that showed that flowers with higher honey bee visits resulted in more seeds per fruit and that squash bee visitation had no effect on the number of seeds. However, previous studies have shown that with increasing pollen deposition, number of seeds also increases (Walters and Taylor 2006, Winsor and Stephenson 1987). Given the positive relationship between female squash bee visits and pollen deposition, the results of my study are consistent with studies that have found that visitors that effectively deposit greater amounts of pollen will ensure a higher number of seeds per fruit.

 In terms of pollen removal, previous studies have reported that honey bees vary in the extent to which they remove pollen from squash flowers. Tepedino (1981), Artz & Nault (2011), and McGrady et al. (2019), for example, have all found that honey bees visit male squash flowers less often compared to pistillate flowers, whereas the opposite pattern holds for squash bees. Vidal et al. (2010), however, found that honey bees often collected pollen from squash flowers and could remove up to two-thirds of the pollen available from squash flowers within two visits. Site-to-site variation in the extent to which honey bees remove pollen from squash flowers may depend on the availability of other sources of pollen in the environment. As with Canto-Aguilar (2011), I found that female squash bees were the most effective in removing pollen. Additionally, Hurd et al. (1974) suggested that squash bees may have an advantage since they are larger and fly faster than honey bees, thereby visiting more staminate flowers and

possibly removing more pollen during a visit compared to other bee species. Behavioral foraging patterns of female squash bees may affect pollen removal. Female squash bees will generally land on the anthers of the flower and move around the anthers as they drink nectar. The constant movement on the anthers and hairier bodies results in passive collection of pollen during a visit, even in cases where no active pollen collection takes place.

Overall, female squash bees appear to be more effective pollinators compared to honey bees for cultivated squash. Davids (2018) showed that single visitation by female squash bees resulted in higher fruit set and pollen deposition than for single visits by honey bees. My study further revealed that increasing visitation and time spent on the flower's stigma increased reproductive success of squash plants. A possible explanation for why my results differs from previous studies, most of which were conducted in agricultural areas, could be due in part to the study site, which was a small plot of squash surrounded by a heterogeneous patchwork of natural and ornamental vegetation. At this study site, honey bees likely have access to a variety of pollen sources and may not be as motivated to collect squash pollen. Furthermore, Williams and Thomson (2003) proposed that even if pollinators had low pollen deposition and pollen removal rates but had high visitation rates than effectiveness per visit would not be an applicable measurement for pollinators. Therefore, to better understand the importance of honey bees and squash bees, determining how overall pollinator visitation and pollen deposition to a flower affect reproductive success may be considered (Sahli and Conner 2007). Additionally, in order to have a better understanding of the pollinator effectiveness of male squash bees, earlier nest emergence by male squash bees should be considered. In this study, flowering began at the end of July, however, previous studies have shown that male squash bees begin to emerge from their

nests as early as mid-June (Tepedino 1981). For this reason, earlier seeding of squash plants should be studied to determine if seasonality has an effect on pollinator effectiveness.

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Tables

Table 1. Results of (i) multiple logistic regression analysis of visitation frequency on fruit set, and (ii) multiple regression analyses of visitation frequency on mean seed mass, number of seeds per fruit, pollen deposition, and pollen removed. Independent variables in these analyses consider pollination date and visitation by *A. mellifera, P. pruinosa* (♀), and *P. pruinosa* (♂). For each analysis the full model is provided under 'A' and the model with the lowest AIC value is provided under 'B.'

	Model 1A						Model 1B				
$N = 103$	AIC		γ^2		\boldsymbol{P}		AIC		γ^2	\boldsymbol{P}	
	116		36.9		< 0.001		110		36.8	< 0.001	
Response variable	Coefficients										
Fruit set	$\beta \pm 1SE$		Wald (χ^2)		\boldsymbol{P}		$\beta \pm 1SE$		Wald (χ^2)	\boldsymbol{P}	
A. mellifera	0.278 ± 0.094		8.69		0.003202		0.275 ± 0.094		8.65	0.003264	
P. pruinosa (\mathcal{Q})	0.674 ± 0.167		16.2		< 0.001		0.666 ± 0.164		16.5	< 0.001	
P. pruinosa (S)	-0.075 ± 0.269		0.077		0.78						
Pollination date (day of year)	-0.066 ± 0.028		5.59		0.018105			-0.065 ± 0.028	5.51	0.018884	
			Model 2A				Model 2B				
$N = 40$	AIC	Adjusted R^2		\mathbf{F}		\boldsymbol{P}	AIC	Adjusted R^2	\mathbf{F}	P	
	335	0.173		3.04		0.02981	330	0.217	11.8	0.00145	
Response Variable	Coefficients										
Mean seed mass	$\beta \pm 1SE$		t-value			\boldsymbol{P}	$\beta \pm 1SE$		t-value	\boldsymbol{P}	
A. mellifera	0.191 ± 0.844		0.226			0.8223					
P. pruinosa (φ)	-1.03 ± 1.16		-0.886			0.3816					
P. pruinosa (S)	0.049 ± 2.13		0.023			0.9818					
Pollination date (day of year)	-0.465 ± 0.246		-1.89			0.0672		-0.607 ± 0.177	-3.43	0.00146	
	Model 3A								Model 3B		
$N = 40$	AIC	Adjusted R^2		$\boldsymbol{\mathrm{F}}$		\boldsymbol{P}	AIC	Adjusted R^2	$\boldsymbol{\mathrm{F}}$	\boldsymbol{P}	
	78.2	0.144		2.10		0.08018	74.4	0.190	3.29	0.02181	
Response variable	Coefficients										
log(Number of seeds)	$\beta \pm 1SE$		t-value			\boldsymbol{P}	$\beta \pm 1SE$		t-value	\boldsymbol{P}	
A. mellifera	0.013 ± 0.035		0.36			0.71845					
P. pruinosa (φ)	0.072 ± 0.047		1.53			0.13563	0.071 ± 0.034		2.07	0.04638	
P. pruinosa (S)	-1.14 ± 0.378		-3.01			0.00504	-1.17 ± 0.358		-3.26	0.00249	
(P. pruinosa $(\text{S})^2$	0.086 ± 0.047			1.93		0.06194	0.088 ± 0.043		2.03	0.05035	
A. mellifera * P. pruinosa (S)	0.210 ± 0.072			2.92		0.00631	0.218 ± 0.067		3.26	0.00249	
Pollination date (day of year)	0.0001047 ± 0.0098			0.011		0.99159					

Table 1. Continued.

Table 2. Results of (i) multiple logistic regression analysis of cumulative duration of stigmatic contact on fruit set, and (ii) multiple regression analyses of duration of stigmatic contact on mean seed mass, number of seeds per fruit, and pollen deposition. Independent variables in these analyses consider pollination date and visitation by *A. mellifera, P. pruinosa* (♀), and *P. pruinosa* (♂). For each analysis the full model is provided under 'A' and the model with the lowest AIC value is provided under 'B.'

		Model 1A		Model 1B			
$N = 101$	AIC		\boldsymbol{P}	AIC	\mathbf{v}^2	\boldsymbol{P}	
	119	27.5	< 0.001	117	27.5	${}< 0.001$	
Response variable	Coefficients						
		Wald			Wald		
Fruit set	$\beta \pm 1SE$	(χ^2)	P	$\beta \pm 1SE$	(χ^2)	\boldsymbol{P}	
A. <i>mellifera</i> stigma time	0.018 ± 0.008	5.68	0.017193	0.018 ± 0.008	5.67	0.0172863	
<i>P. pruinosa</i> (\mathcal{Q}) stigma time	0.015 ± 0.004	17.0	< 0.001	0.015 ± 0.004	17.0	< 0.001	
<i>P. pruinosa</i> (\circ) stigma time	$0.000632 + 0.007$	0.009	0.923943				
$(P. pruinosa \left(\frac{\triangle}{2} \right)$ stigma $time)^2$	-0.0000139 ± 0.0000139 0.00000401	12.0	0.000531	-0.0000139 ± 0.0000139 0.00000272	12.0	< 0.001	
Pollination date (day of year)	-0.057 ± 0.027	4.41	0.035767	-0.057 ± 0.027	4.50	0.0339558	

Table 2. Continued.

Table 3. Results of logistic regression analysis (model 1) and linear regression analyses (model 2 – 5) on the effect of visitation frequency by *A. mellifera* on fruit set, seed set, pollen deposition and pollen removal for squash flowers that were only visited by *A. mellifera*. Akaike information criterion (AIC) was used to estimate best fit model. The number of visits for model 5 was log transformed to reduce variance.

	Coefficient summary				Model summary				
Model		Response variable	$\beta \pm 1SE$	Wald (χ^2)	\boldsymbol{P}	AIC	χ^2		\boldsymbol{P}
1	$N = 58$	Fruit set	0.205 ± 0.086	5.65	0.01749	66.2	6.15		0.01315
			$\beta \pm 1SE$	t-value	\boldsymbol{P}	AIC	Adjusted \mathbb{R}^2	F-value	\boldsymbol{P}
2		$N = 16$ Mean seed mass (mg) 1.024 ± 1.912		0.535	0.601	153	-0.050	0.287	0.6007
3	$N = 16$	Number of seeds	1.38 ± 3.91	0.352	0.7302	176	-0.062	0.124	0.7302
4		$N = 16 \log($ Pollen deposition) 0.067 ± 0.046		1.46	0.166	33.1	0.070	2.131	0.1664
5		$N = 38$ Pollen removed (mg)	4.50 ± 1.95	2.31	0.027	271	0.104	5.31	0.02703

Table 4. Results of logistic regression analysis (model 1) and linear regression analyses (model 2 – 5) on the effect of *A. mellifera* behavior (i.e. total stigma and total anther time) on fruit set, seed set, pollen deposition and pollen removal for squash flowers that were only visited by *A. mellifera*. Total stigma time and anther time were log transformed to better meet regression analysis assumptions.

	Coefficient summary				Model summary				
Model		Response variable	$\beta \pm 1SE$	Wald (χ^2)	\boldsymbol{P}	AIC	χ^2		\boldsymbol{P}
1	$N = 58$	Fruit set	0.466 ± 0.271	2.94	0.08624	69.1	3.19		0.7395
			$\beta \pm 1SE$	t-value	\boldsymbol{P}	AIC	Adjusted \mathbb{R}^2	F-value	\boldsymbol{P}
2		$N = 16$ Mean seed mass (mg)	1.08 ± 6.63	0.163	0.872609	153	-0.069	0.027	0.872
3	$N = 16$	Number of seeds	14.4 ± 13.0	1.11	0.286	174	0.015	1.23	0.2856
4	$N = 16$	$log($ Pollen deposition)	0.391 ± 0.132	2.96	0.0104	27.7	0.340	8.74	0.01043
5		$N = 38$ Pollen removed (mg)	2.68 ± 0.921	2.91	0.00613	268	0.168	8.48	0.006126

		Model 1A			Model 1B				
$N = 102$	AIC	Adjusted R^2	F	\boldsymbol{P}	AIC	Adjusted \mathbb{R}^2	F	\boldsymbol{P}	
	739	0.294	6.26	< 0.001	736	0.298	8.15	< 0.001	
Response variable	Coefficients								
Pollen removed		$\beta \pm 1SE$	t-value	\boldsymbol{P}		$\beta \pm 1SE$	t-value	\boldsymbol{P}	
A. mellifera	1.16 ± 0.429		2.70	0.00830	1.13 ± 0.406		2.78	0.006524	
P. pruinosa (\mathcal{Q})	8.24 ± 1.80		4.58	< 0.001	7.96 ± 1.68		4.73	< 0.001	
P. pruinosa (S)		-1.24 ± 3.94	-0.316	0.75282					
$(P. pruinosa (\tfrac{Q}{r}))^2$		-0.709 ± 0.194	-3.65	< 0.001		-0.705 ± 0.186	-3.79	0.000268	
$(P. pruinosa \, (\text{d}^2))^2$		3.00 ± 1.80	1.67	0.09818		2.36 ± 0.980	2.41	0.017901	
A. mellifera * <i>P.</i> pruinosa (φ)		-0.415 ± 0.230	-1.81	0.07411		-0.427 ± 0.221	-1.93	0.05628	
A. mellifera * P. pruinosa (S)		-1.37 ± 0.531	-2.58	0.01148		-1.368 ± 0.496	-2.76	0.006931	
Pollination date (day of year)		-0.101 ± 0.086	-1.78	0.24206					

Table 5. Results of multiple regression analyses on the effect of visitation frequency and the total time species spent on the anthers by each species on pollen removed. Independent variables in these analyses consider pollination date, visitation and cumulative time spent on anthers by *A. mellifera, P. pruinosa* $(\circled{?})$, and *P. pruinosa* $(\circlearrowleft).$ For each analysis the full model is provided under 'A' and the model with the lowest AIC value is provided under 'B.'

Figures

Figure 1. Partial regression coefficients (β ± 95% CI) from Model 1B, 4B, and 5B (Table 1) for the effect of visitation frequency of *A. mellifera* and *P. pruinosa* (♀) on (A) fruit set, (B) pollen grains deposited onto stigmas, and (C) the amount of pollen removed from anthers. Brackets indicate significant difference between regression coefficients. Asterisks refer to significance of coefficients within a model (*** < 0.001; * < 0.01; * < 0.05)

Figure 2. Mean time spent on stigma or anthers by second visitor. Mean stigma times of second visitors (*A. mellifera* or *P. pruinosa* (♀)) showed no significant difference in (A) pistillate flowers when the first visitor was either *A. mellifera* (blue) or *P. pruinosa* (♀) (orange). Likewise, there was no significant difference in mean anther times in (B) staminate flowers. ns: not significant.

Appendices

Appendix 1. Soil Moisture Reading Data. This table summarizes the mean soil moisture readings for each plant per date. Soil moisture readings were collected three times a week from July 8th to September 6th. Each plant received four soil moisture readings per date, one hour after watering ended. The mean of all soil moisture readings throughout the project are listed under Over Entire Season.

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Appendix 2. Staminate flowers open to bees daily. Pollination bags were removed from a portion of staminate flowers before visitation trials began to supply visitors with enough pollen for deposition on pistillate flowers.

Appendix 3. Protocols for behavior analysis of single visit and multiple visits (3- 6 visits and 9 to 12) to pistillate and staminate flowers

Single visits to pistillate flowers

A visit was defined as beginning when the bee entered the cup of the flower or touched the stigma of the pistillate flower and ended when the bee had left the cup of the flower completely. A spreadsheet was created for each video to quantify stigma behaviors and nectar behaviors. For a behavior to be noted, the behavior had to be at least one second since behaviors were analyzed in one second increments. For stigma behaviors, analysts recorded the duration the bee spent touching the stigma, the duration the bee was moving while on the stigma, and the number of stigma lobes the bee touched throughout its visit. Movement on the stigma was defined as any time the bee was moving its legs while on the stigma, swaying was not counted as movement. For nectar behaviors, analysts recorded the duration the bee spent drinking nectar. Nectar behaviors were recorded when the bee's head or tongue was visibly in the nectary. If the bee did not exhibit either of these during its visit then its behavior was defined as entered flower cup. After all behaviors were recorded, stigma and nectar behaviors were then added respectively to calculate total times for each behavior and proportion of behavior over total time bee spent in flower.

Single visits to staminate flowers

A visit was defined as beginning when the bee entered the cup of the flower or touched the anthers of the staminate flower and ended when the bee had left the cup of the flower completely. A spreadsheet was created for each video to quantify pollen, anther, and nectar behaviors. For pollen behaviors, analysts recorded the duration the bee spent collecting pollen either directly from the anthers or from the base of the flower cup. Honey bees typically collect

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pollen with their front legs and then place pollen into pollen baskets on their back legs. Squash bees generally sit on the anthers and will collect pollen using their back legs. For anther behavior, analysts recorded the duration the bee spent touching the anthers and the amount of time they spent moving on the anthers. Like single visits to pistillate flowers, duration of nectar behavior was recorded when the bees head or tongue was visibly in the nectary. If bee did not exhibit any of these behaviors the bee's behavior was defined as entered flower cup. Once all behaviors were recorded, pollen, anther, and nectar time were added respectively to calculate total times for each behavior and proportion of behavior over total time the bee spent in the flower.

Multiple visits to pistillate and staminate flowers

Protocols for single visit pistillate and staminate flowers were adapted to for multiple visit videos to pistillate and staminate flowers. Definition of a visit was kept the same, however, a bee could visit multiple times. This meant that if a bee left and re-entered the flower cup, this would count as a new visit. Stigma and nectar behavior were recorded for each bee that visited the female flower as well as visitors that only entered the flower cup but did not exhibit stigma or nectar behavior. For staminate flowers duration for pollen, anther, and nectar behaviors were also recorded. The species of bee and the visitor number were also noted for each behavior. After all visits occurred total times for behaviors were recorded for each bee species that visited the flower. Any aggressive behavior or mating between bees was taken note of but not quantified. As stated above for a behavior to be recorded the behavior needed to last at least one second. However, an exception was made for bees that entered the flower cup and left the flower cup in less than one second since a visit was defined as entering the cup of a flower.

Appendix 4. Fruit set data of pistillate flowers visited by 1 visitor (SA = single *A. mellifera*, SPF $=$ single *P. pruinosa* (\circ), or SPM = single *P. pruinosa* (\circ)), 3 – 6 visitors, and 9 – 12 visitors.

Appendix 4. Fruit set data, Continued

Appendix 4. Fruit set data, Continued

Appendix 5. Pollen deposition data for pistillate flowers that set fruit. Pollination treatments: 1 visitor (SA = single *A. mellifera*, SPF = single *P. pruinosa* (♀), or SPM = single *P. pruinosa* (3) , 3 – 6 visitors, and 9 – 12 visitors.

	Pollination			Number of visits				
	date (day of Pollination			P. pruinosa	P. pruinosa	First	Second	pollen count
Plant	year)	treatment	A. mellifera	(φ)	$(\textcircled{3})$	count	count	(grains)
$\overline{4}$	209	9 to 12	$\boldsymbol{7}$	$\mathbf{1}$	$\overline{2}$	896	815	856
6	209	SA	1	$\boldsymbol{0}$	$\boldsymbol{0}$	82	70	76
7	237	9 to 12	$\overline{4}$	6	$\boldsymbol{0}$	1406	1343	1375
9	211	SPF	$\boldsymbol{0}$	$\mathbf{1}$	$\mathbf{0}$	874	864	869
10	214	3 to 6	$\overline{2}$	$\mathbf{1}$	$\mathbf{0}$	252	235	244
14	242	3 to 6	$\mathbf{1}$	5	$\boldsymbol{0}$	494	489	492
18	242	9 to 12	4	5	3	743	760	752
21	239	3 to 6	$\mathbf{1}$	3	$\boldsymbol{0}$	293	308	301
25	220	SPF	$\boldsymbol{0}$	$\mathbf{1}$	$\boldsymbol{0}$	199	203	201
30	213	SPF	$\boldsymbol{0}$	$\mathbf{1}$	$\boldsymbol{0}$	1236	1235	1236
32	222	9 to 12	$\overline{4}$	5	$\boldsymbol{0}$	521	514	518
37	230	3 to 6	3	\overline{c}	1	279	271	275
38	230	9 to 12	\mathfrak{Z}	6	$\mathbf{1}$	205	222	214
39	213	3 to 6	$\overline{4}$	$\boldsymbol{0}$	$\boldsymbol{0}$	370	334	352
40	224	9 to 12	τ	$\overline{\mathcal{L}}$	$\boldsymbol{0}$	1020	1003	1012
47	219	9 to 12	\mathfrak{Z}	5	1	980	954	967
49	241	9 to 12	$\,8\,$	$\mathbf{1}$	$\boldsymbol{0}$	181	181	181
52	232	3 to 6	$\boldsymbol{2}$	$\overline{2}$	$\mathbf{1}$	112	99	106
53	236	SPF	$\boldsymbol{0}$	$\mathbf{1}$	$\boldsymbol{0}$	409	418	414
56	209	3 to 6	$\sqrt{2}$	$\boldsymbol{0}$	$\mathbf{1}$	215	179	197
59	234	9 to 12	$\mathbf{1}$	8	$\mathbf{0}$	577	541	559
61	218	9 to 12	$\boldsymbol{6}$	$\overline{\mathcal{L}}$	$\boldsymbol{0}$	311	306	309
66	246	9 to 12	$\mathbf{1}$	$\sqrt{ }$	$\mathbf{1}$	1066	1070	1068
70	221	9 to 12	τ	\overline{c}	$\boldsymbol{0}$	678	679	679
72	214	SPF	$\boldsymbol{0}$	$\mathbf{1}$	$\boldsymbol{0}$	1217	1114	1166
73	241	9 to 12	9	3	$\mathbf{0}$	717	698	708
77	250	9 to 12	$\boldsymbol{0}$	10	$\boldsymbol{0}$	674	676	675
78	233	3 to 6	\overline{c}	\overline{c}	$\boldsymbol{0}$	189	181	185
79	219	9 to 12	3	\overline{c}	6	392	400	396
82	239	9 to 12	6	5	$\boldsymbol{0}$	765	789	777
87	243	9 to 12	$\,8\,$	3	$\boldsymbol{0}$	2392	2338	2365
97	235	3 to $6\,$	$\mathbf{2}$	3	$\boldsymbol{0}$	299	285	292
101	208	3 to $6\,$	6	$\boldsymbol{0}$	$\boldsymbol{0}$	272	266	269
115	245	9 to 12	$\sqrt{5}$	$\sqrt{ }$	$\boldsymbol{0}$	654	697	676
117	245	9 to 12	1	8	$\boldsymbol{0}$	722	644	683
120	219	SPF	$\boldsymbol{0}$	$\mathbf{1}$	$\boldsymbol{0}$	160	157	159
121	226	9 to 12	10	$\boldsymbol{0}$	$\boldsymbol{0}$	1075	1011	1043

Appendix 5*.* Pollination deposition data, Continued

Appendix 6. Fruit data. Fruits resulted from single *A. mellifera* (SA) visit, single *P. pruinosa* (♀) (SPF) visit, $3-6$ visits by A. *mellifera* and *P. pruinosa* (\bigcirc and \bigcirc), and $9-12$ visits by A. *mellifera* and *P. pruinosa* (\mathcal{Q} and \mathcal{Z}).

Appendix 7. Behavioral analysis of videos data for *A. mellifera* visits to pistillate flowers that also received *P. pruinosa* $(\frac{6}{5})$ visits. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, pf = single *P. pruinosa* (\circ) , or pm = single *P. pruinosa* (\Diamond), 3to6 = 3 – 6 visitors, and 9to12 = 9 – 12 visitors]. Pollination date). See appendix 7 and 8 for behavioral analysis of videos for *P. pruinosa* $(\frac{6}{7}$ and $\frac{3}{7})$ respectively.

Appendix 8. Behavioral analysis of videos data for *P. pruinosa* (φ) visits to pistillate flowers that also received *A. mellifera* or *P. pruinosa* (I) visits. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, pf = single *P. pruinosa* $(\circled{)}$, or pm = single *P. pruinosa* ($\circled{3}$), 3to6 = 3 – 6 visitors, and 9to12 = 9 – 12 visitors]. Pollination date). See appendix 6 and 8 for behavioral analysis of videos for *A. mellifera* and *P. pruinosa* (\vec{c}) respectively.

Appendix 9. Behavioral analysis of videos data for *P. pruinosa* (°) visits to pistillate flowers that also received *A. mellifera* or *P. pruinosa* $(\frac{6}{2})$ visits. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, pf = single *P. pruinosa* $(\circled{)}$, or pm = single *P. pruinosa* ($\circled{3}$), 3to6 = 3 – 6 visitors, and 9to12 = 9 – 12 visitors]. Pollination date). See appendix 6 and 7 for behavioral analysis of videos for *A. mellifera* and *P. pruinosa* (\mathcal{Q}) respectively.

Appendix 10. Pollen removal data for staminate flowers. Pollination treatments: 1 visitor (SA = single *A. mellifera*, SPF = single *P. pruinosa* $(\frac{6}{7})$, or SPM = single *P. pruinosa* $(\frac{3}{7})$, 3 – 6 visitors, and $9 - 12$ visitors. Pollen removal was calculated by subtracting pollen remaining from mean pollen available.

Appendix 11. Behavioral analysis of videos data for *A. mellifera* visits to staminate flowers that also received *P. pruinosa* $(\frac{6}{5})$ visits. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, pf = single *P. pruinosa* (\circ) , or pm = single *P. pruinosa* ($\circled{3}$), 3to6 = 3 – 6 visitors, and 9to12 = 9 – 12 visitors]. Pollination date). See appendix 11 and 12 for behavioral analysis of videos for *P. pruinosa* $(\varphi$ and φ) respectively

Appendix 12. Behavioral analysis of videos data for *P. pruinosa* (φ) visits to staminate flowers that also received *A. mellifera* or *P. pruinosa* (°) visits. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, pf = single *P. pruinosa* $(\circled{2})$, or pm = single *P. pruinosa* ($\circled{3}$), 3to6 = 3 – 6 visitors, and 9to12 = 9 – 12 visitors]. Pollination date). See appendix 10 and 12 for behavioral analysis of videos for *A. mellifera* and *P. pruinosa* (\vec{c}) respectively

Appendix 13. Behavioral analysis of videos data for *P. pruinosa* (°) visits to staminate flowers that also received *A. mellifera* or *P. pruinosa* $(\frac{6}{2})$ visits. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, pf = single *P. pruinosa* $(\circled{2})$, or pm = single *P. pruinosa* ($\circled{3}$), 3to6 = 3 – 6 visitors, and 9to12 = 9 – 12 visitors]. Pollination date). See appendix 10 and 11 for behavioral analysis of videos for *A. mellifera* and *P. pruinosa* (\mathcal{Q}) respectively.

Appendix 14. Fruit set data of pistillate flowers visited by *A. mellifera.* Pollination treatment: $SA = single A$. *mellifera* visit, $3 - 6$ visits, and $9 - 12$ visits.

Appendix 15. Fruit data. Fruits resulted from single *A. mellifera* (SA) visit, 3 – 6 visits by *A. mellifera* , and 9 – 12 visits by *A. mellifera.*

Appendix 16. Pollen deposition data for pistillate flowers that set fruit. Pollination treatments: SA = single *A. mellifera*, 3 – 6 visits by *A. mellifera*, and 9 – 12 visits by *A. mellifera*

Appendix 17. Behavioral analysis of videos data for *A. mellifera* visits to pistillate flowers that only received visits by *A. mellifera*. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, to $6 = 3 - 6$ visits, and 9 to $12 = 9 - 12$ visits]. Pollination date).

Appendix 18. Behavioral analysis of videos data for *A. mellifera* visits to staminate flowers that only received visits by *A. mellifera*. Where Video label = (Plant number. Flower number. Pollination treatment [apis = single *A. mellifera*, $3\text{to}6 = 3 - 6$ visits, and $9\text{to}12 = 9 - 12$ visits]. Pollination date)

			Pollen		
	Flowering date Pollination		remaining	Mean pollen	Calculated pollen
Plant 3	(day of year) 210	treatment 3 to 6	(mg) 8	available (mg) 21.5	removed (mg) 13.5
6	214	SA	23	11.5	$\boldsymbol{0}$
8					
	243 246	SA	15 6	15.0 23.0	$\boldsymbol{0}$
14		3 to 6 SA	14		17
15	217			20.7 23.5	6.7
21	219	3 to 6	24		$\boldsymbol{0}$ $\boldsymbol{0}$
27	227	SA	27	22.7	
29	233	SA	18	22.5	4.5
34	219	SA	30	24.7	$\boldsymbol{0}$
36	234	SA	28	29.0	$\mathbf{1}$
37	209	3 to 6	11	26.5	15.5
52	241	3 to 6	$\overline{4}$	27.0	23
56	214	3 to 6	20	25.0	5
58	221	SA	25	23.0	$\boldsymbol{0}$
59	214	9 to 12	10	20.0	10
68	214	SA	10	31.7	21.7
73	233	9 to 12	15	10.0	$\boldsymbol{0}$
76	222	3 to 6	6	35.0	29
78	211	3 to 6	13	29.0	16
79	219	9 to 12	20	17.0	$\boldsymbol{0}$
87	225	9 to 12	17	32.0	15
89	221	3 to $6\,$	14	25.0	11
90	225	SA	16	25.0	9
92	223	3 to 6	5	33.0	28
96	236	SA	24	21.0	$\boldsymbol{0}$
104	215	SA	40	34.0	$\boldsymbol{0}$
106	222	SA	22	12.0	$\boldsymbol{0}$
112	229	9 to 12	5	7.0	$\boldsymbol{2}$
114	234	3 to 6	15	21.0	6
117	210	9 to 12	3	12.0	9
118	210	SA	12	22.5	10.5
121	223	9 to 12	11	32.0	21
123	222	SA	29	26.0	$\boldsymbol{0}$
130	238	3 to $6\,$	14	23.0	9
131	225	SA	32	26.7	$\boldsymbol{0}$
139	227	9 to 12	12	23.0	11
141	210	3 to 6	13	16.0	3
149	226	SA	21	29.0	$8\,$

Appendix 19. Pollen removal data for staminate flowers. Pollination treatments: SA = single *A. mellifera*, 3to6 = 3 – 6 *A. mellifera* visits, and 9to12 = 9 – 12 *A. mellifera* visits. Pollen removal was calculated by subtracting pollen remaining from mean pollen available. $P₁₁$

Appendix 20. Identity of first and second visitor, and stigma time of second visitor for pistillate flowers. Where Video label = (Plant number. Flower number. Pollination treatment $[3\text{to } 6 = 3 - 6$ visits, and 9to12 = 9 – 12 visits]. Pollination date). Visitors: A = *A. mellifera* ; PF = *P. pruinosa* (φ)

Appendix 21. Identity of first and second visitor, and stigma time of second visitor for staminate flowers. Where Video label = (Plant number. Flower number. Pollination treatment $[3$ to $6 = 3 - 6$ visits, and 9to12 = 9 – 12 visits]. Pollination date). Visitors: A = *A. mellifera* ; PF = *P. pruinosa* Second visitor

Appendix 22. R code for statistical analyses.

Code used for Table 1: Visitation

library("lmtest") library("rcompanion")

```
female.flowers \le- read.csv("final.version.female.flowers.update.csv", header = T)
```

```
male.flowers <- read.csv("final.version.male.flowers.update.csv", header = T)
```
female.flowers\$a2 <- female.flowers\$apis^2 female.flowers\$pf2 <- female.flowers\$pf^2 female.flowers\$pm2 <- female.flowers\$pm^2 female.flowers\$af <- female.flowers\$apis*female.flowers\$pf female.flowers\$am <- female.flowers\$apis*female.flowers\$pm female.flowers\$fm <- female.flowers\$pf*female.flowers\$pm female.flowers\$afm <- female.flowers\$apis*female.flowers\$pf*female.flowers\$pm

#----Model 1----

```
Model1A \leq- glm(fruit \sim apis + pf + pm + j.date, data = female.flowers, family = binomial(link = "logit"))
summary(Model1A)
Anova(Model1A, type="II", test="Wald")
lrtest(Model1A)
```

```
Model1B \langle- glm(fruit \sim apis + pf + j.date, data = female.flowers, family = binomial(link = "logit"))
summary(Model1B)
Anova(Model1B, type="II", test="Wald")
lrtest(Model1B)
```
 $p.1B \le$ - linearHypothesis(Model1B,"apis = pf")

#----Model2----

fruit <- female.flowers[c(1:41),] fruit. $2 <$ - fruit $[-c(14),]$

Model2A \lt - lm(mean.seed.weight \sim apis + pf + pm + j.date, data = fruit.2) summary(Model2A) AIC(Model2A)

Model2B \leq - lm(mean.seed.weight \sim j.date, data = fruit.2) summary(Model2B) AIC(Model2B)

#----Model 3---

```
Model3A \lt- lm(log(seed.number) \sim apis + pf + pm + pm2 + am + j.date, data = fruit.2)
summary(Model3A)
AIC(Model3A)
Model3B \langle- lm(log(seed.number) \sim pf + pm + pm2 + am, data = fruit.2)
summary(Model3B)
AIC(Model3B)
p.3A <- linearHypothesis(Model3A,"apis = pf")
#----Model 4----
Model4A \lt- lm(log(average.pollen.count) \sim apis + pf + pm + a2 + j.date, data = fruit)
summary(Model4A)
AIC(Model4A)
Model4B \leq- lm(log(average.pollen.count) \sim apis + pf + a2, data = fruit)
summary(Model4B)
AIC(Model4B)
p.4B <- linearHypothesis(Model4B,"apis = pf")
#----Table 3: Model 2A----
male.flowers$a2 <- male.flowers$apis.visit.count^2
male.flowers$pf2 <-male.flowers$pf.visit.count^2male.flowers$pm2 <- male.flowers$pm.visit.count^2
male.flowers$af <- male.flowers$apis.visit.count*male.flowers$pf.visit.count
male.flowers$am <- male.flowers$apis.visit.count*male.flowers$pm.visit.count
male.flowers$fm <- male.flowers$pf.visit.count*male.flowers$pm.visit.count
male.flowers$afm <-
male.flowers$apis.visit.count*male.flowers$pf.visit.count*male.flowers$pm.visit.count
Model5A \lt- lm(estimated.pollen.removed \sim apis.visit.count + pf.visit.count + pm.visit.count + pf2 + pm2
+ af + am + j.date, data = male-flowers)summary(Model5A)
AIC(Model5A)
Model5B <- lm(estimated.pollen.removed \sim apis.visit.count + pf.visit.count + pf2 + pm2 + af + am, data
= male.flowers)
summary(Model5B)
AIC(Model5B)
p.5B \leq- linearHypothesis(Model5B, "apis.visit.count = pf.visit.count")
#----Figure 1 for Models 1B, 3A, 4B, 5B (used 3A instead of 3B because 3B does not include apis 
visitation)------
```
library("ggExtra") library("ggplot2") library("gridExtra") library("grid") library("sjlabelled") library("sjmisc") library("sjPlot") library("sjstats")

 $\text{coef} \leq \text{tidy}(\text{Model1B}, \text{conf.int} = \text{TRUE})$

 $\text{coef} < \text{coef}$ [c(2,3),]

 $p \leq -$ ggplot(coef, aes(x = term, y = estimate)) + geom_point(color = c("darkblue", "orange")) + geom_errorbar(aes(ymin = conf.low, ymax = conf.high), color = c("darkblue","orange"), width = 0.1) + labs(title = "(A) Fruit set", $x=$ ("Visitors"), $y = (expression(beta)))$

 $p1 \leq p + scale_x_d$ discrete(name = "Visitor", labels=c("apis" = expression(italic("A.mellifera")), "pf" = expression(italic("P. pruinosa")))) + ylim(-0.25, 2) + geom_hline(yintercept=0, linetype="dashed", color $=$ "black") + theme_sjplot2() + geom_text(x = 1, y = 0.75, label = "**") + geom_text(x = 2, y = 1.25, $label =$ "***") + theme(axis.title.y = element_text(angle = 0, vjust = 0.5), axis.line = element_line(color = "black"), axis.ticks = element_line(color = "black"))

 $p1 < -p1 + geom_text(x = 1.5, y = 1.7, label = "p = 0.0309") + annotate("segment",$ $x = c(1,1,2)$, xend=c(1,2,2), y= c(1.4,1.45,1.45), yend=c(1.45,1.45,1.4))

 $\text{coef}.4B \leq \text{tidy}(\text{Model}4B, \text{conf.int} = \text{TRUE})$

 $\text{coef}.4B \leq \text{coef}.4B[\text{c}(2,3),]$

 $k \le$ - ggplot(coef.4B, aes(x = term, y = estimate)) + geom_point(color = c("darkblue", "orange")) + geom_errorbar(aes(ymin = conf.low, ymax = conf.high), color = c("darkblue","orange"), width = 0.1) + labs(title = "(C) log Pollen deposition", $x =$ ("Visitors"), $y =$ (expression(beta)))

 $p3 < -k$ + scale x discrete(name ="Visitor", labels=c("apis" = expression(italic("A.mellifera")), "pf" = $expression(italic("P. pruinosa"))$) + ylim(-0.6, 0.6) + geom_hline(yintercept=0, linetype="dashed", color $=$ "black") + theme_sjplot2() + geom_text(x = 2, y = 0.25, label = "***") + theme(axis.title.y = element_text(angle = 0, vjust = 0.5), axis.line = element_line(color = "black"), axis.ticks = $element_line(color = "black")$

 $p3 < -p3 + geom_text(x = 1.5, y = 0.5, label = "p = 0.0153") + annotate("segment",$ $x = c(1,1,2)$, xend=c(1,2,2), y= c(0.3,0.4,0.4), yend=c(0.4,0.4,0.3))

 $\text{coef}.5B \leq \text{tidy}(\text{Model5B}, \text{conf.int} = \text{TRUE})$

 $\text{coef}.5B \leq \text{coef}.5B[\text{c}(2,3),]$

 $m < -$ ggplot(coef.5B, aes(x = term, y = estimate)) + geom_point(color = c("darkblue", "orange")) + geom_errorbar(aes(ymin = conf.low, ymax = conf.high), color = c("darkblue","orange"), width = 0.1) + labs(title = "(D) Pollen removed", $x =$ ("Visitors"), $y =$ (expression(beta)))

 $p4 \le m + scale_x_d$ discrete(name = "Visitor", labels=c("apis.visit.count" = expression(italic("A.mellifera")), "pf.visit.count" = expression(italic("P. pruinosa")))) + ylim(-1, 15) + geom_hline(yintercept=0, linetype="dashed", color = "black") + theme_sjplot2() + geom_text(x = 1, y = 2.5, label = "**") + geom_text(x = 2, y = 11.7, label = "***") + theme(axis.title.y = element_text(angle = 0, vjust = 0.5), axis.line = element_line(color = "black"), axis.ticks = element_line(color = "black"))

 $p4 \leq p4 +$ geom text(x = 1.5, y = 14, label = "p ≤ 0.001 ") + annotate("segment", $x = c(1,1,2)$, $x = c(1,2,2)$, $y = c(12,5,13,13)$, $y = c(13,13,12.5)$

grid.arrange(p1, p3, p4, nrow = 1, ncol = 3)

Table 2: Behavior (Stigma time and anther time)

library("lmtest") library("rcompanion")

female.flowers <- read.csv("final.version.female.flowers.update.csv", header = T)

male.flowers <- read.csv("final.version.male.flowers.update.csv", header = T)

female.flowers\$a2 <- female.flowers\$total.a.stigma^2 female.flowers\$pf2 <- female.flowers\$total.pf.stigma^2 female.flowers\$pm2 <- female.flowers\$total.pm.stigma^2 female.flowers\$af <- female.flowers\$total.a.stigma*female.flowers\$total.pf.stigma female.flowers\$am <- female.flowers\$total.a.stigma*female.flowers\$total.pm.stigma female.flowers\$fm <- female.flowers\$total.pf.stigma*female.flowers\$total.pm.stigma female.flowers\$afm < female.flowers\$total.a.stigma*female.flowers\$total.pf.stigma*female.flowers\$total.pm.stigma #Removed large pf stigma times

female.flowers2 <- female.flowers[$-c(3,31)$,]

#----Model 1----

Model1A <- glm(fruit ~ total.a.stigma + total.pf.stigma + total.pm.stigma + pf2 + j.date, family = $binomial(int = "logit"), data = female.flowers2)$ summary(Model1A) Anova(Model1A, type="II", test="Wald") lrtest(Model1A)

```
Model1B \leq- glm(fruit \sim total.a.stigma + total.pf.stigma + pf2 + j.date, family = binomial(link = "logit"),
data = female.flowers2)summary(Model1B)
Anova(Model1B, type="II", test="Wald")
lrtest(Model1B)
```
#----Model 2----

fruit \le - female.flowers[c(1:41),]

fruit.1 <- fruit[-c(3,14,31),]

Model2A <- lm(mean.seed.weight \sim total.a.stigma + total.pf.stigma + total.pm.stigma + pm2 + am + fm + $j.data$, data = fruit.1) summary(Model2A) AIC(Model2A)

Model2B \leq - lm(mean.seed.weight \sim total.pf.stigma + total.pm.stigma + pm2 + am + fm + j.date, data = fruit.1) summary(Model2B) AIC(Model2B)

#----Model 3----

```
Model3A <- lm(log(seed.number) ~ total.a.stigma + total.pf.stigma + total.pm.stigma + j.date, data =
fruit.1)
summary(Model3A)
AIC(Model3A)
```
#----Model 4----

```
Model4A \lt- lm(average.pollen.count \lt total.a.stigma + total.pf.stigma + total.pm.stigma + j.date, data =
fruit.1)
summary(Model4A)
AIC(Model4A)
```
Model4B \langle - lm(average.pollen.count \sim total.pf.stigma, data = fruit.1) summary(Model4B)

AIC(Model4B)

Table 3

```
apis.only \langle- read.csv("pure.apis.data.update.csv", header = T)
```
Model14 \langle - glm(fruit \sim apis, data = apis.only, family = binomial(link = "logit")) summary(Model14) AIC(Model14) #66.17484 # apis = 0.017490 plot(Model14) Anova(Model14, type="II", test="Wald") lrtest(Model14) apis.only\$log.a.stigma <- log(apis.only\$total.a.stigma + 1) Model14.1 \leq glm(fruit \sim log.a.stigma, data = apis.only, family = binomial(link = "logit")) summary(Model14.1) AIC(Model14.1) #69.13124 #not significant plot(Model14.1) Anova(Model14.1, type="II", test="Wald") lrtest(Model14.1) apis.fruit <- apis.only[c(2,3,9,14,16,17,18,22,26,28,32,35,37,40,47,49),] apis.fruit\$apis.log <- log(apis.fruit\$apis +1) Model14.2 \lt - lm(seed.number \sim apis, data = apis.fruit) summary(Model14.2) AIC(Model14.2) #175.5616 #not significant Model14.3 \lt - lm(mean.seed.weight \sim apis, data = apis.fruit) summary(Model14.3) AIC(Model14.3) #152.6368 #not significant Model14.5 \lt - lm(log(average.pollen.count) \sim apis, data = apis.fruit) summary(Model14.5) AIC(Model14.5) #33.1495 #not significant Model14.7 \lt - lm(mean.seed.weight \sim log.a.stigma, data = apis.fruit) summary(Model14.7) AIC(Model14.7) #152.9453 #not significant

Model14.8 \lt - lm(seed.number \lt log.a.stigma, data = apis.fruit)

summary(Model14.8) AIC(Model14.8) #174.1071 #not significant

Model14.9 \langle - lm(log(average.pollen.count) \sim log.a.stigma, data = apis.fruit) summary(Model14.9) AIC(Model14.9) #213.1466

Table 4

apis.only.male.flowers \langle - read.csv("apis.only.male.flowers.csv", header = T)

Model14.10 \leq - lm(estimated.pollen.removed \sim log(apis.visit.count+1), data = apis.only.male.flowers) summary(Model14.10) AIC(Model14.10) #272.8481 #marginally significant 0.0918 plot(Model14.10)

apis.only.male.flowers\$log.a.anther <- log(apis.only.male.flowers\$total.a.anther + 1)

Model14.11 \leq lm(estimated.pollen.removed \sim log.a.anther, data = apis.only.male.flowers) summary(Model14.11) AIC(Model14.11) #267.8456 # significant

#----Table 5: Model 2B----

male.flowers\$log.a.anther <- log(male.flowers\$total.a.anther+1) male.flowers\$log.pf.anther <- log(male.flowers\$total.pf.anther+1) male.flowers\$log.pm.anther <- log(male.flowers\$total.pm.anther+1)

```
male.flowers$a.al2 <- male.flowers$log.a.anther^2
male.flowers$pf.al2 <- male.flowers$log.pf.anther^2
male.flowers$pm.al2 <- male.flowers$log.pm.anther^2
male.flowers$af.al <- male.flowers$log.a.anther*male.flowers$log.pf.anther
male.flowers$am.al <- male.flowers$log.a.anther*male.flowers$log.pm.anther
male.flowers$fm.al <- male.flowers$log.pf.anther*male.flowers$log.pm.anther
male.flowers$afm.al <-
male.flowers$log.a.anther*male.flowers$log.pf.anther*male.flowers$log.pm.anther
```
#Removed high pf anther time male.flowers $2 <$ - male.flowers $[-c(83),]$

```
Model5A \leq- lm(estimated.pollen.removed \sim log.a.anther + log.pf.anther + log.pm.anther + a.al2 + af.al +
j.data = male.flowers2)summary(Model5A)
AIC(Model5A)
```
Model5B \leq - lm(estimated.pollen.removed \sim log.pf.anther + log.pm.anther + a.al2 + af.al, data = male.flowers2) summary(Model5B)

AIC(Model5B)

#Figure 2: Effect of first visitor on second visitor

#Effect of first visitor on second visitor stigma or anther time visitor.order <- read.csv("first.identity.effect.on.second.csv",header = T) visitor.order\$log.second.visitor.stigma.time <- log(visitor.order\$second.visitor.stigma.time + 1)

#effect of first visitor on second visitor stigma time if second visitor is apis.

pf.a <- visitor.order\$log.second.visitor.stigma.time[c(18:29)]

a.a <- visitor.order\$log.second.visitor.stigma.time $[c(1:17)]$

t.test(a.a, pf.a, alternative = "two.sided", var.equal = $FALSE$)

#effect of first visitor on second visitor stigma time if second visitor is pf pf.pf <- visitor.order\$log.second.visitor.stigma.time[c(37:56)]

a.pf <- visitor.order\$log.second.visitor.stigma.time[c(30:36)]

t.test(a.pf, pf.pf, alternative = "two.sided", var.equal = $FALSE$)

visitor.order.mf <- read.csv("first.visitor.effect.on.second.mf.csv",header = T) visitor.order.mf\$log.second.visitor.anther.time <- log(visitor.order.mf\$second.visitor.anther.time +1)

#effect of first visitor on second visitor stigma time if second visitor is apis.

pf.a <- visitor.order.mf\$log.second.visitor.anther.time[c(23:32)]

a.a <- visitor.order.mf\$log.second.visitor.anther.time[c(1:22)]

t.test(a.a, pf.a, alternative = "two.sided", var.equal = $FALSE$)

#effect of first visitor on second visitor stigma time if second visitor is pf

pf.pf <- visitor.order.mf\$log.second.visitor.anther.time[c(40:54)]

a.pf <- visitor.order.mf\$log.second.visitor.anther.time[c(33:39)]

t.test(a.pf, pf.pf, alternative = "two.sided", var.equal = $FALSE$)

#Figure 2

b1 <- ggplot(visitor.order, aes(fill= first.visitor.identity, y=log.second.visitor.stigma.time, $x = secondv}$.visitor.identity)) + geom_boxplot() + xlab("Second visitor") + ylab("log Mean stigma time $(sec)''$) + scale_fill_manual(values=c("darkblue","orange"), name ="First visitor", labels=c("apis" = expression(italic("A.mellifera")), "pf" = expression(italic("P. pruinosa")))) + scale_x_discrete(labels=c("apis" = expression(italic("A.mellifera")), "pf" = expression(italic("P. pruinosa")))) + theme_classic() + scale_y_continuous(expand = $c(0,0)$, limits = $c(0,8)$) + geom_text(x = 1, y = 3, label = "ns") + geom_text(x = 2, y = 5.5, label = "ns") + labs(title = "(A)")

b2 <- ggplot(visitor.order.mf, aes(fill= first.visitor.identity, y=log.second.visitor.anther.time, $x = secondv.·sistor.identity) + geom_boxplot() + xlab("Second visitor") + ylab("log Mean another time)$ $(sec)''$) + scale fill_manual(values=c("darkblue","orange"), name ="First visitor", labels=c("apis" = expression(italic("A.mellifera")), "pf" = expression(italic("P. pruinosa")))) + scale_x_discrete(labels=c("apis" = expression(italic("A.mellifera")), "pf" = expression(italic("P. pruinosa")))) + theme_classic() + scale_y_continuous(expand = c(0,0), limits = c(0,8)) + geom_text(x = 1, y = 2, label = "ns") + geom_text(x = 2, y = 6, label = "ns") + labs(title = "(B)")

grid.arrange(b1, b2, nrow = 2, ncol = 1)