Title

Interactions between squash bees (Peponapis prinosa) and honey bees (Apis mellifera) on winter squash (Cucurbita pepo)

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Interactions between squash bees (*Peponapis prinosa)* and honey bees (*Apis mellifera)* on winter squash (*Cucurbita pepo*)

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Sara Shell Sandoval

Committee in charge:

Professor David Holway, Chair
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Professor Joshua Kohn

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The Thesis of Sara Shell Sandoval is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Interactions between squash bees (*Peponapis prinosa)* and honey bees

(*Apis mellifera)* on a winter squash (*Cucurbita pepo)*

by

Sara Shell Sandoval

Master of Science in Biology

University of California San Diego, 2018

Professor David Holway, Chair

This study focuses on interactions between the non-native western honey bee (*Apis mellifera*), which is a super generalist pollinator, and the native squash bee (*Pepanapis pruinosa*), which is specialist that solely depends on squash pollen to reproduce. This pollination system provides an opportunity to compare the behavior of specialist (*Peponapis*) and generalist (*Apis*) pollinators and how interactions between the two affect plant reproductive success. Using videos to record the behavior of *Peponapis* and *Apis* in the flowers of acorn squash (*Cucurbita pepo)*, we found that compared to squash bees, honey bees had higher visitation rates and more frequently occupied flowers with multiple individuals. Accordingly, intra-floral interactions between honey bee individuals (both aggressive and non-aggressive) were more common than were interactions between squash bees and honey bees. Honey bees increased their visitation rate

in response to increasing nectar volume unlike squash bees, which exhibited an independent relationship between visitation rate and foraging. Interestingly, honey bee foraging appeared to have both positive and negative effects on plant reproductive success. Seed number was negatively related to the cumulative time that honey bees spent on stigmas, whereas mean seed weight and fruit volume both increased with the frequency of aggressive interactions between honey bee individuals.

Introduction

An estimated 85% of flowering plants depend on insects, birds, or mammals for pollination services (Ollerton et al. 2011). Plants attract pollinators with nectar and pollen, and in turn pollinators transfer pollen among plants. Some pollinators are specialists, which means they primarily to exclusively visit flowers of their host plant of a given species. Specialist bees rely entirely on floral resources (nectar and pollen) provided by their host plant for reproduction and survival. This strategy contrast with generalist pollinators, which visit numerous plant species. Given that flowering plant species are commonly visited by numerous pollinator taxa (Waser et al. 1996), specialist and generalist pollinators may often interact with one another while visiting flowers.

In this study we examine interactions between specialist squash bees and generalist honey bees. *Peponapis pruinosa* is native to the Americas and is solitary and oligolectic, specializing on squash plants in the genus *Cucurbita* (Hurd et al. 1970). Squash bees occasionally collect nectar from other plant species, but female bees require squash pollen to rear their larvae (Hurd and Linsley 1964). The range of *P. pruinosa* has expanded north from Central Mexico through much of North America with the spread of cultivated *Cucurbita* species (Bischoff et al. 2009). Squash bees are efficient pollinators of *Cucurbita* species because of their ability to collect heavy pollen grains with the modified hairs on their legs (Hurd and Linsley 1964). Squash bees visit *Cucurbita* species early in the morning, often before other bees appear (Hurd et al. 1970).

The generalist in this system is the western honey bee (*Apis mellifera*), which is native to Europe, Africa, and the Middle East, but introduced to the New World (Han et al. 2012). *Apis mellifera* is a eusocial species with a flexible diet (Requier, et al., 2015). Honey bees were first introduced to the New World to make honey and wax, but now primarily serve as pollinators for a wide variety of agricultural crops, some which are largely to almost entirely dependent on honey bees to provide pollination services (Klein et al. 2007). Honey bees typically forage within 1 km of the colony, but depending on the time of year, long-range foraging up to 10 km is possible (Beekman and Ratnieks 2000). Honey bee colonies are active year-round and can be transported wherever they are needed to provide pollination services for agricultural crops. For these reasons, honey bees are now the most broadly-used, managed pollinator of crops in the United States (Morse and Calderone 2000).

Given that honey bees are widely introduced outside of their native range, numerous studies have sought to evaluate the extent to which competition between honey bees and native bees affects native bee populations and plant reproductive success (Schaffer et al. 1983, Kato et al. 1999, Goulson 2003). In a review of this topic, Paini (2004) reported that 11% of reviewed studies found no impact of honey bees on native bees but identified common limitations of these studies, including low replication and confounding variables, that make it difficult to determine whether honey bees directly or indirectly impact native pollinators. Exploitative competition between honey bees and native bees is commonly studied and there is a large focus on resource depletion (Carneiro and Martins 2012, Herbertsson et al. 2016).

This study focuses on interactions between the non-native western honey bee (*Apis mellifera*), which is a super generalist, and a native, squash specialist bee (*Pepanapis pruinosa*). Using videos to record the behavior of *Peponapis* and *Apis* in the flowers of squash (*Cucurbita pepo),* we address three research questions: (i) Do floral traits influence bee visitation and does this vary between the two focal species? (ii) Do certain bee species interact more aggressively or non-aggressively while in flowers? and (iii) Do interactions between bees predict plant reproductive success (e.g., seed set and fruit volume)? Answers to these questions will clarify how non-native honey bees affect the mutualism between squash and squash bees.

Methods

Study System

We worked with *Cucurbita pepo*, which comes in several cultivated varieties of pumpkin, winter squash, and summer squash. Our study focused on acorn squash (variety: honey bear). *Cucurbita pepo* is monoecious with separate male and female flowers. Flowers of both sexes are large and bright yellow-orange in color, but female flowers produce more nectar compared to male flowers (Nepi et al. 1996). Individual flowers open during early morning and close by afternoon; consequently, the flowers only live for one day. The short lifespan of flowers restricts pollinators to a narrow window of time to collect pollen and nectar.

We examined pollinator behavior on female flowers produced by 20 different plants grown from seeds at the Biology Field Station on the campus of UC San Diego between June and August 2016. Plants were grown in rows with 1.22 m spacing between them. Squash plants flowered from late June to late August. As plants started to flower, we measured the following: corolla size, nectar volume and nectar sugar content. Using digital calipers, we measured corolla size (in mm) as the widest dimension of the open flower. Nectar volume was measured using 200 **μ**L pipettes, while sugar content (% BRIX = strength of solution as percentage by mass) was measured with a refractometer. We focused on one female flower per plant for our observations, but each plant produced an average of 31 total female and male flowers (range 7-75). Focal flowers featured in this study all bloomed in July, which coincided with the peak of both flower production and bee visitation. Fruits were allowed to develop for 50 days from when the flower was open and thus pollinated by bees, prior to harvesting. Mature fruits produced by focal flowers were harvested and weighed beginning in late August. We measured the volume of each fruit and counted and weighed each fruit’s seeds.

The plants considered in this study were grown under variable levels of drought and temperature. Although these physical conditions altered some aspects of plant growth and reproductive allocation, none of the reproductive variables measured in this study were affected by drought and temperature in our sample (n = 20) of female flowers. See Appendix 1 for details.

To record behavioral interactions of honey bees and squash bees in *C. pepo* flowers, we used Black Box DareDVL Wifi Mini Waterproof Sports Action Dash cameras. Video recordings were made between 0600 and 0800 hours each morning. We bagged flowers the day before (*i.e.*, when they were floral buds) to ensure that bees had not yet visited flowers before the start of each recording. Video cameras were placed 15 cm away from the open corolla of a flower and recorded pollinator visitation for two hours. We recorded the following data from videos: rate of visitation by *A. mellifera* (visits/min), rate of visitation by *P. pruinosa* (visits/min), the maximum number of *A. mellifera* and *P. pruinosa* present in the flower (standardized for length of time), and the type of intraspecific and interspecific interactions between bees.

We classified interactions between bees as either aggressive or non-aggressive. We considered aggressive interactions to be those in which an initiator pushed or pulled a receiver away from the stigma, bit its abdomen, head or legs, or lunged at or grappled with a receiver. Aggressive interactions typically resulted in the retreat of the receiver from the flower. Non-aggressive interactions involved any contact between bees that did not result in the retreat of the receiver and that involved the initiator contacting but not fighting with the receiver. Receivers in non-aggressive interactions would often remain in the flowers, where they continued to drink nectar, rest, or groom. Non-aggressive interactions also included non-aggressive touching or climbing over other bees. For example, bees often contacted each other with their legs or heads while moving around in flowers.

We also quantified the amount of time bees contacted stigmas to determine if the duration of stigmatic contact affected plant reproductive success: fruit volume, seed number, and mean seed weight. The duration of stigmatic contact was the cumulative time honey bees or squash bees spent in contact with floral stigmas during an entire video and included the time bees were moving or stationary on the stigma.

Data analysis

We used regression analysis to examine associations (i) between floral traits and bee behaviors, and (ii) between bee behaviors and the reproductive output of flowers. Resource traits included corolla size, nectar volume, and sugar content. Bee behaviors included visitation rate, maximum number of bees visiting, rate of aggressive and non-aggressive interactions, and total stigma visit time (sec). Plant reproductive variables included mean seed weight (mg), mean fruit volume (g), and number of seeds per fruit.

Results

Visitation

Honey bees visited squash flowers more often than did other pollinators. Visitation by honey bees was more than five times higher than that of squash bees (Table 1; paired *t*-test: $t_{18} = 6.49$, $P = 0.001$). Likewise, the maximum number of honey bees in individual flowers was nearly four times greater than the maximum number of squash bees (Table 1; paired *t*-test: $t_{19} = 5.64$, $P < 0.001$). Visits by honey bees and squash bees made up 99% of all visits to squash flowers; the other 1% of visits were made by native bees such as *Bombus* spp. and *Halictus* spp.

Honey bee visitation was more strongly related to nectar volume and sugar content than to corolla size. This relationship was not seen with squash bees (Table 2). We focused on nectar volume because of its slightly more relatedness to honey bee visitation. Honey bees and squash bees differed with respect to the relationship between visitation rate and nectar volume (Fig. 1). Honey bee visitation increased with nectar volume (simple linear regression: $F_{1,16} = 6.10$, $P = 0.03$, $R^2 = 0.23$), but there was no relationship between visitation rate and nectar volume for squash bees (simple linear regression: $F_{1, 16} = 0.19$, $P = 0.67$, $R^2 = 0.01$). The maximum number of honey bees simultaneously present in a flower increased with increasing visitation rate (simple linear regression: $F_{1, 17} = 7.67$, $P = 0.013$, $R^2 = 0.31$), but this relationship did not hold for squash bees (simple linear regression: $F_{1, 17} = 0.75$, $P = 0.40$, $R^2 = 0.04$). The frequency of non-aggressive and aggressive interactions between honey bees increased with increasing honey bee visitation rate (simple linear regression: $F_{1, 17} = 14.5$, $P = 0.001$, $R^2 = 0.46$; $F_{1, 17} = 5.32$, $P = 0.03$, $R^2 = 0.24$). Total honey bee stigma time increased as honey bee visitation rate increased (simple linear regression: $F_{1, 17} = 9.85$, $P = 0.01$, $R^2 = 0.37$).

Bee species behaved differently from one another while interacting in flowers. Interactions between *Peponapis* and *Apis* were shorter in duration compared to intraspecific interactions involving *Apis*, with respect to aggressive interactions (Table 3; paired *t*-test: t_{19} = 3.42, P = 0.003) and non-aggressive interactions (paired *t*-test: t_{19} = 6.87, P < 0.001). Compared to *Peponapis, Apis* initiated more aggressive (Table 3; paired *t*-test: t_{18} = 4.60, P < 0.001) and non-aggressive (Table 3; paired *t*-test: t_{19} = 2.95, P = 0.01) interactions.

Interactions and Reproductive Success

To assess how bee behavior affected plant reproductive success, we tested two relationships: (i) whether or not the duration of time spent on squash flower stigmas by honey bees and squash bees were related to fruit volume, seed number, and seed weight, and (ii) whether or not honey bee-honey bee interactions were related to fruit volume, seed number, and seed weight.

Only time spent on stigmas by honey bees significantly predicted the number of seeds of the squash flower while time spent on stigmas by honey bees and squash bees did not affect the seed weight or fruit volume (Table 4). There was no relationship between honey bee visitation rate and any measure of reproductive success (simple linear regression: seed weight: $F_{(1,17)}$=0.64, P=0.44, R^2=0.04; seed number: $F_{1,17}$=0.24, P=0.63, R^2=0.01; fruit volume: $F_{1,17}$=0.19, P=0.67, R^2=0.01), so we focused on time spent on stigma and interactions between the bees. As time spent on stigmas by honey bees increased, the seed number decreased, but this relationship was no longer significant when two outliers for total stigma time are removed (Figure 3; $F_{1,17}$=5.10, P=0.04, R^2=0.23). We removed two plants due to the immense time spent on stigma by honey bees and the fact that the stigmas on those two plants were abnormal in stigma shape and size.

The total time spent on the stigma of squash flowers was related to specific bee behaviors as well. As the rate of *Apis* visitation increased, the total time spent on the stigma also increased ($F_{1,16}$ = 5.42, P = 0.03, R^2 = 0.21). There were more aggressive and non-aggressive interactions between honey bees when the visitation rate of honey bees increased (Figure 2; simple linear regression: $F_{1, 17}$ = 5.32, P = 0.03, R^2 = 0.24; $F_{1, 17}$ = 14.5, P = 0.001, R^2 = 0.46). Moreover, receivers walked away significantly more often when there were more aggressive or non-aggressive interactions (simple linear regression: $F_{1, 18}$ = 13.84, P = 0.002, R^2 = 0.43; $F_{1, 18}$ = 4.52, P = 0.05, R^2 = 0.20), which could affect how much time was spent in contact with the stigma. However, we found that the number of bees that walked away from aggressive or non-aggressive interaction did not predict total time spent in contact with the stigma (simple linear regression: $F_{1.17}$ = 0.08, P = 0.78, R^2 = 0.004; $F_{1,17}$ = 2.54, P = 0.13, R^2 = 0.13).

When looking at aggressive and non-aggressive interactions we saw that aggressive interactions predicted some measures of reproductive success. Fruit volume increased with the frequency of aggressive interactions between honey bees (Figure 4; simple linear regression: $F_{1, 18}$ = 4.84, P = 0.04, R^2 = 0.21). Seed weight also increased with an increasing frequency of aggressive interactions between honey bees (Figure 4; simple linear regression: $F_{1, 18}$ = 8.56, P = 0.01, R^2 = 0.32). These relationships held even after outliers were removed.

Discussion

The squash pollination system provides an opportunity to compare the behavior of specialist (*Peponapis*) and generalist (*Apis*) pollinators and how these interactions affect plant reproductive success. Compared to squash bees, honey bees had higher visitation rates and more frequently occupied flowers with multiple individuals. Accordingly, intra-floral interactions between honey bee individuals (both aggressive and non-aggressive) were more common than were interactions between squash bees and honey bees. Honey bees increased their visitation rate in response to increasing nectar volume unlike squash bees, which exhibited an independent relationship between visitation rate and foraging success. Interestingly, honey bee foraging appeared to have both positive and negative effects on plant reproductive success. Seed number was negatively related to the cumulative time that honey bees spent on stigmas, whereas mean seed weight and fruit volume both increased with the frequency of aggressive interactions but not with honey bee visitation rate by itself.

Since bee species differ in their morphology and lifestyles, they also differ in their behavior. *Apis mellifera* exhibits a variety of behaviors while in flowers including feeding, grooming, and even fighting (Santa et al. 2017). Behaviors of *Peponapis* have not been documented as extensively as honey bees, but they are known to spend most of their time collecting pollen from *Cucurbita* (Hurd and Linsley 1964). These different behaviors may affect how each bee species acts in an encounter with each other.

Intraspecific aggression of honey bees at a food source is known from interactions at artificial feeders (Nieh 2010), but interspecific aggression towards larger, native bees on artificial feeders has not been reported to our knowledge. In a study between Africanized honey bees and stingless bees, Roubik (1980) found that honey bees repelled other bee species by vibrating their wings but did not directly attack them. In another study where flowers were used as a food source, honey bees displayed aggressive behavior towards native bees that in some cases led to native bees leaving the flower (Gross & Mackay, 1997). In our system aggressive interactions between honey bees and squash bees were seldom observed and when they were observed squash bees more often initiated the interaction.

Squash flowers provide an abundance of floral resources as evidenced by our observation of up to seven bees occupying a single flower at once. In stingless bees, interspecific aggression increases with increasing sugar concentration with less aggressive species excluded from these high-quality resources (Johnson and Hubbell 1974). Johnson & Hubbell (1974) suggested that the more bees present on a flower or bait, the more likely fighting will occur due the attack pheromone secreted by the mandibular gland of the fighting bees. We observed a similar result with respect to aggression and visitation rate. Our study saw a similar result for aggression between honey bees and visitation rate of honey bees as increased flower nectar volume increased bee visitation, which increased the rate of aggressive interactions.

We did observe that intraspecific interactions between honey bees increased the time that honey bees spent on floral stigmas, but the frequency of these interactions also increased visitation rate, making it impossible to separate their separate effects. That said, visitation rate alone did not predict fruit and seed set; we only saw that increased honey bee visitation led to more contact with the stigma by honey bees. By comparing total time spent on stigma of squash bees and honey bees, we observed that seed number decreased as time spent on the stigma by honey bees increased. Gross & Mackay (1997) reported a similar result in cases where honey bees were the last visitors to the flower. Gross and Mackay (1997) further concluded that honey bees were inefficient pollinators and should be removed from reserve areas. To clarify, we did not analyze stigma contact time of honey bees or squash bees during or immediately after any interactions or visits, so we cannot make this same conclusion.

Aggression between bees has also been linked to plant reproductive success. Sampson et al. (2016) reported that aggressive interactions among individuals of a specialist bee species (*Ptilothrix bombiformis)* in *Hibiscus* flowers increased stigmatic pollen deposition. Our results suggest something similar; it is possible that aggressive interactions among *Apis* individuals might allow them to encounter the stigma more often and affect the squash plant seeds and fruit.

Some studies mention that honey bees are a ecologically disruptive introduced species (Goulson 2003), and they have been labeled as a supergeneralist pollinator for their ability to visit a wide range of plant species (Richardson et al. 2000). In our system, honey bees visited flowers more often than did squash bees and increased their rate of visitation with increasing nectar volume. Honey bees always outnumbered squash bees and were often the only pollinator present. This type of observation leads to the one of the controversies surrounding honey bees. How is visitation by honey bees affecting the reproductive success of crops and native plants?

The high visitation rate by honey bees observed in this study suggests the possibility of over visitation. For example, high rates of visitation to raspberry flowers by *Bombus terretris* and *Apis mellifera* led to damaged floral styles (Sáez et al. 2014). We observed a potentially similar result in this study that requires additional study. While we did not assess potential damage to squash flowers, we did observe a negative relationship between the cumulative time that honey bees spend on the floral stigma and seed number. Unlike the flowers of raspberry, squash flowers are larger and probably sturdier. In videos floral styles appeared intact even after many visits by honey bees.

Our results illustrate the potential importance of examining intraspecific and interspecific interactions between floral visitors as a factor influencing plant reproductive success. Single-visit measures of pollinator effectiveness, which are commonly employed to compare pollinator species, do not incorporate the effects of multiple visits or interactions that take place inside of flowers. Clarifying the importance of repeated visits by pollinators and how their interactions affect plant reproductive success has an obvious applied importance. Honey bees provide benefits as an introduced species, but their effectiveness as pollinators varies greatly from species to species, both in agricultural and non-agricultural systems (Hung et al. 2018). Given the annual value of global crops ranging from $235 billion to $577 billion (IPBES 2016), the fact that many of those crops depend on pollinators (Potts et al. 2016), and the loss of pollinators can cause cascading effects (Kearns and Inouye 1997), it seems important to clarify the effects of honey bees as pollinators.

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Tables

Table 1. Mean (± 1 SE) visitation rate and maximum number of individual *Apis mellifera* and *Peponapis pruinosa* in squash flowers.

	Visits/min	Max number of bees/60 min
Apis mellifera	0.45 ± 0.01	4.25 ± 0.44
Peponapis pruinosa	0.08 ± 0.01	1.10 ± 0.30

Table 2. Simple linear regressions of honey bee and squash bee visitation as function of three different floral traits: nectar volume, nectar BRIX, and corolla size.

	Nectar volume	Nectar BRIX	Corolla Size
Honey bee visits/min	*P* 0.03*	*P* 0.03*	*P* 0.38
	$F_{1,16}$ 6.10	$F_{1,16}$ 6.06	$F_{1,16}$ 0.80
	R^2 0.28	R^2 0.27	R^2 0.05
Squash bee visits/min	*P* 0.67	*P* 0.59	*P* 0.57
	$F_{1,16}$ 0.19	$F_{1,16}$ 0.30	$F_{1,16}$ 0.33
	R^2 0.01	R^2 0.02	R^2 0.02

Table 3. Summary statistics of the frequency and duration of behavioral interactions (aggressive/non-aggressive) between honey bees (*Apis mellifera*) and squash bees (*Peponapis pruinosa*). Interaction type includes honey bees as both the initiator and receiver (H-H), a honey bee as the initiator and a squash bee as the receiver (H-S), and a squash bee as the initiator and a honey bee as the receiver (S-H).

Interaction Type	Aggressive interactions/min	Duration of aggressive interactions (sec)	Non-aggressive interactions/min	Duration of non-aggressive interactions (sec)
H-H	0.17 ± 0.04	3.75 ± 0.92	1.02 ± 0.24	3.00 ± 0.55
H-S	0.001 ± 0.001	0.30 ± 0.22	0.02 ± 0.01	0.45 ± 0.23
S-H	0.01 ± 0.01	0.70 ± 0.42	0.05 ± 0.02	1.00 ± 0.39

Table 4. Plant reproductive success as a function of stigma time for honey bees and squash bees.

Reproductive Success	Honey bee stigma time	Squash bee stigma time
Seed weight	$F_{1,17} = 0.06$ $P = 0.81$ $R^2 = 0.05$	$F_{1,17} = 2.46$ $P = 0.14$ $R^2 = 0.08$
Seed number	$F_{1,17} = 5.10$ $P = 0.037$* $R^2 = -0.19$	$F_{1,17} = 0.45$ $P = 0.51$ $R^2 = 0.03$
Fruit volume	$F_{1,17} = 2.71$ $P = 0.12$ $R^2 = 0.09$	$F_{1,17} = 2.96$ $P = 0.10$ $R^2 = 0.10$

Figures

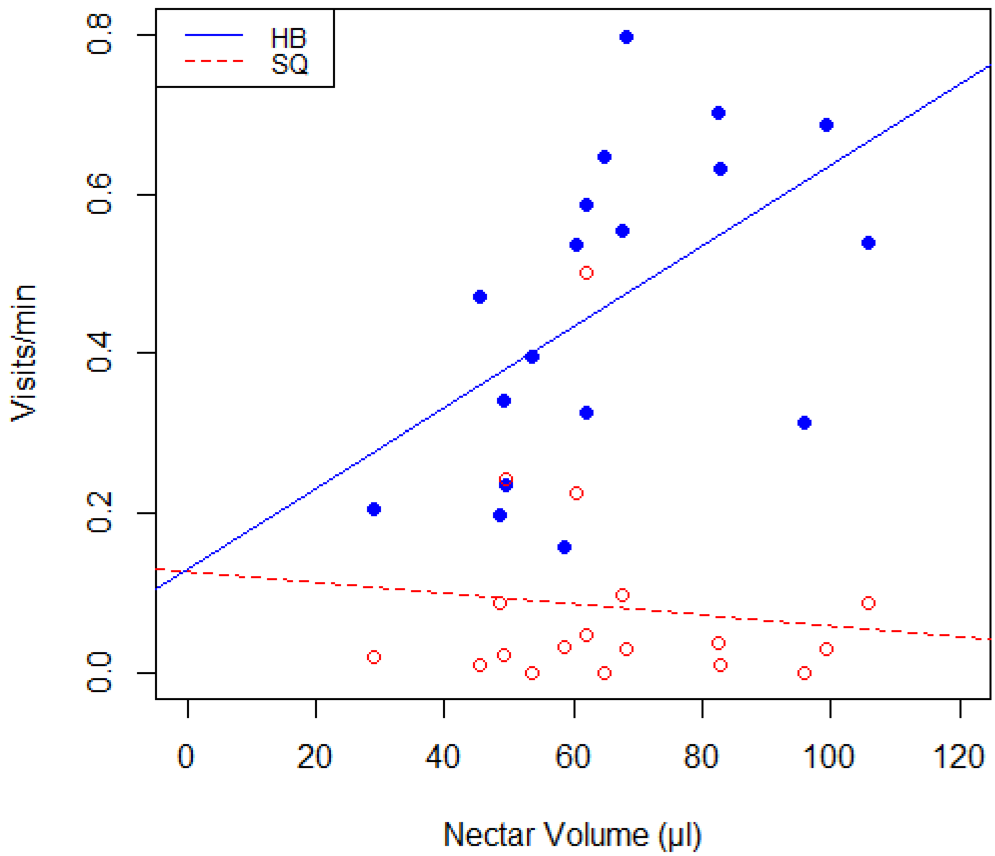


Figure 1. Honey bee (*Apis mellifera*) visitation rate increased with nectar volume of the squash (*Cucurbita pepo*) flowers, whereas squash bee (*Peponapis pruinosa*) visitation rate was independent of nectar volume.

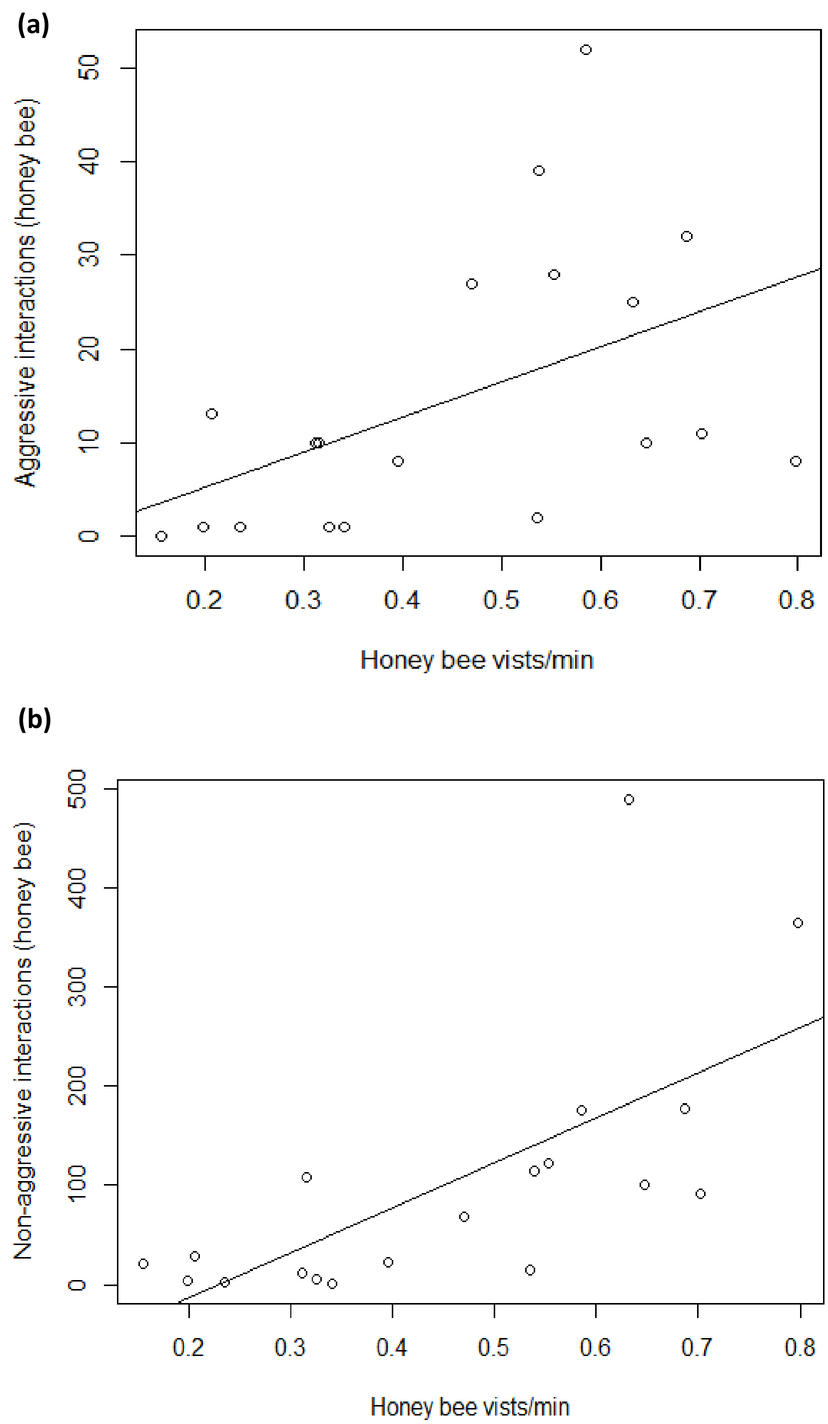


Figure 2. Aggressive (a) and non-aggressive (b) interactions between honey bee individuals increased with visitation rate.

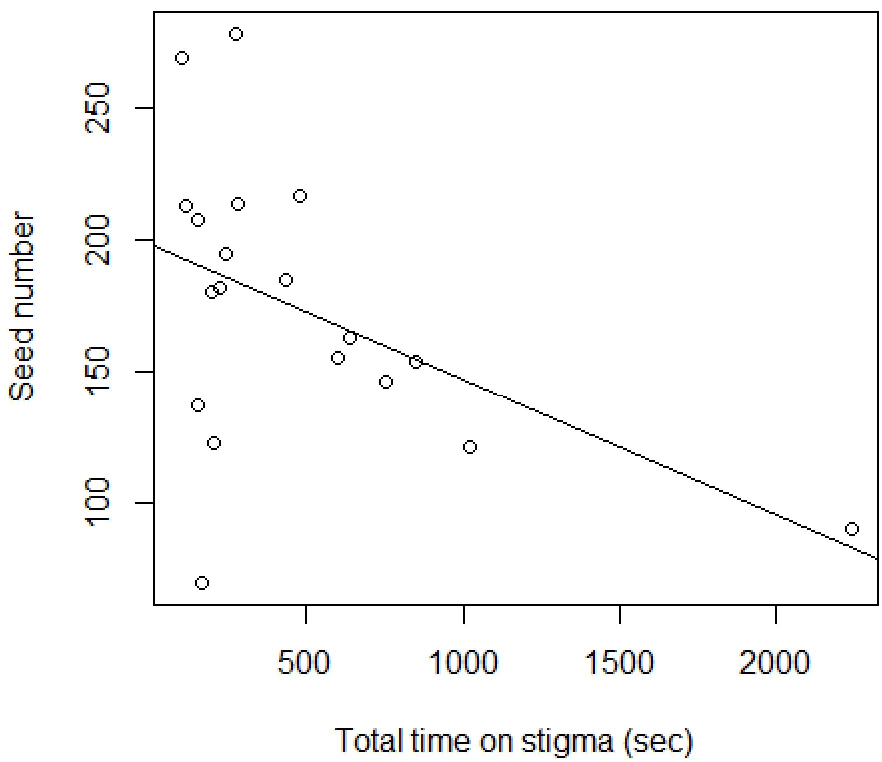
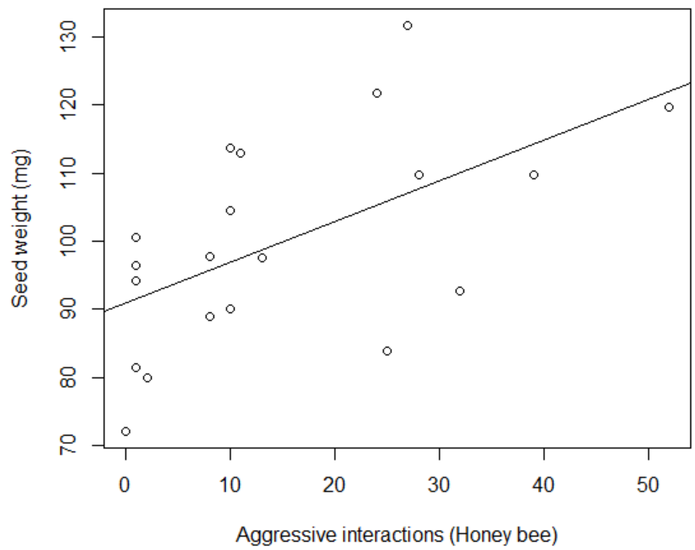


Figure 3. Seed number decreases as the total time on stigma by *Apis* increases.

(a)



(b)

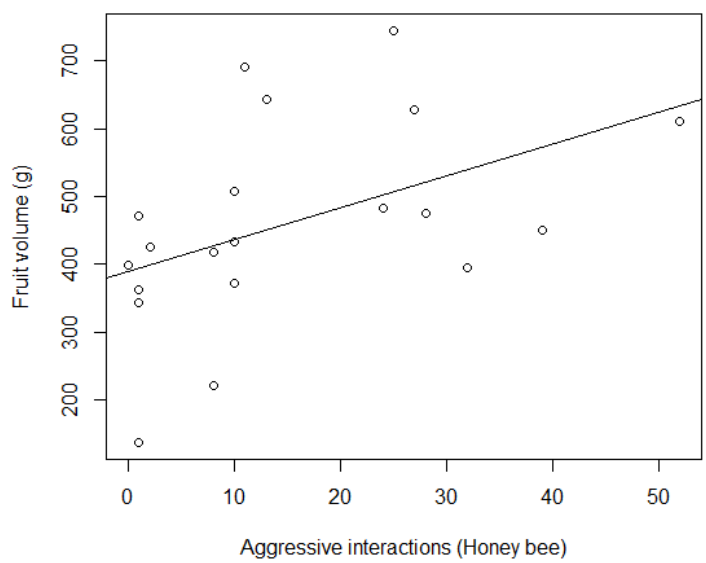


Figure 4. Seed weight (a) and fruit volume (b) increased with an increasing frequency of *Apis*-*Apis* aggressive interactions.

Appendices

Appendix 1. Physical conditions representing drought and reproductive variables measured.

ANCOVA results listed below (df = 1,16).

	Fruit volume (g)	Seed number	Seed weight (mg)
Soil moisture	$P = 0.36$ $F = 0.91$	$P = 0.44$ $F = 0.63$	$P = 0.30$ $F = 1.13$
Temperature treatment	$P = 0.26$ F=1.34	$P = 0.20$ $F = 1.82$	$P = 0.95$ $F = 0.01$
Soil moisture ~ temperature treatment	$P = 0.67$ F= 0.19	$P = 0.61$ $F = 0.26$	$P = 0.55$ $F = 0.37$