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Plant-pollinator mutualisms in the face of environmental change: an experimental test with Cucurbita and its bee pollinators

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Plant-pollinator mutualisms in the face of environmental change: an experimental test with *Cucurbita* and its bee pollinators

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requirements for the degree Doctor of Philosophy

in

Biology

by

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2020

The dissertation of Jess Gambel is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2020

EPIGRAPH

Bees do have a smell, you know, and if they don't they should,
for their feet are dusted with spices from a million flowers.

Ray Bradbury

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ABSTRACT OF THE DISSERTATION

Plant-pollinator mutualisms in the face of environmental change: an experimental test with *Cucurbita* and its bee pollinators

by

Jess Gambel

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Professor David Holway, Chair

Plant-pollinator mutualisms provide essential services to both wild and cultivated ecosystems across the globe. Yet these important interactions face many environmental threats that could impact the ability of pollinators to effectively mediate reproduction in plant hosts. Climate change is one such threat. Past studies have investigated how abiotic stress can lead to mismatches in phenology and distribution in plant-pollinator relationships, but less research has focused on the effects of altered environmental conditions on plant floral traits. This dissertation centers on the impacts that warming and drought stress have on the production of floral resources, how bee pollinators respond to those changes, and how plant reproduction is ultimately affected. For this work, we utilized the *Cucurbita* system, including both cultivated squash (*Cucurbita pepo*) and free-living gourd (*C. foetidissima*), which is pollinated by generalist honey bees (*Apis mellifera*) as well as specialist squash bees (*Eucera*). In Chapter 1, we examined how the combined effects of warming and drought altered the *C. pepo* system and found that water stress increased pollen limitation in bee-pollinated plants due to (i) decreased pollen competition caused by low-levels of stigmatic pollen deposition, (ii) reduced viability of pollen produced by plants grown under low soil moisture conditions, and (iii) a reduced capacity of self-pollinated fruits to increase seed set in response to increasing soil moisture. In Chapter 2, we investigated how generalist and specialist bees responded when given a choice between *C. pepo* plants grown at varying soil moistures, and discovered that only generalist honey bees increased visitation with plant soil moisture, thereby increasing deposition of pollen from well-watered plants and increasing seed set. In Chapter 3, we focused on comparing the effectiveness of honey bees and squash bees as pollinators of both *C. pepo* and *C. foetidissima* and assessed that, overall, squash bees removed more pollen, deposited more pollen, and contributed more to fruit set and seed set on both *Cucurbita* species. The results of this dissertation may be applied to other plant-pollinator systems and reveal that the contributions of generalist and specialist pollinators to pollination services may be impacted in an altered climate.

CHAPTER 1

Pollinators mediate effects of experimental drought on squash (*Cucurbita pepo*) reproduction

by

Jess Gambel and David Holway

ABSTRACT

Rising temperatures and changing precipitation patterns accentuate arid conditions experienced by plants. Warming and drought directly affect plant performance and survival, but plants may also be affected by how pollinators respond to floral traits compromised by abiotic stress. A specific aim of this study lies in understanding how pollinators mediate effects of experimental warming and drought on plants that require animal-mediated pollination. Field manipulations in cultivated squash (*Cucurbita pepo*) revealed that warming and drought independently affected floral traits (e.g., flower size, nectar volume). When effects on reproductive allocation were evident in this experiment, low soil moisture levels always had an inhibitory effect. By examining responses of squash pollinated by hand *versus* by bees, we observed that bee-pollinated plants experienced increasing levels of pollen limitation (estimated based on seed set) in response to decreasing soil moisture. Additional soil moisture manipulations indicated that drought-induced pollen limitation resulted, at least in part, from (i) decreased pollen competition caused by low-levels of stigmatic pollen deposition, (ii) reduced viability of pollen produced by plants grown under low soil moisture conditions, and (iii) a reduced capacity of self-pollinated fruits to increase seed set in response to increasing soil

moisture. Although this study focuses on only a single plant species, drought-induced pollen limitation could be a common phenomenon based on the frequency with which drought stress affects reproductive allocation in other plant species.

INTRODUCTION

Since the pre-industrial era, the rise in atmospheric carbon dioxide concentrations caused by human activities has raised global temperatures, with the greatest rate of increase occurring in the 21st century (IPCC 2014). In fact, the years 2015 – 2019 were the warmest on record (NOAA 2020). As temperature increases drive climatic instability, average precipitation has become more variable with a growing contrast between wet and dry regions (Collins et al. 2013). In drier areas, aridity is predicted to increase to the level of severe drought by mid-century (Dai 2011). Such environmental effects could greatly impact ecosystem services, including pollination (Giannini 2017, Gérard 2020, Wagner 2020). Most research (Willmer 2011, Gilman et al. 2011, Bartomeus et al. 2011, Hegland et al. 2009, Memmott et al. 2007, Petanidou et al. 2014) examining the impacts of warming and drought on plant-pollinator interactions has focused on phenological, distribution, or population-level responses of plants and pollinators (Waser & Price, 2016, Descamps et al. 2018). Less research has focused on how drought and warming affect physiological mechanisms underlying the production of floral resources (Scaven & Rafferty 2013, Descamps et al. 2018), how changes in reproductive allocation influence pollinators, and how changes in pollinator behavior affect plant reproductive success.

A limited number of studies have investigated how warming or drought independently affect floral traits in insect-pollinated systems (Al-Ghzawi et al. 2009, Hoover et al. 2012, Rader et al. 2013, Burkle & Runyon 2016, Gallagher & Campbell 2017, Descamps et al. 2018, Glenny et al. 2018). These studies have found that higher temperatures can reduce flower size (Hoover et al. 2012, Descamps et al. 2018), increase flower (Hoover et al. 2012) and nectar (Hoover et al. 2012) production, and decrease pollen viability (Koti et al. 2005), while water stress also reduces flower size (Galen 1999, Halpern et al. 2010, Burkle & Runyon 2016, Gallagher & Campbell

2017, Descamps et al. 2018) and pollen viability (Al-Ghzawi et al. 2009, Descamps et al. 2018), but diminishes flower (Al-Ghzawi et al. 2009, Burkle & Runyon 2016, Descamps et al. 2018) and pollen production (Waser & Price 2016) as well as nectar volume (Halpern et al. 2010, Waser & Price 2016, Gallagher & Campbell 2017) and sugar content (Waser & Price 2016, Descamps et al. 2018).

By modifying floral resources, drought and warming could impact rates of pollinator visitation and outcrossing pollination. Bees may exhibit preferences for larger flowers (Galen & Newport 1987), more viable pollen (Robertson et al. 1999), or increased nectar volume and concentration (Thomson 1986). Descamps et al. (2018), for example, found that warming and drought stress reduced rates of bumblebee visitation to flowers of *Borago officinalis*. Waser and Price (2016) determined that reductions in pollen deposition in *Ipomopsis aggregate*, despite high pollinator visitation, most likely resulted from drought reducing nectar and pollen availability for hummingbird pollinators. Al-Ghzawi et al. (2009) found that drought-induced reductions in flower number, pollen quantity, and pollen quality led to 70% fewer honey bee visits and, consequently, fewer pods and seeds produced in *Trigonella moabitica*. Gallagher and Campbell (2017) determined that although flower size and nectar production in *Mertensia ciliata* increased linearly with water availability, bumblebee visitation was highest at intermediate corolla length, which coincided with intermediate moisture levels. Interestingly, seed set was greatest at peak water availability and not at peak pollinator visitation (Gallagher & Campbell 2017), showing that plant reproduction may be dependent to varying degrees on resource availability and pollination. Past studies, however, do not explicitly attempt to separate effects of abiotic stress on plant reproductive performance from those effects that that are mediated by pollinators. Diminished visitation by pollinators resulting from altered floral rewards may subsequently increase the likelihood of pollen limitation (Thomson 1986), which may, in turn, reduce fruit and seed production (Burd 1994). As many plants rely on pollinators to provide outcrossing pollination, declines in pollinator abundance or diversity could alter how plants respond to changing physical conditions. Understanding the effects of a changing climate on ecosystems requires the identification of pollinator function in providing pollination services under altered environmental conditions.

To address the extent to which pollinators mediate the effects of abiotic stress on plant reproduction, we focus on cultivated squash (*Cucurbita pepo*). This system is well suited to our experimental investigation. First, squash and gourd plants require pollinators to move pollen from male (staminate) flowers to female (pistillate) flowers (Tepidono 1981, Hoehn et al. 2008, Artz & Nault 2011). Changes in reproductive allocation (e.g., nectar production) resulting from drought and warming could thus influence how pollinators interact with plants and in ways that modify plant reproductive success. *Cucurbita* also produce large flowers in which floral traits are easy to measure and pollinators are readily observed. Moreover, the short period of time that flowers remain viable (< 1 day) simplifies efforts to quantify pollinator behavior and to measure different aspects of plant reproductive success. Lastly, interactions between *Cucurbita* and its pollinators are well studied (Hurd et al. 1971, Tepedino 1981, Artz & Nault 2011), economically important (McGrady et al. 2020), and susceptible to environmental change (Hoover et al. 2012).

A specific aim of this research lies in understanding the degree to which pollinators mediate effects of experimental warming and drought on plants that require insect pollination for reproduction. As our interests rest in how pollination changes due to an altered climate, we established our experiment in such a way as to not allow pollinators to choose among plants in different treatment combinations of drought and warming. This method ensured that pollinators only made trips to and from plants experiencing the same physical conditions, thus emulating a climate scenario in which pollinators would likely not be presented with options. Furthermore, we focus on how these floral visits mediate changes in plant reproduction through effects on stigmatic pollen deposition. Differences in the quantity or quality of pollen deposited by pollinators impact fruit and seed set. Winsor (1987), for example, documented that pollen competition in *C. pepo* increases with stigmatic pollen deposition. Under high pollen loads, only the most viable pollen grains appear to contribute to pollination as a result of increased pollen competition; seed set and offspring vigor thus increase with increasing amounts of deposited pollen (Winsor 1987). In contrast, smaller pollen loads appear to reduce pollen competition and thus provide an opportunity for less viable pollen grains to achieve fertilization, thereby decreasing seed set (Winsor 1987). For small pollen loads, pollen quality may become increasingly important for plant reproductive success. Pollen quality may be affected by altered environmental conditions (Koti et al. 2005, Al-Ghzawi et al. 2009, Descamps et al. 2018) or by pollen source, e.g. pollen from self *versus* non-self plants (Hayes et al. 2005). Although *C. pepo* can set fruit from self-pollination (Loy 2004, Abu-Hammour & Wittmann 2010), measures of female fitness, such as seed set, in the wild gourd, *C. pepo* spp. *texana*, decrease as a result of inbreeding depression (Hayes et al. 2005). More importantly, the extent of inbreeding depression in fitness traits may in turn become exacerbated due to environmental stress (Hayes et al. 2005). No research to date has shown the effects of selfing in drought-stressed domesticated *C. pepo* plants. Moreover, instances of reduced pollen competition on pollinated stigmas may further expose differences in viability of self *versus* non-self pollen under varying degrees of water stress.

In the present study, we manipulated soil moisture and temperature experienced by cultivated squash to investigate how variation in these conditions (i) affected different aspects of reproductive allocation relevant to pollination, and (ii) how these changes in turn influenced pollinator-mediated aspects of plant reproduction. We first tested how soil moisture and temperature affected floral traits (of both male and female flowers) likely to affect pollinator visitation. To examine the specific influence of pollinators, we compared fruit and seed set between plants pollinated by bees and plants pollinated by hand. The results of this first experiment led us to focus on how soil moisture variation affected the interaction between plants and pollinators in terms of pollen viability and the availability of non-self pollen. Given that drought stress commonly affects reproductive allocation in other plant species in ways that influence pollinator visitation, we would anticipate that pollinators could play an important role in influencing how plants respond to abiotic stress. Moreover, isolating the effects of pollinator performance from plant function in response to variation in resource availability provides a novel approach in determining how pollination systems may be impacted by a changing climate.

METHODS

We grew *Cucurbita pepo* (Honey Bear F1 acorn squash seed from Johnny's Selected Seeds$^{©}$) at the University of California, San Diego Biology Field Station (32°53'08.8" N, 117°13'47.2" W) in the summers of 2016, 2017 and 2018. Squash were germinated from seeds planted directly in the ground in 1.2 m x 1.2 m plots with each plot containing a single plant. Jobe's Organic Vegetable Fertilizer$^{©}$ (2% nitrogen, 5% phosphorus, 3% potassium) was added (130 g/plant) to the soil during seeding to aid in plant establishment. After plants matured, we sprayed them with neem oil 2-3 times during each season as needed to reduce pest and fungus infestations. In each year of this study squash plants flowered from early July into early September. Manipulations included temperature and irrigation (2016) and irrigation only (2017, 2018). In each year, plants in different treatment groups were spatially interspersed with respect to one another. Details of each experiment are described below with additional information in Appendix 1.1.

At our study site squash were primarily pollinated by squash bees and honey bees. Solitary squash bees (e.g., *Eucera (Peponapis) pruinosa, E. (Xenoglossa) strenua* (Dorchin et al. 2018)) specialize on *Cucurbita*; the former species provides a unique example of a pollinator that has spread widely in North America in response to the cultivation of *C. pepo* (López-Uribe et al. 2016). The western honey bee (*Apis mellifera*) is a eusocial generalist that commonly visits *Cucurbita* (Hurd et al. 1974, Tepedino 1981, Artz & Nault 2011). Honey bees that visited squash flowers at our study site likely represent a mix of managed colonies (n = 16) kept at this location for research and feral colonies, which are locally common (Kono & Kohn 2015).

Temperature x irrigation experiment

From June through September 2016, we established a fully crossed, two-way factorial temperature (warmed, ambient) by irrigation (high, low) experiment with 20 *C. pepo* in each of the four treatment groups (Appendix 1.1). To elevate temperature for plants in the warmed group, we used passive, open-top warming chambers (Godfree et al. 2011) that fit over the semi-bush squash plants. Thermochron iButtons© that recorded hourly temperature readings (Godfree et al. 2011) for each plant throughout the season indicated that mean daily temperatures were 2.5˚C higher for plants in the warmed treatment group compared to plants in the ambient treatment group (one-way ANOVA: $F_{1,78} = 501.80$, $P < 0.0001$; Table A.1.1.1). The irrigation treatment included two levels: 2.0 L water/plant/day (high) and 0.67 L water/plant/day (low). We used a drip-line system to irrigate plants every morning. For the first three weeks following the germination of seeds, all plants received 2.0 L water/plant/day to encourage establishment. Plants in the low irrigation group thereafter received 0.67 L water/plant/day. We used a FieldScout TDR 100© soil moisture meter to take 2 - 4 replicate measurements of volumetric water content (VMC%) in the soil at 20 cm below each plant one hour after watering, 2 - 3 times per week (Waser & Price 2016). Mean volumetric water content was 10% lower for plants in the low irrigation group compared to plants in the high irrigation group (two-way ANOVA: $F_{1,76} = 93.92$, $P < 0.0001$; Table A.1.1.1). Soil moisture levels, however, were highly variable among plots. Mean volumetric water content, for example, varied from 36 - 56% (high irrigation) to 27 - 51% (low irrigation). For this reason, we treat soil moisture as a continuous variable in all statistical analyses (see also Gallagher & Campbell 2017) but retain the use of the four experimental groupings in the organization of the experiment.

For every plant, we measured traits of female and male flowers from which insect visitors were excluded. In the afternoons, we bagged flowers ready to open the next morning by placing a Seedburo Treated S27 Shoot Pollinating Bag (5cm x 2.5cm x 18cm) around each floral bud (Stoner & Eitzer 2012). Bags prevented insects from entering flowers. On the morning that the unvisited flowers opened, we detached them from the plants while still bagged. We measured flower size (male flowers n = 456, female flowers n = 390), nectar volume (male flowers n = 383, female flowers n = 349), nectar concentration (male flowers n = 357, female flowers n = 356), pollen mass (male flowers n = 440), and pollen viability (male flowers n = 410). Appendix 1.1 describes methods used to measure these floral traits.

Flowers not used to measure floral traits were left open to pollinators to assess how temperature and irrigation affected pollen deposition, fruit set, and seed set. In each of the four experimental groups, we randomly assigned plants to a bee-pollinated group (n = 15 plants) and a hand-pollinated group (n = 5). Plants in these two groups were spatially interspersed with respect to one another. Bee-pollinated plants were open to pollinators subject to the constraint that only flowers from plants within the same experimental group were accessible on a given day; all other open flowers were bagged to exclude pollinators. This approach ensured that bee-pollinated plants consequently received pollen only from other bee-pollinated plants within the same experimental group. As applicable, we temporarily removed warming chambers from the plants being observed that morning so as not to inhibit bee visitation. For each open flower, we recorded all floral visitors observed in five, 15-min surveys spread out across a 5-h time span between 0600 and 1100 (Hurd et al. 1974). We also video recorded a subset of open flowers for 1.5 hours each morning to examine bee visits in more detail than was possible from direct observation. Methods used to videotape flowers are provided in Appendix 1.1.

To estimate pollen limitation, hand-pollinated plants were used as a basis for comparison to bee-pollinated plants. In the afternoons, we used Seedburo Treated S27 Shoot Pollinating Bags (5 cm x 2.5 cm x 18 cm) to exclude bees from flowers ready to open the next morning (Stoner & Eitzer 2012). To hand pollinate each female flower, we mixed together pollen from two male flowers from different plants and then used a cotton swab to generously apply the abundance of mixed pollen to the entire stigma of the female flower (Winsor et al. 2000). Just as bees were allowed to visit plants only within the same experimental group on a given day, hand-pollinated plants only received pollen from plants within the same experimental group. After hand pollination, we re-bagged flowers with breathable mesh bags (Paper Mart standard gold organza (15 cm x 23 cm)) to exclude pollinators while simultaneously permitting the flower to experience conditions like those experienced by flowers open to bees (Kearns & Inouye 1993).

After the bee-pollinated and hand-pollinated flowers closed (i.e., by early afternoon of the day that they opened), we randomly selected flowers to estimate either (i) pollen deposition, or (ii) fruit and seed set. To estimate pollen deposition, we first used a single-edged razor blade to remove bee and hand-pollinated floral stigmas, which we immediately placed in a solution of 70% ethanol (Winsor et al. 2000) and basic fuchsin dye (Kearns & Inouye 1993). We randomly selected two stigmatic lobes (stigmas usually have 6 lobes total), counted all pollen grains present on these lobes under a dissecting microscope at 50x magnification, and then calculated the mean pollen count between both lobes (Artz & Nault 2011). We calculated the total stigmatic pollen count as the mean pollen deposited per lobe plus any stained grains present in the ethanol solution. As expected, pollen deposition was higher in hand-pollinated plants: total pollen count on stigmas pollinated by hand (916 ± 119 (mean ± SE)) was more than two-fold higher compared to that on stigmas pollinated by bees (403 ± 25 grains) (general linear model: $F_{1,55}$ =

37.19, $P < 0.0001$; $\log_{10}$ transformed to improve normality). Flowers that were not sacrificed to estimate pollen deposition were monitored to determine fruit set. We harvested fruit 50 days after pollination (Loy 2004). Developed seeds from mature fruit were dried and weighed. We report total seed mass per fruit per plant as a measure of seed set. Mean seed mass did not depend on seed number in this experiment (simple linear regression: $F_{1,72} = 0.058$, $r^2_{adj} = -0.013$, $P = 0.81$; square transformed to improve normality).

For all statistical analyses performed in this study, we used R version 3.6.1 (R Core Team, 2019) and used the package *ggplot2* to prepare figures (Wickham 2009). We inspected q-q plots to test for normality and used Bartlett tests to assess homogeneity of variances. We also tested the residuals of each model with the Shapiro-Wilk test for normality. For bee-pollinated plants in the *Temperature x irrigation experiment*, we used general linear models with two fixed factors, soil moisture (mean volumetric water content) and temperature (warmed, ambient), to examine their direct effects on individual floral traits (response variables): total flower number, self and non-self flower number, flower size, nectar volume, nectar concentration, pollen mass, and pollen viability. We also used general linear models with soil moisture and temperature as fixed factors to examine bee visitation and behavior rates in flowers. We conducted a second set of analyses with an additional fixed factor (bee pollination, hand pollination) to test whether or not differences in fruit set (general linear model, family = binomial) and seed set (general linear model) resulting from soil moisture and temperature variation could be mediated by interactions with pollinators. For all analyses in the *Temperature x irrigation experiment*, plant is the experimental unit of analysis. All plants were given at least one chance to set fruit from a pollinated female flower. For plants that set more than one fruit (n = 23), we used mean values for total seed mass.

For the floral trait analyses, the response variables of total female flowers and total available self male flowers were right-skewed and thus $\log_{10}$ transformed to improve normality of the residuals. Pollen mass in male flowers was right-skewed (to a lesser degree) and thus square-root transformed to improve normality, although pollen mass still did not have equal variances after transformation. Nectar concentration in female flowers and proportion viable pollen in male flowers were left-skewed and thus square transformed to improve normality, although pollen viability still did not have equal variances after transformation. Variances were marginally equal for nectar volume in male flowers. For the bee visitation analyses, all rates of bee visits per flower per minute were proportions and thus arcsine square root transformed to improve normality; however, except for *Apis* visit rate to female flowers, the residuals were still not normal even after transformation. For the bee behavior analyses, all behavior rates were right-skewed and thus $\log_{10}$ transformed to improve normality of the residuals; however, the residuals were still not normal for *Eucera* behaviors in male flowers and the variances were still not equal for *Apis* pollen collection per min. For the fruit and seed set analyses, the residuals were not normal for fruit set.

To determine if bee-pollinated plants experienced pollen limitation, we calculated pollen limitation using the pollen limitation (PL) index, $L = 1 - (B/H)$ (Larson & Barrett 2000). In this equation, B represents total seed mass for a bee-pollinated plant, and H equals total seed mass for a hand-pollinated plant. For each hand-pollinated plant in the equation, we matched a corresponding bee plant that had similar mean plant soil moisture and was from within the same experimental group. For hand ($n = 5$) and bee ($n = 3$) plants with more than one fruit in July, we used mean values of total seed mass for each plant. We used a general linear model containing soil moisture and temperature to examine their direct effects on pollen limitation. Soil moisture values used in the analysis are those of the matched bee-pollinated plants. We also estimated pollen limitation using a second method in which we used all bee-pollinated plants and the mean values of their matched hand-pollinated plant treatment groups. This second method yielded results that are qualitatively similar (Table A.1.1.2).

Irrigation experiment I (pollinator transfer of self versus non-self pollen)

From June through September 2017, we tested how irrigation level affected the degree to which bee pollinators of squash transfer self pollen *versus* non-self pollen. This particular research focus was stimulated by one of the results of the *Temperature x irrigation experiment*, namely that the availability of non-self, male squash flowers (i.e., at the scale of our experimental garden) increased with increasing soil moisture experienced by individual plants (Table 1.1). Given this result, pollinators may be transferring increasing amounts of self pollen with decreasing soil moisture, which may cause reduced seed set. In *Irrigation experiment I* we grew squash in a similar manner to that in the *Temperature x irrigation experiment*, but in the present experiment we grew *C. pepo* plants (n = 56) under different levels of irrigation and did not manipulate temperature. We used a drip-line system to irrigate plants every morning; the irrigation treatment included two levels: 1.3L water/plant/day (high) and 0.38 L water/plant/day (low). We monitored soil moisture levels as in the *Temperature x irrigation experiment* and found that volumetric water content decreased by an average of 17% for plants in the low irrigation treatment (one-way ANOVA: $F_{1,94} = 158.60$, $P < 0.0001$). Soil moisture levels again exhibited substantial variation within each experimental group. Mean volumetric water content, for example, varied from 38 - 63% (high) to 10 - 54% (low). For this reason, we again consider soil moisture as a continuous variable in all statistical analyses but use treatment group designations in the organization of the experiment. All analyses are restricted to data collected from July 28 - August 25 during the height of flowering and pollinator visitation.

To test for differences in the transfer of self pollen and non-self pollen as a function of irrigation level, we used florescent powdered pigments to track bee movements among plants (Ordway et al. 1987). On each day of the experiment, we identified 1-2 focal plants that each had one female flower and at least one male flower open that day. Before allowing bees access to flowers, we used flat toothpicks to apply powdered DayGlo© fluorescent pigments (colors: saturn yellow, aurora pink, and horizon blue) to the anthers of focal male flowers (Waser 1988). On a given day, the male flowers of each focal plant received its own unique pigment color; all male flowers on non-focal plants received a different pigment color. Each day we switched the pigment color assignments between focal and non-focal plants to mitigate for any color preferences exhibited by bees. When bees contacted the anthers of a male flower with pigment, pollen grains as well as pigment particles adhered to the bees' bodies. Therefore, the number of pigment particles acted as a proxy for the number of pollen grains transported by bees to the stigmas of the female flowers (Waser 1988).

We estimated pollen deposition as follows. Following pollination, removed stigmas were immediately placed in 100% ethanol (Winsor et al. 2000). Prior to adding basic fuchsin solution to dye pollen grains, we used a dissecting microscope to count pigment particles under 40x magnification (Waser 1988). We then added basic fuchsin solution (Kearns and Inouye 1993) and used a dissecting microscope to count the number of pollen grains on the entire stigma and in the ethanol solution containing the stigma (Artz & Nault 2011). Mean total pigment particle count on bee-pollinated stigmas was highly correlated with mean total pollen grain deposition (Pearson correlation: $r_{29} = 0.83$, $P < 0.0001$). To assess whether or not irrigation level affects the deposition by bees of self *versus* non-self pollen, we used general linear models to compare pigment and pollen deposition as a function of soil moisture.

Irrigation experiment II (effects of self versus non-self pollen deposition)

From June through September 2018, we conducted a field experiment that involved irrigation and hand-pollination to test (i) how pollen source (i.e., soil moisture experienced by plants that produce the pollen), and (ii) pollen identity (i.e., self pollen *versus* non-self pollen) affect the seed set of plants grown under different levels of soil moisture (Recart et al. 2019). To hand pollinate female squash flowers, we used identical methods as those employed in the *Temperature x irrigation experiment*, except that we reduced the amount of pollen deposited to stigmas (see methods in Appendix 2) (i) to emulate levels observed in bee-pollinated flowers, and (ii) to decrease pollen competition below levels that likely occurred in the hand-pollination treatment in the *Temperature x irrigation experiment*. When stigmatic pollen deposition is adjusted to levels comparable to those delivered by bees, reduced pollen competition would presumably allow the majority of pollen grains to germinate and not just those that were most viable (Winsor 1987). This scenario should thus provide greater sensitivity to detect differences in how plants respond (in terms of seed set) to self or non-self pollen under varying degrees of water stress.

Squash rearing was performed in a similar manner to that in the *Temperature x irrigation experiment*, but in this experiment we grew *C. pepo* plants (n = 46) under different levels of irrigation and did not manipulate temperature. We used a drip-line system to irrigate plants every morning; the irrigation treatment included two levels: 2.4 L water/plant/day (high) and 0.38 L water/plant/day (low). Mean volumetric water content for plants in the low irrigation treatment was 11% lower than that of plants in the high irrigation treatment (one-way ANOVA: $F_{1,44} = 152.40$, $P < 0.0001$). The volumetric water content for plants in the high irrigation treatment ranged from 41 - 54%, whereas that of plants in the low irrigation treatment ranged from 31 - 41%. All analyses are restricted to data collected from August 3 - September 8 during the height of flowering and pollinator visitation. Once squash plants began to flower, we hand-pollinated female flowers with either self pollen or non-self pollen from plants within the same irrigation group. We measured pollen deposition as in *Irrigation experiment I* and fruit set and seed set as in the *Temperature x irrigation experiment.*

To test how soil moisture affects the seed set of flowers pollinated with pollen that varied in terms of its source and its identity, we considered two different measures of soil moisture. First, we used soil moisture values of the plants that produced focal female flowers. Second, we used the mean soil moisture experienced by the plants that provided pollen used for hand pollination (self: same plant; non-self: 1-2 different plants). In the fruit set analysis, we did not use mean soil moisture experienced by the plants donating pollen because some plants had multiple chances to produce non-self fruits, and each of these chances utilized pollen from different plants with different soil moistures. To test how flowers pollinated with self pollen *versus* non-self pollen responded to soil moisture variation in terms of fruit set, we ran separate general linear models (family = binomial), each containing soil moisture as a fixed factor. Given the number of zero values in our data set (i.e., flowers that did not set fruit despite the opportunity), we used a zero-inflated negative binomial (ZINBI) model using package *gamlss* to analyze seed set, which included zero values. This model contained two fixed factors, soil moisture and pollen type (self or non-self), and one random factor (plant). We adopted this approach because (i) as mentioned previously, plants in this experiment were given multiple opportunities to set fruit, and (ii) plants had the opportunity to grow both self and non-self fruits. We ran these models first using the plant's soil moisture and then again using the mean soil moisture of the two plants donating pollen for the non-self fruits.

Irrigation experiment III (effects of pollen deposition from high versus low moisture plants)

From June through September 2018, we conducted a field experiment that involved irrigation and bee-pollination to test how pollen source (i.e., soil moisture experienced by plants that produced pollen) affected the seed set of plants grown under different levels of soil moisture. For this experiment, we allowed bee visitation on plants grown under a gradient of soil moistures. Therefore, bees could move freely between flowers in both high-irrigation and low-irrigation treatment groups and, consequently, deposit pollen from both high and low moisture plants on stigmas of plants grown under different levels of soil moisture.

Squash rearing was performed in a similar manner to that in the *Temperature x irrigation experiment*, but in this experiment we grew *C. pepo* plants (n = 61) under different levels of irrigation and did not manipulate temperature. We used a drip-line system to irrigate plants every morning; the irrigation treatment included two levels: 2.2 L water/plant/day (high) and 0.35 L water/plant/day (low). Mean volumetric water content for plants in the low irrigation treatment was 10% lower than that of plants in the high irrigation treatment (one-way ANOVA: $F_{1,89} = 143.30$, $P < 0.0001$). The volumetric water content for plants in the high irrigation treatment ranged from 38 - 51%, whereas that of plants in the low irrigation treatment ranged from 24 - 48%. All analyses are restricted to data collected from July 21 - August 21 during the height of flowering and pollinator visitation.

To test for differences in seed set due to pollen deposition from high and low moisture plants, we used florescent powdered pigments to track bee transfer of pollen from male flowers of plants in high and low irrigation treatments to female flowers of plants in both treatment groups (Ordway et al. 1987). Before allowing bees access to flowers in the morning, we applied powdered DayGlo© fluorescent pigments (colors: aurora pink and horizon blue) to male flowers as in *Irrigation experiment I*. Male flowers on plants in the high-irrigation treatment received one pigment color, male flowers on plants in the low-irrigation treatment received the other pigment color. Each day we switched the color assignments to mitigate for any color preferences exhibited by bees. As in *Irrigation experiment I*, the number of pigment particles acted as a proxy for the number of pollen grains transported by bees to the stigmas of the female flowers (Waser 1988). We measured pollen and pigment deposition as in *Irrigation experiment I* and seed set as in the *Temperature x irrigation experiment.* Mean total pigment particle count on bee-pollinated stigmas was highly correlated with mean total pollen grain deposition (Pearson correlation: $r_{46} = 0.80$, $P < 0.0001$). To determine the effect of soil moisture from plants donating pollen on seed set, we used a general linear model with one fixed factor, proportion of deposited pigment from plants grown under low irrigation. For all analyses in *Irrigation experiment III,* plant is the experimental unit of analysis. All but five plants were given at least one chance to set fruit from a pollinated female flower. For plants that set more than one fruit (n = 3), we used mean values for total seed mass.

RESULTS

Temperature x irrigation experiment

Reproductive allocation in bee-pollinated squash plants was affected by the manipulation of temperature and soil moisture, but these factors generally had independent effects on floral traits (Table 1.1). When effects on allocation were evident, low soil moisture levels were always inhibitory. For both flower sexes, flower size increased with increasing soil moisture and decreased under warmed conditions (warmed: 51.97 ± 1.22 mm, ambient: 56.14 ± 1.56 mm) (Fig. 1.1A and Fig. 1.1B). Nectar volume increased with increasing soil moisture and increased temperature (warmed: 39.08 ± 2.86 mL, ambient: 32.55 ± 1.96 mL) for male flowers, but this effect was not significant in female flowers (Fig. 1.1C and Fig. 1.1D). Warmed conditions also stimulated male flowers to produce more pollen (warmed: 18.45 ± 1.05 mg, ambient: 15.36 ± 0.83 mg) (Table 1.1). In addition, nectar concentration increased with soil moisture in ambient temperature male flowers (simple linear regression: $F_{1,28} = 10.82$, $r^2_{\text{adj}} = 0.25$, $P < 0.01$) but not in female flowers (Table 1.1). There were no effects of temperature or soil moisture on the proportion of viable (i.e. stained red or purple) pollen grains in male flowers (Table 1.1). Lastly, per plant flower production was unaffected by the manipulation of temperature and soil moisture, except for the potential availability of non-self flowers (Table 1.1).

The main pollinators observed visiting squash flowers in this experiment were honey bees (*Apis mellifera*) and squash bees (*Eucera* (*Peponapis) pruinosa*). Together these two species accounted for 97% of all floral visits. In general, pollinator visitation rates did not differ with respect to variation in temperature and soil moisture experienced by squash plants (Table A.1.1.3), but this experiment was not designed to examine pollinator choice. The one exception with respect to visitation was that we observed that the cumulative pollen collecting per min for

Apis mellifera was higher in warmed plants (Table A.1.1.3). There was also a significant interaction between soil moisture and temperature for visits per female flower per minute for *Apis* and *Eucera* (Table A.1.1.3); however, these results were not significant when broken down between warmed and ambient treatments.

Fruit set and seed set of squash plants increased with increasing soil moisture (Table 1.2). Aside from soil moisture, no other measured variable, including pollination type, affected the likelihood of fruit set (Table 1.2). In contrast, seed set increased with increasing soil moisture but only for bee-pollinated plants (Table 1.2, Fig. 1.2A). The seed set of fruits that resulted from hand-pollination did not depend on soil moisture and was almost 27% higher (hand: 22,259 ± 1872 mg, bee: 17,595 ± 1100 mg) compared to that from fruits that resulted from bee pollination (Table 1.2). Based on seed set measurements, the pollen limitation index increased with decreasing soil moisture (Fig. 1.2B). There was no effect of temperature on pollen limitation (two-way ANCOVA: $F_{1,14} = 1.60$, $P = 0.23$).

Irrigation experiment I (pollinator transfer of self versus non-self pollen)

Based on fluorescent pigment deposition, we found no evidence that non-self pollen transfer by bees increased with increasing soil moisture. Non-self pigment deposition per stigma per plant was independent of soil moisture (simple linear regression: $F_{1,29} = 0.37$, $r^2_{adj} = -0.021$, $P = 0.55$). Mean total pollen deposition per stigma per plant was also independent of soil moisture (simple linear regression: $F_{1,29} = 0.076$, $r^2_{adj} = -0.032$, $P = 0.79$).

Irrigation experiment II (effects of self versus non-self pollen deposition)

The likelihood of fruit set did not increase with plant soil moisture for female flowers hand pollinated with either self pollen (general linear model: $z = 1.82$, $P = 0.069$) or non-self pollen (general linear model: $z = 1.75$, $P = 0.080$). Seed set increased with increasing plant soil moisture (Table 1.3A) and with increasing soil moisture experienced by the pollen donors (Table 1.3B), but these significant effects were only observed in fruits that resulted from pollination with non-self pollen (Fig. 1.3A and Fig. 1.3B). For fruits that resulted from pollination with self pollen, we observed no significant effects of soil moisture on total seed mass per fruit (Fig. 1.3A and Fig. 1.3B). Moreover, seed set increased with total stigma pollen deposition (Table 1.3C) in fruits that resulted from pollination with non-self pollen (Fig. 1.3C) but decreased with total stigma pollen deposition in fruits that resulted from pollination with self pollen (Fig. 1.3C).

Irrigation experiment III (effects of high versus low moisture pollen deposition)

Seed set decreased with increased proportion of stigmatic deposition of pigment from plants grown in the low irrigation treatment (Fig.1.4). Thus, stigmatic deposition of more pigment (i.e. pollen) from low moisture plants led to reduced seed set in developed fruits.

DISCUSSION

We assessed the degree to which pollinators influence how experimental warming and drought affect the reproduction of cultivated squash (*Cucurbita pepo*). For those floral traits that responded to the experimental manipulations, low soil moisture levels always resulted in inhibitory effects: smaller flower size, reduced nectar volume and nectar concentration (male flowers only), and a (potentially) reduced availability of non-self, male flowers. By comparing responses of squash that were either pollinated by bees or by hand, we observed that bee-pollinated plants experienced increasing levels of pollen limitation as a function of decreasing soil moisture. This result illustrated that pollinators mediate how soil moisture variation affects the outcome of plant reproduction. Additional soil moisture manipulations indicated that drought-induced pollen limitation results, at least in part, from (i) decreased pollen competition resulting from low-levels of stigmatic pollen deposition, (ii) reduced viability of pollen produced by plants grown under low soil moisture conditions, and (iii) a reduced capacity of self-pollinated fruits to respond to increasing soil moisture.

In the present study levels of warming were realistic based on current climate projections (IPCC 2014), but mean summer temperatures at our coastal study site were mild (2016 - 2018 season mean: 25˚C) and even experimentally warmed conditions likely did not result in prolonged physiological stress for squash plants, which are often grown in areas with higher mean summer temperatures compared to those at our study site (Molinar et al. 1999, Loy 2004, Wien et al. 2004). In contrast to previous studies that have documented reduced reproductive allocation to warming (Descamps et al. 2018), two of the three significant responses to temperature in the *Temperature x irrigation experiment* represented increases in performance (e.g., increased nectar volume, increased pollen production). Our study is not unique in this respect; Hoover et al. (2012) also report elevated nectar production by pumpkins experiencing experimental warming. Aside from flower size, which decreased under warmed conditions, all of the measured aspects of reproductive allocation in this study either did not respond to the elevated temperature treatment or apparently benefitted from it. The lack of interactive effects observed in *Temperature x irrigation experiment* may have thus been partly due to a temperature treatment that did not physiologically challenge plants, but other studies also report that the effects of temperature and soil moisture can act independently of one another (Descamps et al. 2018).

In contrast to the effects of temperature, we found that soil moisture variation affected more reproductive traits and that these responses were all negative (see also Descamps et al. 2018). Observed effects mirror those reported in previous studies. Known effects of drought, for example, include reductions in flower size (Burkle & Runyon 2015, Gallagher & Campbell 2017, Descamps et al. 2018) and nectar volume (Waser & Price 2016, Gallagher & Campbell 2017). Changes in floral traits caused by drought stress can also reduce visitation by pollinators (Burkle & Runyon 2015, Waser & Price 2016, Gallagher & Campbell 2017), but this trend seems to exhibit context specificity and is not universal. Although the responses observed in our system might be expected to reduce visitation by pollinators, our experiment was not designed to measure pollinator choice as open flowers on a given day were all from the same treatment group. Pollinator visitation is the focus of Chapter 2, in which we explicitly test the consequences of floral choice made by specialist (squash bees) and generalist (honey bees) pollinators in an experimental setting where bees can simultaneously visit squash flowers produced by plants grown under different levels of soil moisture. An additional reproductive trait that varied with soil moisture was the availability of non-self, male flowers. Previous studies have also reported changes in flower production in response to drought stress. Al-Ghzawi et al. (2010), Philips et al. (2018), Descamps et al. (2018), for example, all report examples of drought-induced reductions in flower production, but this finding is not universal and likely depends on the timing of floral development relative to the onset of drought stress (Gallagher & Campbell 2017).

To examine the role of pollinators in mediating how soil moisture variation affects plant reproductive success, we compared seed set for plants pollinated by bees versus those pollinated by hand and found that bee-pollinated plants grown under low soil moisture levels were pollen limited. To our knowledge, drought-induced pollen limitation has not previously been reported in the literature but could be common given well-known effects of non-lethal, drought stress on various aspects of plant reproductive allocation (Al-Ghzawi et al. 2010, Burkle & Runyon 2015, Waser & Price 2016, Gallagher & Campbell 2017, Philips et al. 2018, Descamps et al. 2018). Although pollen limitation commonly results from inadequate visitation by pollinators, this mechanism seems unlikely to explain our result given that bee visitation did not increase with increasing soil moisture level. Differential pollen production can also be ruled out as an underlying mechanism for drought-induced pollen limitation given that the amount of pollen produced was independent of soil moisture. Given that we observed an apparent reduction in the availability of non-self, male flowers as a function of low soil moisture levels, the transfer of self pollen might have been higher for plants grown under low soil moisture conditions. However, we found no evidence for this type of effect through the use of pigments to estimate patterns of pollinator movement.

Our results suggest that drought-induced pollen limitation in part stemmed from reduced pollen competition on stigmas that received pollen from bees. Winsor (1987), for example, found that seed set and offspring vigor in *C. pepo* increased with increasing pollen load and concluded that more intense pollen competition was the underlying cause of this effect. Like Winsor (1987), we observed that seed set increased with increasing pollen deposition (Table 1.3C, Fig. 1.3C). In the *Temperature x irrigation experiment*, the greater than two-fold higher pollen loads on hand-pollinated stigmas, compared to bee-pollinated stigmas, would have heightened pollen competition and allowed only the fastest-growing pollen tubes to achieve fertilization. Consistent with this hypothesis is the fact that the seed set of hand-pollinated plants was independent of the soil moisture levels that these plants experienced while growing. In contrast, the smaller pollen loads deposited by bees would have led to relatively lower levels of pollen competition and a greater opportunity for self pollen or pollen of reduced viability to achieve fertilization.

Relaxed pollen competition in bee-pollinated plants could help to explain why we observed drought-induced pollen limitation, but for this mechanism to reduce seed set pollen deposited on the stigmas of must have been of inferior quality. Two lines of evidence support this hypothesis. First, we observed a negative relationship between seed set and the amount of pigment from the male flowers of plants grown under low soil moisture conditions (Fig. 1.4). Moreover, we found that seed set increased with increasing levels of soil moisture experienced by plants contributing non-self pollen (Fig. 1.3B). These results taken together suggest that the pollen viability assays used in the *Temperature x irrigation experiment* may not have been sensitive enough to measure more subtle aspects of pollen viability as measured here. Previous studies that did find significant effects of drought on pollen viability used different assays (Al-Ghzawi et al. 2009: pollen germination; Descamps et al. 2018: fluorescein diacetate) than the one performed here. Second, we observed that the seed set produced by flowers pollinated with non-self pollen increased with soil moisture, whereas the seed set resulting from flowers pollinated with self pollen did not respond to increasing soil moisture. Although the reduced availability of non-self, male flowers for plants grown under low soil moisture conditions was on some level an artifact of our experimental design (e.g., the size of our experimental garden), it would seem common for plant species to grow in discrete enough patches for the availability of self pollen versus non-self pollen might influence the outcome of pollination. For example, buffalo gourd, which is the local, native congener of *C. pepo*, grows in patches of up to five individuals with hundreds of meters separating patches (see Chapter 3).

In conclusion, our results reveal that pollinators can mediate effects of warming and drought on plant reproduction by increasing pollen limitation as a result of (i) decreased pollen competition caused by low-levels of stigmatic pollen deposition, (ii) reduced viability of pollen produced by plants grown under low soil moisture conditions, and (iii) a reduced capacity of self-pollinated fruits to increase seed set in response to increasing soil moisture. Although this study focuses on only a single plant species, drought-induced pollen limitation could be a common phenomenon based on the frequency with which drought stress affects reproductive allocation in other plant species. Water stress, for example, commonly lowers pollen viability (Prasad et al. 2008, Smith & Zhao 2016, Su et al. 2013). As the occurrence of drought proliferates globally (Dai 2013, IPCC 2014) due to increased aridity resulting from anthropogenic climate change (Cayan et al. 2010), drought-induced pollen limitation as mediated by pollinators may become a common and pressing concern in ecosystems worldwide. The degree of pollen limitation partly depends on how pollinators move, and thus transfer pollen, amongst flowers within and between plants. For example, increased pollinator visitation within the same plant could increase instances of self-pollination, which may heighten inhibitory effects of drought on reproduction. But if pollinators increase rates of outcrossing or preferentially forage on plants producing higher quality pollen, then pollen limitation resulting from reduced water availability may be alleviated.

Chapter 1, in part, is currently being prepared for submission for publication of the material. Gambel, Jess; Holway, David A. The dissertation author was the primary investigator and author of this material.

TABLES

Table 1.1. The effects of temperature, irrigation, and their interaction on floral traits in bee-pollinated *Cucurbita pepo* plants (n = 60) in the *Temperature x irrigation experiment*. *P* values in boldface are significant at α = 0.05.

Response Variable	Treatment	df	*F*	*P*	r^2_{adj}
Total male flowers	Mean soil moisture	1,56	0.30	0.59	0.039
	Temperature	1,56	2.1	0.15	
	Soil moisture x temperature	1,56	2.94	0.092	
Total female flowers	Mean soil moisture	1,56	0.0030	0.96	-0.045
	Temperature	1,56	0.020	0.89	
	Soil moisture x temperature	1,56	0.4	0.52	
Male flower size (corolla width in mm)	Mean soil moisture	1,56	22.72	**<0.0001**	0.32
	Temperature	1,56	5.72	**0.020**	
	Soil moisture x temperature	1,56	2.22	0.14	
Female flower size (corolla width in mm)	Mean soil moisture	1,55	21.11	**<0.0001**	0.33
	Temperature	1,55	10.12	**0.0024**	
	Soil moisture x temperature	1,55	0.029	0.87	
Total self male flowers available	Mean soil moisture	1,56	2.46	0.12	0.015
	Temperature	1,56	0.46	0.50	
	Soil moisture x temperature	1,56	1.16	0.29	
Total non-self male flowers available	Mean soil moisture	1,56	3.61	0.063	0.067
	Temperature	1,56	1.22	0.28	
	Soil moisture x temperature	1,56	2.44	0.12	
Nectar volume (μL) in male flowers	Mean soil moisture	1,56	6.11	**0.017**	0.13
	Temperature	1,56	4.09	**0.048**	
	Soil moisture x temperature	1,56	1.65	0.20	
Nectar volume (μL) in female flowers	Mean soil moisture	1,55	3.25	0.077	0.078
	Temperature	1,55	3.11	0.084	
	Soil moisture x temperature	1,55	1.58	0.21	
Nectar concentration (BRIX) in male flowers	Mean soil moisture	1,56	2.04	0.16	0.14
	Temperature	1,56	0.46	0.50	
	Soil moisture x temperature	1,56	10.28	**0.0022**	
Nectar concentration (BRIX) in female flowers	Mean soil moisture	1,55	0.0040	0.95	-0.00082
	Temperature	1,55	2.91	0.094	
	Soil moisture x temperature	1,55	0.038	0.85	
Pollen mass (mg) in male flowers	Mean soil moisture	1,56	0.10	0.75	0.045
	Temperature	1,56	5.09	**0.028**	
	Soil moisture x temperature	1,56	0.60	0.44	
Proportion viable (stained red/purple) pollen in male flowers	Mean soil moisture	1,56	0.62	0.43	0.0060
	Temperature	1,56	2.72	0.11	
	Soil moisture x temperature	1,56	0.019	0.89	

Table 1.2. The effects of temperature, irrigation, pollination type (bee or hand), and their interactions on fruit set and seed set for *Cucurbita pepo* plants (n = 80) in the *Temperature x irrigation experiment*. *P* values in boldface are significant at α = 0.05.

Response Variable	Treatment	Estimate	*SE*	*z*	*P*
Fruit Set	Meansoil moisture	0.10	0.049	2.09	**0.037**
	Temperature	1.27	2.49	0.51	0.61
	Pollination	-0.12	3.83	-0.033	0.97
	Soil moisture x temperature	-0.056	0.062	-0.90	0.37
	Soil moisture x pollination	-0.010	0.097	-0.11	0.91
	Temperature x pollination	0.37	5.21	0.071	0.94
	Soil moisture x temperature x pollination	0.0064	0.13	0.05	0.96

Response Variable	Treatment	df	*F*	*P*	r^2_{adj}
Seed Set (mean total seed mass (mg) per fruit per plant)	Mean soil moisture	1,71	4.26	**0.043**	0.12
	Temperature	1,71	0.70	0.41	
	Pollination	1,71	4.21	**0.044**	
	Soil moisture x temperature	1,71	0.0020	0.97	
	Soil moisture x pollination	1,71	7.82	**0.0067**	
	Temperature x pollination	1,71	0.25	0.62	
	Soil moisture x temperature x pollination	1,71	0.0020	0.97	

Table 1.3. Effects of irrigation and pollination treatments on seed set in *Cucurbita pepo* plants in *Irrigation experiment II*. Included are results from zero-inflated negative binomial models testing for the main and interactive effects of soil moisture and pollination (self or non-self) on seed set of self (n = 30) and non-self (n = 89) pollinated female flowers in hand-pollinated plants. *P* values in boldface are significant at $\alpha = 0.05$.

Response Variable	Treatment	Estimate	*t*	*P*	Cox-Snell R^2
(A) Soil moisture experienced by plant contributing female flower					
Total seed mass (mg) per fruit	Mean soil moisture	$\mu = 0.061$	4.75	**<0.0001**	0.31
	Pollination	$\mu = 2.95$	3.25	**0.0016**	
	Soil moisture x pollination	$\mu = -0.061$	-3.13	**0.0023**	
	μ coefficient intercept (link = log)	$\mu = 6.47$	11.02	**<0.0001**	
	σ coefficient intercept (link = log)	$\sigma = -2.56$	-12.23	**<0.0001**	
	ν coefficient intercept (link = logit)	$\nu = 0.50$	2.63	**0.0099**	
(B) Soil moisture experienced by plant contributing pollen					
Total seed mass (mg) per fruit	Mean soil moisture	$\mu = 0.076$	4.61	**<0.0001**	0.31
	Pollination	$\mu = 5.35$	5.25	**<0.0001**	
	Soil moisture x pollination	$\mu = -0.11$	-5.19	**<0.0001**	
	μ coefficient intercept (link = log)	$\mu = 5.76$	7.58	**<0.0001**	
	σ coefficient intercept (link = log)	$\sigma = -2.61$	-12.51	**<0.0001**	
	ν coefficient intercept (link = logit)	$\nu = 0.50$	2.63	**0.0099**	
(C) Pollen deposition experienced by plant contributing female flower					
Total seed mass (mg) per fruit	Total stigma pollen deposition	$\mu = 0.00055$	4.07	**<0.0001**	0.31
	Pollination	$\mu = 0.88$	2.53	**0.013**	
	Pollen deposition x pollination	$\mu = -0.0071$	-2.82	**0.0059**	
	μ coefficient intercept (link = log)	$\mu = 8.59$	48.40	**<0.0001**	
	σ coefficient intercept (link = log)	$\sigma = -2.74$	-13.12	**<0.0001**	
	ν coefficient intercept (link = logit)	$\nu = 0.50$	2.63	**0.0099**	

FIGURES

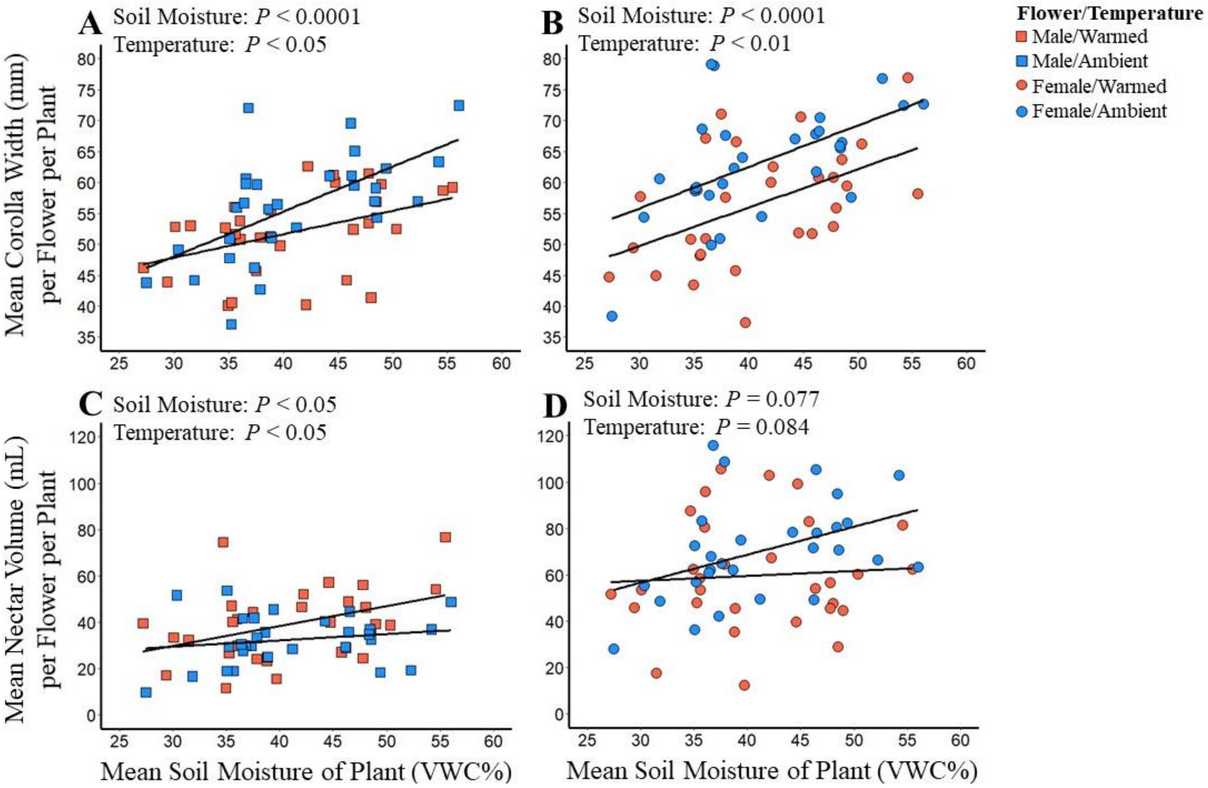

Figure 1.1. The effects of temperature and soil moisture variation on flower size (A, B) and nectar production (C, D) for male (A, C) and female (B, D) flowers of cultivated squash (*Cucurbita pepo*). Table 1.1 provides additional statistical information relevant to these analyses.

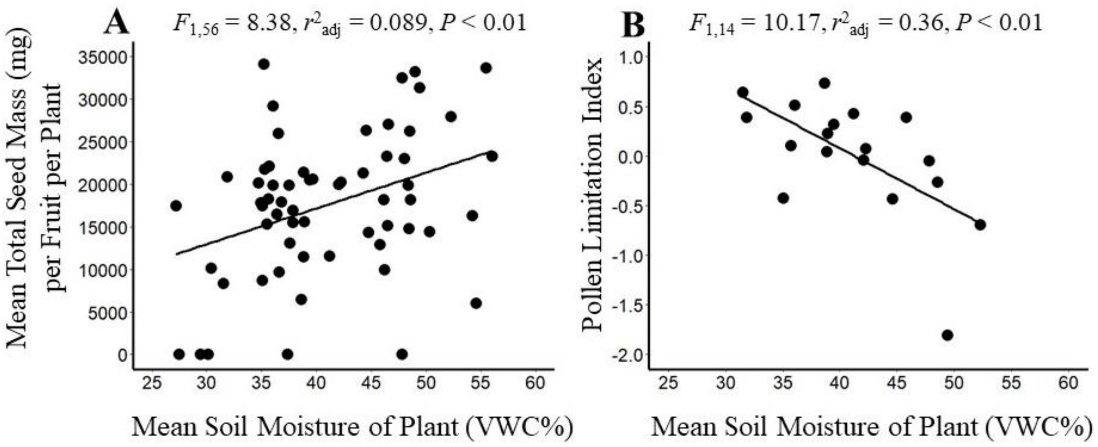

Figure 1.2. The effects of soil moisture variation on (A) seed set and (B) pollen limitation for fruits of cultivated squash (*Cucurbita pepo*) that resulted from bee pollination. Table 1.2 provides additional statistical information relevant to these analyses.

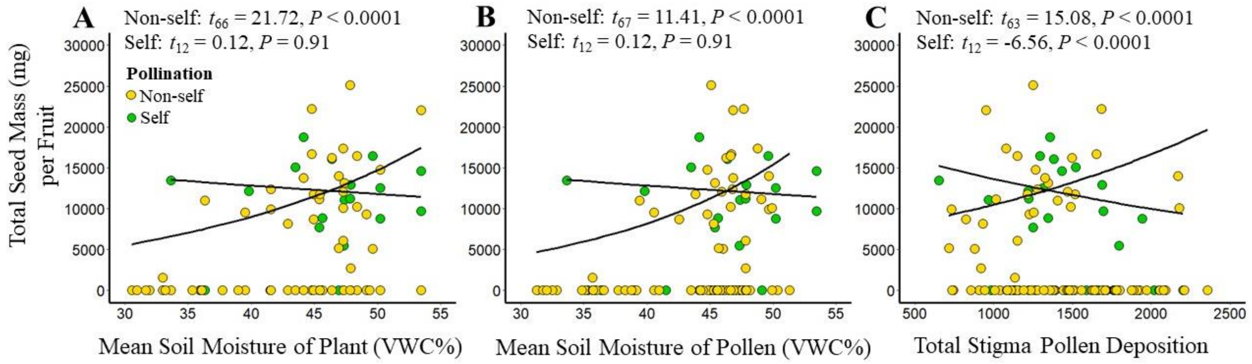

Figure 1.3. The effects of soil moisture variation and pollen deposition on seed set from flowers pollinated with either self pollen or non-self pollen. Effects are from (A) the soil moisture experienced by the plant contributing the female flower, (B) the soil moisture experienced by the plant contributing pollen, and (C) pollen deposition experienced by the plant contributing the female flower. Zero values are included in these analyses because (i) plants were given multiple opportunities to set fruit, and (ii) plants had the opportunity to grow both self and non-self fruits. Table 1.3 provides additional statistical information relevant to these analyses.

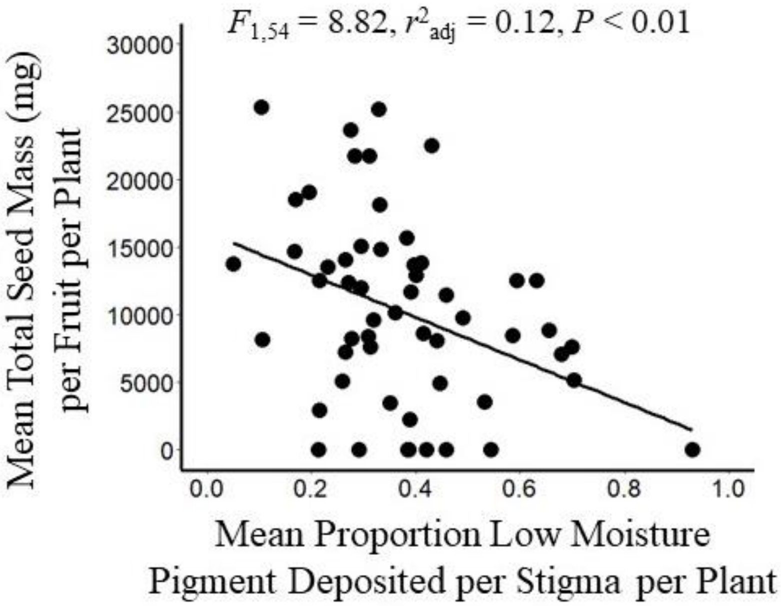

Figure 1.4. The effect of stigmatic deposition of pigment (a proxy for pollen) from plants grown at low irrigation on seed set of fruits of cultivated squash (*Cucurbita pepo*) that resulted from bee pollination.

APPENDICES

Appendix 1.1. Temperature x irrigation experiment

Figure A.1.1.1: *Temperature x irrigation experiment* on *Cucurbita pepo* plants grown at the UC San Diego Biology Field Station during June - September 2016. We used a drip irrigation system to water plants and simulated warming using passive, open-top warming chambers. The drip irrigation lines traversed each individual plot, watering the base of each plant. We monitored temperature readings at each individual plant using Thermochron iButtons$^{©}$ housed in PVC piping.

Floral traits

We measured flower size as the open floral corolla width (Hoover et al. 2012). We used a 2µL pipette to collect nectar from each flower (Stoner & Eitzer 2012). We measured the nectar volume by first centrifuging and then measuring the collected sample using a 200 µL pipette. To measure nectar concentration, we used a 10 µL pipette to remove a 2 µL-sized sample from this collected nectar and placed the sample on a refractometer (Corbet 1978) to measure the BRIX (0-50%) of that sample. We measured up to three samples to obtain a clear reading, which usually occurred with the first sample. We also measured pollen mass and viability for each unvisited male flower. Using a metal spatula, we gently scraped pollen grains off the anthers of the collected male flower (Stoner & Eitzer 2012) into a tared petri dish, which we then weighed using a scale. We tested pollen viability by spreading 1 mg of pollen from each sample onto a microscope slide and then applying 40 µL MTT solution (thiazolyl blue) (Khatun & Flowers 1995). MTT tests pollen grains for enzymatic activity: grains stained red to purple are viable, while grains stained black or left unstained are not viable (Khatun & Flowers 1995). Pollen staining with MTT is easily facilitated in the field and correlates well with *in vitro* pollen germination tests (Firmage & Dafni 2000), but care needs to be taken to correctly distinguish the varying color tonalities of stained grains (Rodriguez-Riano & Dafni 2000). Slides were left to dry for several hours and then refrigerated until measured. Using a dissecting microscope at 30x magnification, we counted the number of different colored pollen grains on slides to determine the proportions of viable and nonviable grains per sample.

Video recording

We video-taped the visitation behaviors of bees within a subset of open flowers for 1.5 hours each morning during visitation surveys using Black Box DareDVL Wifi Mini Waterproof Sports Action Dash Cameras. Cameras were placed on tripods approximately 20 – 40 cm away from the flower so as not to obstruct bee visitation. For each female flower video, we estimated the cumulative rates at which bees contacted the stigma per minute video. For each male flower video, we estimated the cumulative rates at which bees contacted the anthers and collected pollen per minute video.

Table A.1.1.1. The effects of temperature and soil moisture manipulations experienced by cultivated *Cucurbita pepo* plants (n = 80) in the *Temperature x irrigation experiment*. Included are results from general linear models testing for effects of the temperature treatment on mean average daily temperature, mean maximum temperature, and mean minimum temperature of the plants, as well as the effects of temperature, irrigation, and their interaction on the mean soil moisture (volumetric water content) of the plants. *P* values in boldface are significant at α = 0.05.

Response Variable	Treatment	df	*F*	*P*
Mean average daily temperature (˚C)	Temperature	1,78	500.80	**<0.0001**
Mean maximum temperature (˚C)	Temperature	1,78	356.10	**<0.0001**
Mean minimum temperature (˚C)	Temperature	1,78	317.40	**<0.0001**
Mean plant soil moisture (VWC%)	Irrigation	1,76	93.92	**<0.0001**
	Temperature	1,76	0.10	0.76
	Irrigation x temperature	1,76	0.058	0.81

Table A.1.1.2. The effects of temperature, irrigation, and their interaction on pollen limitation in *Cucurbita pepo* in the *Temperature x irrigation experiment*. Pollen limitation was estimated using total seed mass for each bee-pollinated plant (n = 55) and the mean total seed mass values of its matched hand-pollinated plant treatment group. Soil moisture values used in the analysis are those of the bee-pollinated plants. *P* values in boldface are significant at $\alpha = 0.05$.

Response Variable	Treatment	df	*F*	*P*	r^2_{adj}
Pollen Limitation	Mean soil moisture	1,51	12.32	**<0.001**	0.15
	Temperature	1,51	0.0030	0.95	
	Soil moisture x temperature	1,51	0.28	0.60	

Table A.1.1.3. The effects of temperature, irrigation, and their interaction on bee visitation to *Cucurbita pepo* in the *Temperature x irrigation experiment*. Included are *Apis mellifera* and *Eucera pruinosa* visit rates (arcsine square root transformed) from daily surveys of plants (n = 60) and behavior rates ($\log_{10}$ transformed) from videotaped male (n = 33) and female (n = 27) flowers. *P* values in boldface are significant at $\alpha = 0.05$.

Response Variable	Treatment	df	*F*	*P*	r^2_{adj}
(A) Male Flowers					
Apis mellifera					
Visits per flower per minute	Mean soil moisture	1,54	0.84	0.36	0.014
	Temperature	1,54	2.46	0.12	
	Soil moisture x temperature	1,54	0.41	0.53	
Cumulative anther contact per minute	Mean soil moisture	1,28	0.21	0.65	0.033
	Temperature	1,28	0.56	0.46	
	Soil moisture x temperature	1,28	3.30	0.080	
Cumulative pollen collecting per minute	Mean soil moisture	1,28	1.22	0.28	0.12
	Temperature	1,28	6.13	**0.020**	
	Soil moisture x temperature	1,28	0.0030	0.96	
Eucera pruinosa					
Visits per flower per minute	Mean soil moisture	1,54	0.28	0.60	0.014
	Temperature	1,54	3.43	0.070	
	Soil moisture x temperature	1,54	0.13	0.72	
Cumulative anther contact per minute	Mean soil moisture	1,28	0.017	0.90	-0.032
	Temperature	1,28	0.0070	0.93	
	Soil moisture x temperature	1,28	2.00	0.17	
Cumulative pollen collecting per minute	Mean soil moisture	1,28	0.47	0.50	-0.069
	Temperature	1,28	0.039	0.85	
	Soil moisture x temperature	1,28	0.50	0.48	
(B) Female Flowers					
Apis mellifera					
Visits per flower per minute	Mean soil moisture	1,55	0.028	0.87	0.085
	Temperature	1,55	2.10	0.15	
	Soil moisture x temperature	1,55	6.27	**0.015**	
Cumulative stigma contact per minute	Mean soil moisture	1,23	1.11	0.30	0.0068
	Temperature	1,23	1.06	0.31	
	Soil moisture x temperature	1,23	1.01	0.33	
Eucera pruinosa					
Visits per flower per minute	Mean soil moisture	1,55	0.22	0.64	0.087
	Temperature	1,55	2.46	0.1	
	Soil moisture x temperature	1,55	5.83	**0.019**	
Cumulative stigma contact per minute	Mean soil moisture	1,23	0.41	0.53	-0.059
	Temperature	1,23	0.068	0.80	
	Soil moisture x temperature	1,23	1.07	0.31	

Appendix 1.2. Irrigation experiment II

Hand pollination

As with the 2016 hand pollination of stigmas, we mixed pollen collected from two male flowers, either from the same plant as the female flower for self pollination or from 1 -2 different plants for non-self pollination. We carefully transferred this mixture into a 1.5mL microcentrifuge tube until pollen levels reached the 0.1mL mark on the tube, and then discarded the remaining pollen. We inserted a cotton swab into the tube and touched the upper surface of the collected pollen just enough to cover the tip of the swab. We then used the swab to apply the mixed pollen to the entire stigma. Mean pollen deposition values (for the entire stigma) using this method (1482 ± 43 grains) were not statistically different (Welch two sample t-test: $t_{1,34} = -1.26$, $P = 0.22$) compared to mean pollen deposition values (for the entire stigma) of bee-pollinated plants from the 2017 *Irrigation experiment I* (1712 ± 177 grains).

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CHAPTER 2

Generalist and specialist pollinators differentially influence plant reproduction under experimentally-induced drought

by

Jess Gambel and David Holway

ABSTRACT

Drought represents an increasingly common consequence of climate change. For flowering plants, drought stress often leads to reduced investment in floral traits. Changes in nectar and pollen production can in turn influence pollinator visitation and, in doing so, affect plant reproductive success. Pollinators can thus mediate how plants respond to drought, and their influence seems likely to depend on their foraging preferences and niche breadth. In the face of drought, for example, specialist pollinators might be expected (by necessity) to exhibit indiscriminate foraging behavior with respect to host plant condition, whereas generalist pollinators could exhibit greater selectivity (e.g., only foraging on host plants in good condition) or switch to alternative floral resources. Here, we use experimentally-induced drought to test how the behavior of specialist and generalist pollinators affects squash (*Cucurbita pepo*) reproduction. Quantification of pollinator visitation rate on plants grown along a soil moisture gradient revealed that floral visitation by specialist squash bees was independent of plant soil moisture, whereas floral visitation by generalist honey bees increased with plant soil moisture. Through the use of fluorescent pigments to track pollen movement by bees and hand pollination to control pollen source, we discovered that the foraging selectivity of generalist honey bees led

to the increased deposition of pollen from well-watered plants and that this behavior, in turn, increased seed set. Given that specialization and generalization are defining features of pollinators, differential responses to drought-induced plant stress as a function of specialization could be a common phenomenon with consequences for plant reproduction and pollination services more generally.

INTRODUCTION

Drought represents an increasingly prevalent abiotic stressor in terrestrial ecosystems (Dai 2011, IPCC 2014). For plants, water stress decreases plant turgor, leaf water potential, and stomatal conductance, leading to reduced photosynthetic activity and slower cell growth (Jaleel et al. 2009). Accordingly, effects of drought can lead to diminished reproductive investment: decreased flower production (Carter et al. 1997, Al-Ghzawi et al. 2009, Burkle & Runyon 2016, Descamps et al. 2018, Phillips et al. 2018), reduced flower size (Galen 1999, Carroll et al. 2001, Halpern et al. 2010, Burkle & Runyon 2016, Gallagher & Campbell 2017, Descamps et al. 2018), reduced pollen production (Al-Ghzawi et al. 2009, Waser & Price 2016) and viability (Al-Ghzawi et al. 2009, Descamps et al. 2018), diminished nectar volume (Carroll et al. 2001, Halpern et al. 2010, Waser & Price 2016, Gallagher & Campbell 2017, Phillips et al. 2018) and sugar content (Waser & Price 2016, Descamps et al. 2018), modification of floral coloration (Warren and Mackenzie 2001), and altered emission or composition of floral volatile organic compounds (Burkle & Ruyon 2016, Glenny et al. 2018). Changes in floral traits caused by drought may subsequently influence pollinator behavior (Al-Ghzawi et al. 2009, Burkle & Ruyon 2016, Gallagher & Campbell 2017, Glenny et al. 2018) and, in turn, plant reproductive success (Al-Ghzawi et al. 2009, Waser and Price 2016, Chapter 1).

The roles of different pollinators in mediating how plants respond to drought will be influenced by their behaviors. Generalist pollinators, for example, visit a diversity of plants, sometimes from many different families, for nectar and pollen, whereas specialists obtain pollen and nectar from a limited number of related (often congeneric) floral hosts and often depend on resources from specific host plants to reproduce (Wcislo & Cane 1996, Minckley & Roulston 2006, Cane & Sipes 2006, Armbruster 2017). Bees provide excellent examples of this continuum. Polylectic bees collect pollen from many unrelated plants, but specialist bees (oligoleges) are restricted to pollen collection from few related species (Minckley & Roulston 2006). Accordingly, specialists may be expected to exhibit higher floral constancy (i.e., consecutively visiting conspecific flowers) (Waser 1986, Cane & Sipes 2006) than generalists, which they do, on average, and this behavior contributes to the effectiveness of specialists as pollinators (Strickler 1979, Cane and Payne 1988, Larsson 2005, Tepedino 2016). However, exceptions also exist (Bernhardt & Weston 1996, Olsen 1996, Castellanos et al. 2003).

As drought stress affects a suite of floral traits, it often results in reduced visitation by pollinators (Al-Ghzawi et al. 2009, Burkle & Ruyon 2016, Gallagher & Campbell 2017, Glenny et al. 2018). Specialist pollinators that depend on pollen from a species or genus of plant will be unable to switch resources as a result of plant-to-plant variation in floral resource quality. Generalists, in contrast, may exhibit selectivity for high-quality floral resources given that they have a broader set of resources available to them. While generalist pollinators are considered more resilient to habitat and climatic disturbance compared to specialists (Biesmeijer et al. 2006, Roberts et al. 2011, Winfree et al. 2011), few studies have compared how specialist and generalist pollinators respond to drought conditions. The fate of specialists clearly seems tied to how host plants respond to water stress. Minckley et al. (2013), for example, found that bees specializing on creosote bush, which does not flower in drought, failed to emerge and visit creosote during a drought year; however, specialists of mesquite, which flowers independently of rainfall because of its ability to use tap roots to access groundwater, were able to remain active. Conversely, Mayer and Kuhlmann (2004) discovered that during a drought year, specialist and generalist bees of *Rediviva* emerged on time with the normal flowering season despite bloom being postponed by five weeks; this asynchrony resulted in the loss of pollen collection by bees.

Here, we use field experiments to examine these relationships for cultivated squash (*Cucurbita pepo*) pollinated by honey bees (*Apis mellifera*), which are generalists, and squash bees (*Eucera (Peponapis) pruinosa, E. (Xenoglossa) strenua* (Dorchin et al. 2018)), which specialize on *Cucurbita* to such an extant that they require pollen from these plants to reproduce. The squash system is highly appropriate for this type of investigation. Given that *Cucurbita* do not self-pollinate (Hurd et al. 1971), they need insects to pollinate their flowers to produce fruit and seeds. Moreover, squash plants have separate male (staminate) and female (pistillate) flowers that are large and short-lived (Hurd et al. 1971, Tepedino 1981); these attributes facilitate examination of pollinator behavior and responses by plants.

We test three predictions concerning how specialists and generalists respond to plants experiencing a gradient of drought stress and how these changes in foraging behavior may affect plant reproduction. First, generalists should exhibit selectivity while specialists should be indiscriminate in terms of displaying preferences in the quality of floral resources. Second, higher selectivity by generalists should translate into increased transfer and deposition of pollen from male flowers of non-drought stressed plants onto floral stigmas of female flowers of non-drought stressed plants (i.e., higher floral constancy amongst non-drought stressed plants). Third, increased deposition of pollen from non-drought stressed plants could increase fruit set and seed set if pollen from drought stressed plants is low quality. In seasonally dry environments, water stress likely affects floral resource quality on a regular basis, and pollinator behavior may play an important role with respect to how those resources move amongst plant species. This study represents a novel investigation into how the effects of drought on plant reproductive success hinges on the behavior of specialist and generalist pollinators.

METHODS

We grew *Cucurbita pepo* (Honey Bear F1 acorn squash seed from Johnny's Selected Seeds©) at the University of California, San Diego Biology Field Station (32°53'08.8" N, 117°13'47.2" W) from June through September in 2018. Squash plants (n = 91) were germinated from seeds planted directly in the ground in 1.2 m x 1.2 m plots with each plot containing a single plant. Jobe's Organic Vegetable Fertilizer© (2% nitrogen, 5% phosphorus, 3% potassium) was added (130 g/plant) to the soil during seeding to aid in plant establishment. After plants matured, we sprayed them with neem oil three times during the season to reduce pest and fungus infestations. Thermochron iButtons© interspersed throughout the plots recorded hourly temperature readings during the season; the mean daily temperature at our site during the experiment was 26.5˚C.

The western honey bee (*Apis mellifera*) is numerically dominant at our study site. Feral honey bees are abundant throughout San Diego County (Kono & Kohn 2015, Hung et al. 2018), and 16 managed honey bee colonies were present at the Biology Field Station during the 2018 growing season. This site and the surrounding environment also support two species of native squash bees: *Eucera* (*Peponapis*) *pruinosa* and *Eucera* (*Xenoglossa*) *strenua*) (Dorchin et al. 2018). In the area surrounding our study site, squash bees visit native buffalo gourd (*C. foetidissima*) as well as backyard plantings of squash (Hurd & Linsley 1964, Hurd et al. 1974, Tepedino 1981, Ordway et al. 1987, Artz & Nault 2011). Other pollinators present include sweat bees (*Lasioglossum* spp. and *Agapostemon texanus*), but these bees do not effectively pollinate *Cucurbita* (Ordway et al. 1987, Ali et al. 2014, Pfister et al. 2017) and are not considered further here.

We used a drip-line system to irrigate plants every morning with the daily amount of irrigation held constant (2.2 L water/plant/day) until plants were three weeks old. After that time, when plants were mature enough to survive drought stress, we divided them into two groups. Plants either received 2.2 L water/plant/day (high irrigation; n = 45 plants) or 0.35 L water/plant/day (low irrigation; n = 46 plants). Plants in the two irrigation groups were spatially interspersed with respect to one another. We used a FieldScout TDR 100$^{©}$ soil moisture meter to take 2 - 4 replicate measurements of volumetric water content (VMC%) in the soil at 20 cm below each plant one hour after watering, 2-3 times per week for the duration of the season (Waser & Price 2016). Volumetric water content (based on time-averaged means for each plant) decreased by an average of 10% for plants in the low-irrigation group compared to the high-irrigation group (one-way ANOVA: $F_{1,89} = 143.30$, $P < 0.0001$). Mean volumetric water content, however, varied from 38 - 51% (high irrigation) to 24 - 48% (low irrigation). Given that soil moisture levels exhibited substantial variation within each irrigation group, we considered soil moisture as a continuous variable (which we refer to hereafter as ‘plant soil moisture’) in most statistical analyses (see also Gallagher & Campbell 2017) but use treatment group designations in the organization of the experiment.

Within the high-irrigation and low-irrigation groups, plants were randomly assigned to either a bee-pollination group (n = 31 high irrigation, n = 30 low irrigation) or a hand-pollination group (n = 14 high irrigation, n = 16 low irrigation). Plants in each pollination group were spatially interspersed. Hand pollination was used to control the source of pollen (i.e., with respect to plant soil moisture) and to examine the subsequent effects in terms of plant reproduction. Once squash plants began to flower in mid-July, flowers on hand-pollinated plants were bagged (Seedburo Treated S27 Shoot Pollinating Bags (5 cm x 2.5 cm x 18 cm)) prior to opening to prevent bee visitation (Stoner & Eitzer 2012).

For bee-pollinated plants, we measured traits on a subset of female and male flowers that we bagged (Seedburo Treated S27 Shoot Pollinating Bags (5 cm x 2.5 cm x 18 cm)) before they opened to prevent bee visitation (Stoner & Eitzer 2012). On the morning that bagged flowers opened, we detached them from the plants while still bagged and then carefully removed each flower from its bag. On each flower, we measured corolla width, nectar volume, nectar concentration, pollen mass (male flowers), and pollen viability (male flowers). Appendix 2.1 describes methods used to measure each floral trait. Throughout the season, we kept daily tallies of the number of male and female flowers open on each plant to assess how soil moisture variation affected plant-level, seasonal flower production.

For bee pollinated plants we (i) video-recorded flowers to quantify pollinator behavior and frequency of visitation, and (ii) used fluorescent pigments to investigate how bees transport pollen among plants grown under different levels of soil moisture. We recorded bee visitation on high-irrigation and low-irrigation plants concurrently; therefore, each day bees could move freely between plants in each irrigation group. Videos of male flowers (n = 39 plants) and female flowers (n = 25 plants) were always recorded between 0700 - 0900. For every video, we set the video camera (Victure Action Camera WIFI 14MP 1080P) close enough (20 - 40 cm) to each flower to fill the frame of the video but far enough away such that bee visitation did not seem to be affected. For each video of a female flower, we counted the number of bee visits per unit time, and for each visit measured the time that each focal bee spent in contact with the stigma and drank nectar. For each video of a male flower, we counted the number of bee visits per unit time, and for each visit measured the time that each focal bee contacted the anthers, collected pollen, and drank nectar. We also conducted plot-level surveys of bee visitation to all open flowers each day (see Appendix 2.1).

We used powdered DayGlo© fluorescent pigments to study patterns of pollen movement by bees (Ordway et al. 1987). We used flat toothpicks to apply pigments (aurora pink or horizon blue) to the anthers of all open male flowers prior to bee access to flowers (Waser 1988). Male flowers in the low-irrigation group received one pigment color, whereas male flowers in the high-irrigation group received the other pigment color. Color assignments were switched daily. On the stigmas of bee-pollinated female flowers, pigment particle counts were positively correlated with pollen counts (Pearson correlation: $r_{46} = 0.80$, $P < 0.0001$). Therefore, the number of pigment particles can serve as a proxy for the amount of pollen moved by bees from the anthers of male flowers to the stigmas of female flowers (Waser 1988) and also reveal patterns of pollen movement among plants in the two irrigation groups. We measured pigment deposition on the stigmas of 56 bee-pollinated plants. For plants with multiple stigma samples (n = 28), we used mean values as data points.

To hand-pollinate female flowers, we first applied fluorescent pigments (corresponding to the pigment colors used for that particular day for bee-pollinated flowers) to two male flowers (one from each irrigation group) as described in the previous paragraph and then removed pigment and pollen from the anthers of these flowers. We combined the pollen-pigment mixture from the two flowers in a petri dish and used a cotton swab to generously apply this mixture to the entire stigma of each female flower (Winsor et al. 2000). After hand-pollination, we re-bagged flowers with breathable mesh bags (Paper Mart standard gold organza (15 cm x 23 cm)) to exclude pollinators while simultaneously permitting flowers to experience similar conditions as those experienced by flowers open to bees (Kearns & Inouye 1993). See Appendix 2.2 for additional information on pollen and pigment deposition on hand-pollinated stigmas.

We allowed pollen to germinate on bee and hand-pollinated stigmas for 24 h before collecting stigmas from female flowers setting fruit. This amount of time allowed for pollen grains to fertilize ovules (*personal observation*). We stored refrigerated stigmas in 100% ethanol (Winsor et al. 2000), and later used a dissecting microscope at 40x magnification to count the number of different colored pigment particles deposited on the stigmas (Waser 1988). We then dyed the stigmas with basic fuchsin solution (Kearns & Inouye 1993) and used a dissecting microscope at 50x magnification to count the number of deposited pollen grains (Artz & Nault 2011). We also counted stained grains present in the ethanol solution to obtain an estimate of the total pollen deposited on the stigma. We estimated pollen deposition on 48 bee-pollinated plants; for plants (n = 23) with multiple stigma samples, we used mean values as data points. Fifty days after pollination (Loy 2004), we harvested mature fruits from 47 bee-pollinated plants and 19 hand-pollinated plants. Seeds were removed from fruits in the lab, and then dried, counted, and weighed. We used total seed weight (g) per fruit to estimate seed set. For plants (n = 3) with multiple fruits, we used mean values as data points.

We used R version 3.6.1 (R Core Team, 2019) for all data analysis and used the package *ggplot2* to prepare figures (Wickham 2009). For all general linear models, we inspected q-q plots to test for normality, used Bartlett tests to assess homogeneity of variances, and tested the residuals of each model with the Shapiro-Wilk test for normality. We used simple linear regressions to assess how soil moisture affected the floral traits listed in Table 2.1. For plants in which more than one male or female flower was measured, we used mean values as data points. For the analyses of floral traits, the following response variables were right-skewed and thus

$\log_{10}$ transformed to improve normality of the residuals prior to analysis: male flowers produced, female flowers produced, male flower size, and nectar volume in male flowers. The proportion of viable pollen was left-skewed and thus square transformed to improve normality.

To examine how bee visitation and behavior changes with respect to plant soil moisture (i.e., from flowers that were videotaped), we used separate general linear models for honey bees (*Apis mellifera*) and squash bees (*Eucera*) with soil moisture and total flowers available in the plot (the day each flower was video recorded) as fixed factors. We added flower availability to the model because the number of flowers available to pollinators increased with plant soil moisture (simple linear regression: $F_{1,59} = 9.33$, $r^2_{\text{adj}} = 0.12$, $P < 0.01$; $\log_{10}$ transformed). For analyses of bee visitation to male flowers, see Appendix 2.2 for information on how variables were transformed to improve normality. For the analysis of visitation to female flowers, *Eucera* visitation rate was right-skewed and thus arcsine square-root transformed to improve normality of the residuals.

We used simple linear regressions to determine how soil moisture affected total pollen deposition, total pigment deposition, pollen deposition per open male flower, and pigment deposition per open male flower on bee-pollinated stigmas. Pollen deposition per open male flower was right-skewed and thus inverse transformed to improve normality. Pigment deposition and pigment deposition per open male flower from high-irrigation plants were right-skewed and thus square-root transformed to improve normality. Pigment deposition and pigment deposition per open male flower from low-irrigation plants were right-skewed and thus $\log_{10}$ transformed (after one high outlier was removed) to improve normality of the residuals.

We used a multiple logistic regression to assess how soil moisture and pollination (bee pollination, hand pollination) affected fruit set, and a general linear model to assess how soil moisture and pollination affected seed set. To assess the effect of bee visitation on fruit set, we used a multiple logistic regression containing two fixed factors: *Apis mellifera* visitation rate and *Eucera* visitation rate. For this analysis, we used visitation rates (visits per female flower per minute) from surveys of bees visiting all plants in the plot (see Appendix 2.1). To determine the effect of pollen and pigment deposition on seed set in bee and hand-pollinated plants, we used separate general linear models containing total pollen deposition or proportion pigment from high-irrigation plants as the fixed factor.

RESULTS

The irrigation treatment affected *Cucurbita pepo* plants to some degree in terms of floral traits. Male flower production and pollen production linearly increased with increasing soil moisture (Fig. 2.1), but no other measured floral trait exhibited a significant response to manipulated soil moisture levels (Table 2.1).

Video recordings confirmed that honey bees (*Apis mellifera*) and squash bees (*Eucera* spp.) were the most frequent visitors of squash flowers (Table 2.2). Although there were no significant effects of plant soil moisture on the visitation rate of *Apis* and *Eucera* on male flowers (Table A.2.2.1), we did observe contrasting patterns of visitation on female flowers (Table A.2.2.2). The visitation rate of *Apis* on female flowers increased with increasing plant soil moisture (Fig. 2.2A). Summed across *Apis* visits, both the time spent drinking nectar and contacting floral stigmas increased with plant soil moisture, as well (Fig. 2.2B-C). For *Eucera*, in contrast, visitation rate on female flowers was independent of soil moisture (Fig. 2.2A); cumulative time spent drinking nectar and contacting floral stigmas followed suit (Fig. 2.2B-C; Table A.2.2.2).

Pollen and pigment deposition on the stigmas of bee-pollinated plants increased with increasing plant soil moisture (Fig. 2.3). For pollen deposition we observed a linear increase with increasing plant soil moisture (Fig. 2.3A), but this relationship was no longer significant when we controlled for the number of open male flowers (and thus total pollen) available to bees on the day that each stigma was collected (Fig. 2.3B). Pigment originating from male flowers on plants in the high-irrigation group was deposited in increasingly greater amounts on the stigmas of female flowers as the soil moisture experienced by recipient plants increased (Fig. 2.3C). This relationship remained significant after we controlled for the availability of male flowers from high-irrigation plants (and thus total pollen from high-irrigation plants) on the day that each measurement was made (Fig. 2.3D). For fluorescent pigment originating from male flowers on plants in the low-irrigation group, pigment transfer to stigmas was independent of the recipient plant's soil moisture (simple linear regression: $F_{1,53} = 3.19$, $r^2_{adj} = 0.026$, $P = 0.080$; and after controlling for male flower availability from low-irrigation plants: $F_{1,53} = 2.75$, $r^2_{adj} = 0.031$, $P = 0.10$). Pollen and pigment deposition for hand-pollinated stigmas are listed in Appendix 2.2.

Fruit set was independent of plant soil moisture (multiple logistic regression: $z = 1.30$, $P = 0.20$) and also did not differ as a function of pollination mode (multiple logistic regression: $z = -0.52$, $P = 0.60$): bee-pollinated mean (59.88 ± 5.09%) and hand-pollinated mean (48.68 ± 7.68%). For bee-pollinated plants, however, the likelihood of fruit set increased with increasing honey bee visitation (multiple logistic regression: $z = 2.41$, $P < 0.05$) but not with increasing squash bee visitation (multiple logistic regression: $z = -0.021$, $P = 0.98$).

Seed set increased with plant soil moisture (Fig. 2.4) and was higher for bee-pollinated plants than for hand-pollinated plants (Fig. 2.4; bee-pollinated mean: 10.15 ± 0.94 g; hand-pollinated mean: 7.14 ± 1.25 g; soil moisture x pollination mode interaction: $F_{1,79} = 1.28$, $P = 0.26$). For bee-pollinated plants, we further observed that seed set increased with the amount of pollen deposited on stigmas (Fig. 2.5A) and with the proportion of pigment originating from plants in the high-irrigation group (Fig. 2.5B). In contrast, neither relationship was significant for hand-pollinated plants (Fig. 2.5C: seed set *versus* pollen deposition; Fig. 2.5D: seed set *versus* pigment deposition).

DISCUSSION

We tested three predictions concerning how specialist and generalist pollinators interact with plants grown under a gradient of soil moisture conditions and how pollinator-specific behaviors affect plant reproduction. We received partial support for our first prediction. The frequency of floral visits by honey bees (*Apis mellifera*) increased with increasing plant soil moisture for female flowers but not for male flowers; visit frequency by squash bees (*Eucera*) was independent of plant soil moisture for both female and male flowers. Moreover, increased visitation by *Apis* in female flowers included increased duration of stigmatic contact with increasing plant soil moisture. Our second prediction was met; stigmatic deposition of pigment from plants in the high-irrigation group increased with increasing plant soil moisture. Although we were not able to directly track pollen deposition from plants in the high-irrigation group, pigment and pollen deposition were highly correlated. Support for our third prediction comes from the comparisons of bee-pollinated versus hand-pollinated plants. As expected, seed set increased with increasing plant soil moisture for both bee-pollinated and hand-pollinated plants. For bee-pollinated plants, seed set increased with increasing pollen deposition and with increasing pigment from plants in the high-irrigation group. Hand-pollinated plants, in contrast, received an even mix of pollen from plants in the low-irrigation and high-irrigation groups irrespective of the plant soil moisture of the recipient plant (see Appendix 2.2). These plants did not exhibit relationships between seed set and pollen deposition nor between seed set and the amount of pigment originating from plants in the high-irrigation group. These results taken together suggest that pollen from plants in the high-irrigation group was of higher quality than pollen from plants in the low-irrigation group (see also Chapter 1).

Given that the goals of this study were to investigate how the behavior of pollinators mediates the reproductive performance of plants that experience a gradient of soil-moisture environments, we did not attempt to identify the mechanisms responsible for why pollinators exhibit selectivity. Although an earlier study in this same system (Chapter 1) found that flower size and nectar production increased with increasing plant soil moisture, we found no evidence for similar responses in the current study and assume that inter-annual differences in temperature (the summer of 2018 was the warmest at our field site between years 2015 – 2020 by at least 0.4˚C; PRISM Climate Group), the timing of planting, or differences in the experiment itself (we studied the combined effects of warming and drought in the earlier experiment) are responsible. One possible explanation for our finding of increased visitation by honey bees to female flowers with increasing plant soil moisture may have been caused by the emission or composition of floral VOCs attractive to *Apis* changing with increasing soil moisture (Burkle and Ruyon 2016, Glenny et al. 2018). Irrespective of the mechanism of attraction, *Apis* often exhibit a preference for female flowers of *C. pepo* (Tepedino 1980, Chapter 3) as well as those of *C. foetidissima* (Chapter 3). Given that pollen production increased in male flowers with increasing soil moisture, increased visitation to female flowers with increasing soil moisture by honey bees is sufficient to explain higher deposition of pollen from plants in the high-irrigation group given that exhibited cumulative duration of stigmatic contact increased with visitation.

We relied on fluorescent pigments to examine pollen movement among plants in this experiment. As a proxy for pollen deposition, the use of pigments is established in the literature (Waser and Price 1982, Ordway et al. 1987, Waser 1988, Adler and Irwin 2006, Rojas-Nossa et al. 2016) and in our study seems well justified given the close correlation between pollen and pigment deposition (see Methods). Nonetheless, this approach, by itself, does not permit direct identification of pollen from plants in low-irrigation and high-irrigation groups once it is deposited on the stigma. In fact, total stigmatic pollen deposition was independent of plant soil moisture after correcting for male flower availability. This result differed from the positive effect of increased soil moisture of the recipient plant on the deposition of pigment (i.e., pollen) from plants in the high-irrigation group after correcting for available male flowers. However, total pollen deposition included pollen from plants in both the low-irrigation and high-irrigation groups. Therefore, the inclusion of pollen from all soil moisture levels in the measurement of total pollen deposition likely canceled out the effects from the high-irrigation pollen, as we found that pigment (i.e., pollen) from plants in the low-irrigation group did not increase with increasing plant soil moisture of the recipient plant (before or after correcting for male flower availability).

As with our analysis of pollen deposition, analysis of fruit set also did not allow for an assessment of the contribution of pollen from different sources. It is noteworthy, however, that fruit set increased with increasing visitation by honey bees and was independent of visitation by squash bees. Other work in this system has revealed that *Eucera* outperform *Apis* with respect to per visit contributions to fruit set (Chapter 3). Thus, the contributions of honey bees to fruit set in the present study provides additional evidence that *Apis* transport pollen from male flowers of plants in the high-irrigation group to the female flowers of plants in the high-irrigation group and in doing so enhance plant reproduction. Squash bees, in contrast, indiscriminately visited flowers with respect to plant soil moisture and likely transported pollen from plants across the soil moisture gradient.

The findings of this study seem likely to apply to other plant-pollinator systems. Generalist bees often forage preferentially for high-quality floral resources, such as more viable pollen (Robertson et al. 1999), pollen with a high protein content (Hanley et al. 2008, Leonhardt

& Blüthgen 2012, Vaudo et al. 2016), nectar of increased volume and concentration (Thomson 1986), and nectar with increased amounts of amino acids (Alm et al. 1990). These preferences, in turn, can benefit plants through increased pollinator visitation and pollen deposition. Kasagi and Kudo (2003), for example, found that preferences exhibited by bumble bees for increased nectar resources resulted in reduced pollen limitation in *Phyllodoce*. However, generalists need to not only be attracted to high-quality resources, but they also need to increase stigmatic deposition of high-quality pollen to augment plant reproduction. Mu et al. (2017), for example, assessed that preferences for flower color by flies and honey bees coincided with increased pollen viability, which led to greater reproductive success in *Gentiana leucomelaena*.

Compared to generalists, specialist pollinators are often considered more effective (Strickler 1979, Cane and Payne 1988, Larsson 2005, Tepedino 2016, Chapter 3). Yet during stressful environmental conditions, such as droughts, generalists may preferentially visit high-quality resources while specialists do not. Exhibiting distinct floral constancy amongst conspecifics of high-quality could thereby allow generalist pollinators to mediate successful reproduction in stressed plants. However, if all conspecifics lack resources of high enough quality for generalists, then these pollinators may disregard stressed plants altogether, leaving specialists as the sole floral visitors with the job of pollination. Reductions in the availability of high-quality floral resources may thus turn out to be detrimental for pollinators as well as their floral hosts. Plants species faced with drought may therefore increasingly trade-off allocation to separate, and perhaps competing, floral functions in order to attract a diversity of pollinators.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Gambel, Jess; Holway, David A. The dissertation author was the primary investigator and author of this material.

TABLES

Table 2.1. The effects of experimentally-manipulated soil moisture on floral traits for bee-pollinated *Cucurbita pepo*. Traits in bold are statistically significant at $\alpha = 0.05$.

Response Variable	df	F	P	r^2_{adj}
Total male flowers produced	**1,59**	**7.45**	**0.0084**	**0.097**
Total female flowers produced	1,59	2.77	0.10	0.029
Male flower size (mm)	1,58	1.49	0.23	0.0082
Female flower size (mm)	1,34	2.17	0.15	0.032
Nectar volume (µL) in male flowers	1,58	0.16	0.69	-0.014
Nectar volume (µL) in female flowers	1,36	0.72	0.40	-0.0075
Nectar concentration (BRIX) in male flowers	1,58	0.41	0.53	-0.010
Nectar concentration (BRIX) in female flowers	1,35	0.0060	0.94	-0.028
Pollen mass (mg) in male flowers	**1,58**	**5.52**	**0.022**	**0.071**
Proportion viable pollen in male flowers	1,59	0.074	0.79	-0.016

Table 2.2. The proportions of different bee visitors in videotaped male and female flowers in *Cucurbita pepo*.

Flower Visitor	Proportion of Visits
Male flowers	
Apis mellifera	0.65
Eucera	0.16
Agapostemon texanus	0.08
Lasioglossum spp.	0.11
Female flowers	
Apis mellifera	0.90
Eucera	0.06
Agapostemon texanus	0.02
Lasioglossum spp.	0.02

FIGURES

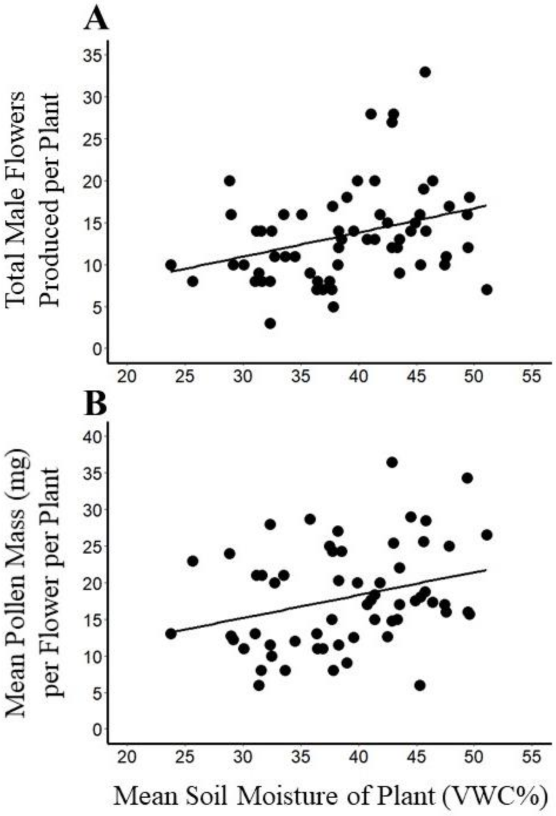


Figure 2.1. Effects of experimentally manipulated soil moisture on (A) male floral production and (B) pollen production per male flower for bee-pollinated *Cucurbita pepo*. Table 2.1 provides additional statistical information relevant to these analyses.

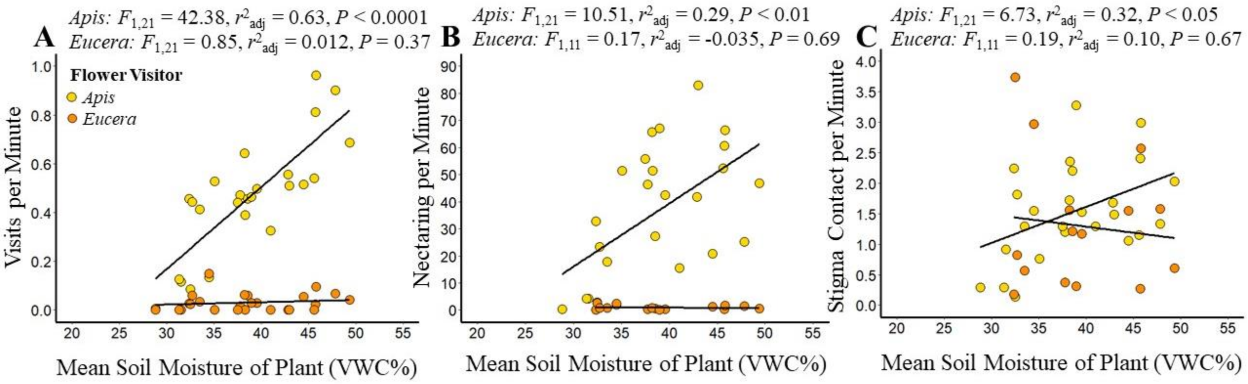


Figure 2.2. Effects of soil moisture on (A) visitation rate (visits/minute), (B) cumulative duration of time spent nectaring/minute, and (C) cumulative duration of time spent in contact with the stigma/minute for honey bees (*Apis mellifera*) and squash bees (*Eucera*) in female flowers of *Cucurbita pepo*.

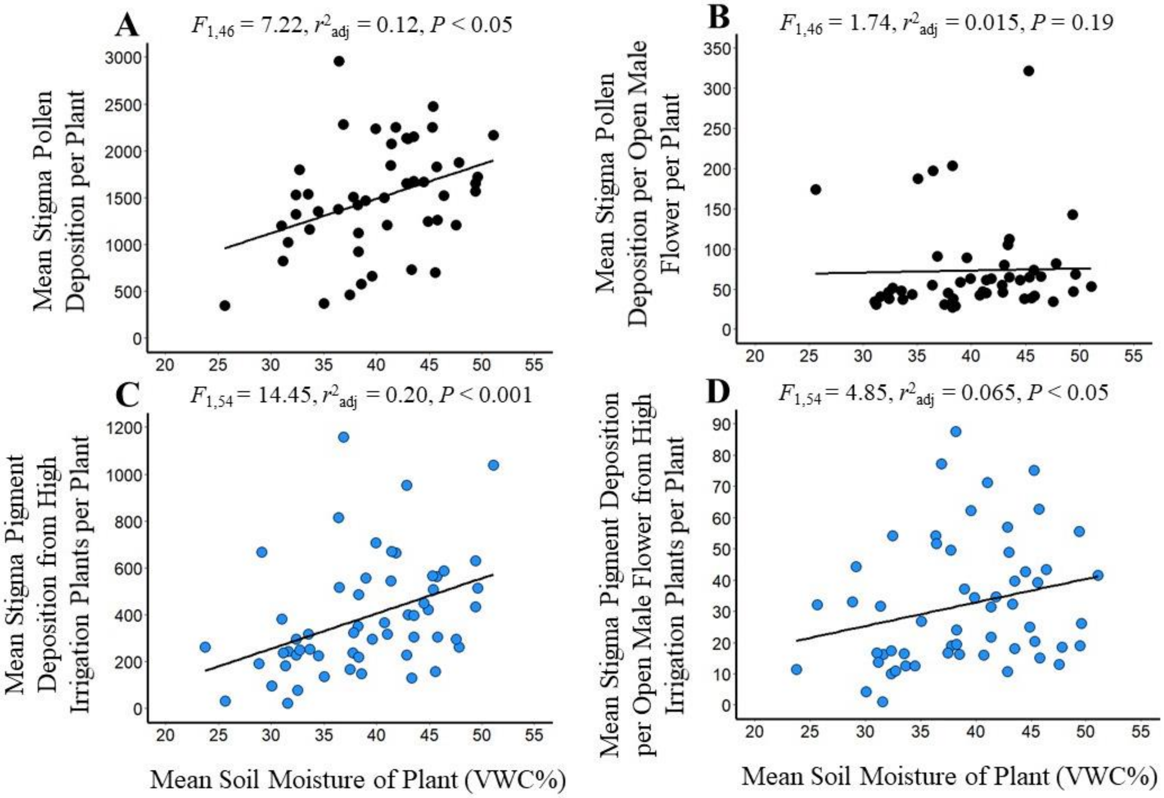
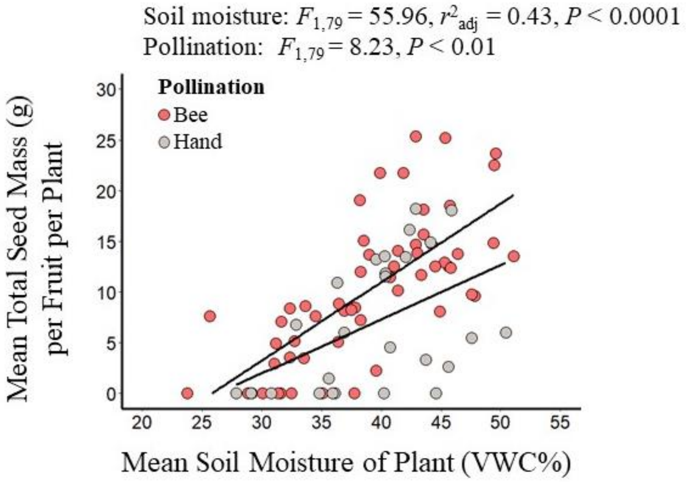


Figure 2.3. Relationship between plant soil moisture and stigmatic deposition of (A) pollen, (B) pollen per open male flower, (C) pigment from high irrigation plants, and (D) pigment per open male flower from high irrigation plants for bee-pollinated *Cucurbita pepo*. Mean plant soil moisture values are those of the recipient plants.



Soil moisture: $F_{1,79} = 55.96$, $r^2_{adj} = 0.43$, $P < 0.0001$

Pollination: $F_{1,79} = 8.23$, $P < 0.01$

Figure 2.4. Seed set versus soil moisture for bee-pollinated and hand-pollinated *Cucurbita pepo*.

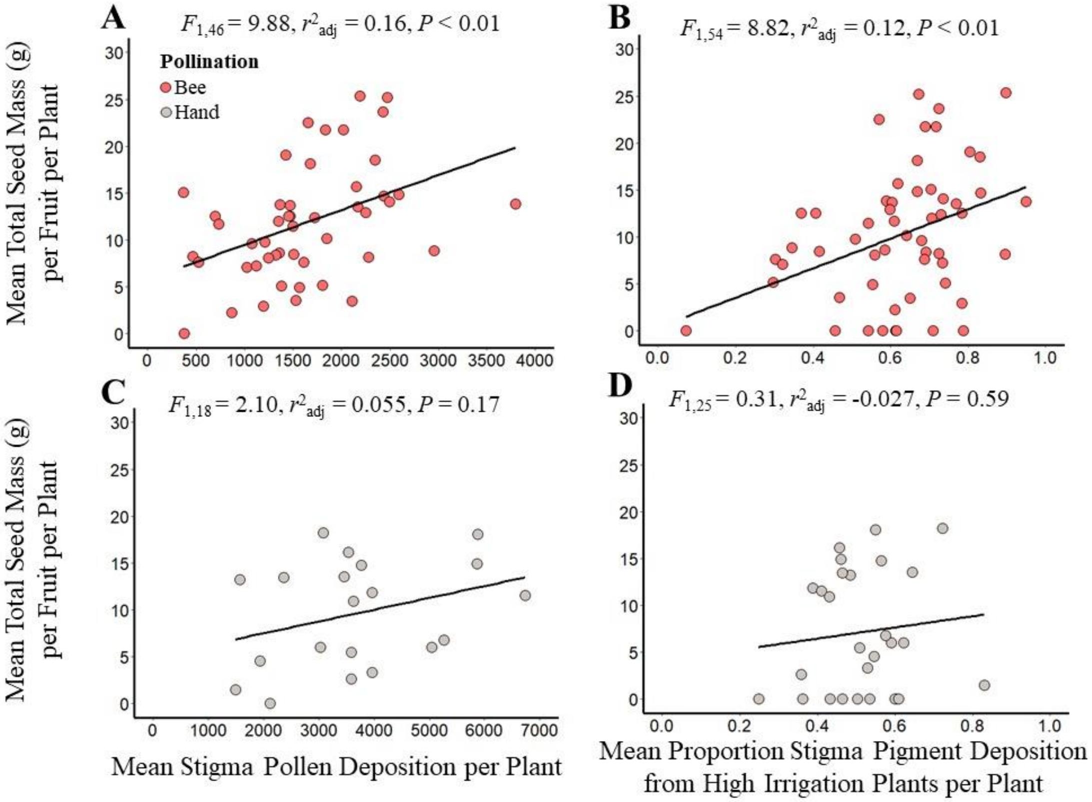


Figure 2.5. The effects of stigmatic pollen deposition (A, C) and proportion stigmatic deposition of pigment from high irrigation plants (B, D) on seed set for bee-pollinated (A, B) and hand-pollinated (C, D) *Cucurbita pepo* plants. Note: the relationship is still significant when the low outlier in (B) is removed.

APPENDICES

Appendix 2.1. Methods

Floral traits

We measured flower size as the open floral corolla width (Hoover et al. 2012). We used a 20 µL pipette to collect nectar from each flower (Stoner & Eitzer 2012). We measured the nectar volume by first centrifuging and then measuring the collected sample using a 200 µL pipette. To measure nectar concentration, we used a 10 µL pipette to remove a 2 µL-sized sample from this collected nectar and placed the sample on a refractometer (Corbet 1978) to measure the BRIX (0-50%) of that sample. We measured up to three samples to obtain a clear reading, which usually occurred with the first sample. We also measured pollen mass and viability for each unvisited male flower. Using a metal spatula, we gently scraped pollen grains off the anthers of the collected male flower (Stoner & Eitzer 2012) into a tared microcentrifuge tube, which we then weighed using a scale. We tested pollen viability by spreading 1 mg of pollen from each sample onto a microscope slide and then applying 40 µL MTT solution (thiazolyl blue) (Khatun & Flowers 1995). MTT tests pollen grains for enzymatic activity: grains stained red to purple are viable, while grains stained black or left unstained are not viable (Khatun & Flowers, 1995). Pollen staining with MTT is easily facilitated in the field and correlates well with *in vitro* pollen germination tests (Firmage & Dafni 2000), but care needs to be taken to correctly distinguish the varying color tonalities of stained grains (Rodriguez-Riano & Dafni 2000). Slides were left to dry for several hours and then refrigerated until measured. Using a dissecting microscope at 30x magnification, we counted the number of different colored pollen grains on slides to determine the proportions of viable and nonviable grains per sample.

Bee surveys

In addition to video-taping bee visitation to individual flowers, each morning between 7AM and 9:30AM we also conducted 5 sets of 15-minute bee visitation surveys (Hurd et al. 1974) of generalist and specialist species in all open flowers in the bee-pollinated plants. We monitored bee visitation on high and low-irrigation plants concurrently. Therefore, bees could move freely between flowers in both treatment groups. Surveys consisted of walking around to all open flowers in the plot and noting all bees visiting those flowers in the allotted time. Each 15-minute survey often consisted of multiple rounds of monitoring the same open flowers.

Appendix 2.2. Results

Table A.2.2.1. The effects of experimentally-manipulated soil moisture, total available flowers, and their interaction on bee behavior rates (per minute video) in videotaped male flowers in *Cucurbita pepo*. Noted traits were transformed to improve normality: *square-root transformed, $^{+}$arcsine square-root transformed, $^{\%}$inverse transformed, $^{\&}$ordered quantile normalization transformed. Residuals for *Apis* nectaring rate and *Eucera* pollen collecting rate were still not normal after transformation.

Response Variable	Treatment	df	*F*	*P*	r^2_{adj}
Apis mellifera					
Visit rate^{+}	Mean soil moisture	1,35	0.69	0.41	-0.056
	Total flowers available	1,35	0.14	0.71	
	Soil moisture x available flowers	1,35	0.16	0.70	
Nectaring rate$^{\%}$	Mean soil moisture	1,34	0.038	0.85	-0.075
	Total flowers available	1,34	0.29	0.60	
	Soil moisture x available flowers	1,34	0.092	0.76	
Pollen collecting rate*	Mean soil moisture	1,34	1.09	0.30	-0.035
	Total flowers available	1,34	0.098	0.76	
	Soil moisture x available flowers	1,34	0.56	0.46	
Anthers contact rate*	Mean soil moisture	1,34	2.91	0.097	0.032
	Total flowers available	1,34	0.44	0.51	
	Soil moisture x available flowers	1,34	0.88	0.36	
Eucera					
Visit rate$^{\&}$	Mean soil moisture	1,35	0.34	0.56	0.017
	Total flowers available	1,35	2.97	0.094	
	Soil moisture x available flowers	1,35	0.36	0.55	
Nectaring rate*	Mean soil moisture	1,25	0.80	0.38	-0.032
	Total flowers available	1,25	1.33	0.26	
	Soil moisture x available flowers	1,25	0.00	1.00	
Pollen collecting rate^{+}	Mean soil moisture	1,25	0.011	0.92	-0.11
	Total flowers available	1,25	0.099	0.76	
	Soil moisture x available flowers	1,25	0.091	0.77	
Anthers contact rate*	Mean soil moisture	1,25	0.47	0.50	-0.043
	Total flowers available	1,25	1.39	0.25	
	Soil moisture x available flowers	1,25	0.00	0.99	

Table A.2.2.2. The effects of experimentally-manipulated soil moisture, total available flowers, and their interaction on bee behavior rates (per minute video) in videotaped female flowers in *Cucurbita pepo*. Results in bold are statistically significant at $\alpha = 0.05$.

Response Variable	Treatment	df	*F*	*P*	r^2_{adj}
Apis mellifera					
Visit rate	**Mean soil moisture**	**1,21**	**42.38**	**<0.0001**	**0.63**
	Total flowers available	1,21	0.0020	0.97	
	Soil moisture x available flowers	1,21	1.82	0.19	
Nectaring rate	**Mean soil moisture**	**1,21**	**10.51**	**0.0039**	**0.29**
	Total flowers available	1,21	0.59	0.45	
	Soil moisture x available flowers	1,21	1.47	0.24	
Stigma contact rate	**Mean soil moisture**	**1,21**	**6.73**	**0.017**	**0.32**
	Total flowers available	1,21	1.61	0.22	
	Soil moisture x available flowers	**1,21**	**5.87**	**0.025**	
Eucera					
Visit rate	Mean soil moisture	1,21	0.85	0.37	0.012
	Total flowers available	1,21	2.18	0.16	
	Soil moisture x available flowers	1,21	0.26	0.61	
Nectaring rate	Mean soil moisture	1,11	0.17	0.69	-0.035
	Total flowers available	1,11	0.0040	0.95	
	Soil moisture x available flowers	1,11	2.35	0.15	
Stigma contact rate	Mean soil moisture	1,11	0.19	0.67	0.10
	Total flowers available	1,11	0.61	0.45	
	Soil moisture x available flowers	1,11	3.81	0.077	

Hand-pollinated stigmas

In addition to measuring bee-pollinated stigmas, we also counted pollen and pigment deposition on collected hand-pollinated stigmas. We counted deposited pollen grains on stigmas from 22 hand-pollinated plants and counted deposited pigments on stigmas from 29 hand-pollinated plants. For plants with multiple stigma samples (pollen: n = 11; pigment: n = 28), we took the mean deposition values of all collected samples for the plant. Mean total pollen (one-way ANOVA: $F_{1,68} = 130.70$, $P < 0.0001$) and pigment deposition (one-way ANOVA: $F_{1,83} = 148.80$, $P < 0.0001$) were both more than two-fold higher in hand-pollinated plants (mean total pollen grains: 3757 ± 227; mean total pigment particles: 1659 ± 86) compared to bee-pollinated plants (mean total pollen grains: 1500 ± 85 grains; mean total pigment particles: 631 ± 42).

During the experiment, we always used the same number of male flowers (2 flowers total: 1 from a high irrigation plant and 1 from a low irrigation plant) when hand-pollinating stigmas, so there was no need to control for male flower availability in pollen and pigment analyses. We found no effect of soil moisture on pollen deposition in hand-pollinated stigmas (simple linear regression: $F_{1,20} = 0.023$, $r^2_{adj} = -0.049$, $P = 0.88$). We also found that there was no relationship between soil moisture of the recipient plant and pigment deposition from high irrigation plants (simple linear regression: $F_{1,27} = 1.42$, $r^2_{adj} = 0.015$, $P = 0.24$) nor from low irrigation plants (simple linear regression: $F_{1,27} = 2.93$, $r^2_{adj} = 0.064$, $P = 0.099$).

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CHAPTER 3

The performance of specialist and generalist pollinators on cultivated squash and wild gourds

by

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ABSTRACT

The western honey bee (*Apis mellifera*) is a super-generalist in terms of floral visitation, but its performance as a pollinator varies widely. Understanding the basis of this variation seems important to clarify, perhaps especially in the introduced range of the honey bee, where this species can achieve numerical dominance in terms of flower visitation frequency. Here, we compared honey bees and specialist squash bees as pollinators of cultivated squash (*Cucurbita pepo*) and free-living buffalo gourd (*C. foetidissima*) in southern California. In single visits, female squash bees removed more pollen from the anthers of male flowers, deposited more pollen on the stigmas of female flowers, and contributed more to fruit set and seed set on both *Cucurbita* species than did honey bees. Multiple regression models testing how 0-12 visits by honey bees and female squash bees (alone or in combination on *C. pepo* only) revealed that pollen removal, pollen deposition, and fruit set linearly increased with the number of visits by female squash bees, whereas only fruit set linearly increased with the number of visits by honey bees. Interspecific differences in pollinator performance stemmed from consistent behavioral differences; female squash bees spent more time per visit in contact with stigmas and anthers than did honey bees. Even though honey bees are numerically dominant as floral visitors in these

Cucurbita systems, they appear less important in terms of contributing to seed production compared to female squash bees. Previous studies on *C. pepo* report that honey bees and squash bees appear more similar to one another than what we report here. These differences may largely stem from past studies pooling together male and female squash bees. More generally, our findings illustrate that behavioral differences that underlie disparities in pollinator performance have clear predictive value as determinants of how species contribute to pollination services.

INTRODUCTION

Variation in specialization is a striking feature of pollination mutualisms. Some species of pollinators visit only a single plant species or genus, while other species forage on plants from many different families and make foraging decisions primarily based on availability (Willmer 2011). Bees exemplify these divergent strategies. Nearly all bees consume a diet of nectar and pollen, but species vary greatly with respect to the diversity of pollen sources in their diets (Danforth et al. 2019). How these differences in niche breadth relate to what pollinators actually contribute to pollination has intrigued ecologists for many years (Motten et al. 1981, Schemske & Horvitz 1984, Larsson 2005, McIntosh 2005). From a plant's perspective, an ideal pollinator will deposit a high proportion of the pollen that it removes, but pollen specialists will retain as much pollen as possible to maximize their own reproduction (Danforth et al. 2019). Effective specialists might thus be expected to represent high-removal high-deposition species in contrast to low-removal, low-deposition generalists (Williams & Thomson 2003). Yet compared to generalist pollinators, specialists do not always contribute the most to pollination (Motten et al. 1981, Parker et al. 2016) and in some cases can even be inferior to generalists, particularly when they are relatively rare (Larsson 2005).

The western honey bee (*Apis mellifera*) is a super-generalist in terms of floral visitation, but its performance as a pollinator varies widely among the plant species that it visits (Hung et al. 2018). Such variation has brought attention to the role of native insects as pollinators in agricultural systems and has raised concerns that honey bees could adversely impact some plant species in non-managed systems. In a meta-analysis of studies in agricultural systems, for example, Garibaldi et al. (2013) found that flower visitation by wild insects was more often associated with an increasing likelihood of fruit set compared to visitation by honey bees, and that wild insects were in general more effective as pollinators. Hung et al. (2018) collated single-visit measures of pollinator effectiveness from crop and non-crop plant species worldwide and found that honey bees were, on average, as efficient as the mean of other pollinators visiting a given species, but less effective compared to the most effective visitor. Underlying these patterns, however, was substantial variation in the performance of honey bees relative to that of other pollinators among different plant species (Hung et al. 2018). Understanding the basis of this variation is required to assess the role that honey bees fill as pollinators, perhaps especially in the introduced range of *Apis mellifera* where this species can achieve numerical dominance through high floral visitation frequency in both managed and unmanaged environments (Bruckman & Campbell 2014, Nabors et al. 2018, Hung et al. 2018).

In the present study we focus on the specialist and generalist pollinators of two species of *Cucurbita* (Cucurbitaceae). *Cucurbita* attracts a variety of insect pollinators, primarily bees, which include generalists, like the western honey bee (*Apis mellifera*), and specialists, such as squash bees (*Eucera* (formerly *Peponapis* and *Xenoglossa*) Dorchin et al. (2018)) that require pollen from cucurbits to reproduce (Hurd et al. 1974). Interactions between *Cucurbita* species and their pollinators are extensively studied (Hurd et al. 1971, 1974, Tepedino 1981, Hoehn et al. 2008, Artz & Nault 2011, Hoover et al. 2012, Delgado-Carrillo et al. 2018), in large part because of the economic importance of cultivated squash (Gallai et al. 2009, McGrady et al. 2019). The *Cucurbita* pollination system is also popular because of its tractability. All species of *Cucurbita* produce separate male (staminate) and female (pistillate) flowers with large, colorful corollas and that provide ample, sugar-rich nectar (Hurd et al. 1971). Individual flowers remain open and receptive to pollinators for only one morning, so flowers can be collected and measured the same day (Tepedino 1981). Owing to the production of separate male and female flowers, all

Cucurbita species require pollinators to produce fruit and seeds (Hurd et al. 1971 Hoehn et al. 2008, Artz & Nault 2011).

Given the commercial importance of cultivated species of *Cucurbita* (e.g., *C. pepo, C. maxima, C. moschata*), explicit comparisons between specialist and generalist pollinators are best known from agricultural settings where visitation by honey bees is common (Nicodemo et al. 2009, Delgado-Carrillo et al. 2018, Pfister et al. 2017, Xie et al. 2016). Tepedino (1981), for example, found that single visits by honey bees and the squash bee *E. pruinosa* were comparable in terms of stigmatic pollen deposition and fruit set for *C. pepo* at an agricultural research station in northern Utah. Likewise, on pumpkin (*C. pepo*) farms in New York, Artz and Nault (2011) found that single visits by honey bees and squash bees (*E. pruinosa*) did not differ from one another in terms of stigmatic pollen deposition, fruit set, and seed set. Neither Tepidono (1981) nor Artz and Nault (2011), however, separated squash bees by sex. Cane et al. (2011) found that at least five male squash bees were required to set *C. pepo* fruit and seed as successfully as one female squash bee. Moreover, as noted by Tepidino (1981), studies conducted outside of the native range of squash bees may reach different conclusions from studies conducted where squash bees are native (and where they might be more abundant). Indeed, studies conducted in Mexico that separate male and female bees found that single visits by female squash bees resulted in higher stigmatic pollen deposition and fruit set compared to single visits by honey bees (Canto-Aguilar et al. 2010, Delgado-Carrillo et al. 2018). However, all previous studies that compare honey bees and squash bees as pollinators of *Cucurbita* have focused on managed plots of cultivated squashes. Squash farms represent rich but homogeneous patches of floral resources and are often situated in environments that are highly modified by human activity. Clearly, an understanding of the relationships between *Cucurbita* and their specialist pollinators would benefit from information about how these species interact under more natural conditions.

Here we compare honey bees and squash bees as pollinators of two *Cucurbita* species in southern California: the free-living native perennial buffalo gourd (*C. foetidissima*) and cultivated squash (*C. pepo*). Buffalo gourd occurs in arid environments throughout southwestern North America and supports a variety of squash bees, including *E. pruinosa*, which is also a common pollinator of cultivated squash and has spread widely in North America in response to the cultivation of *C. pepo* (López-Uribe et al. 2016). For both *Cucurbita* species we used single-visit trials to test whether or not honey bees and female squash bees differ in their ability to remove pollen from male flowers, deposit pollen on the stigmas of female flowers, and contribute to fruit and seed set. Given that single-visit trials, by definition, cannot account for the effects of multiple visitation by more than one bee species, we used multiple regression to examine if a small number of visits (< 12) by each species (alone or in combination) predicts pollen removal, pollen deposition, fruit set, and seed set on *C. pepo*. The results of these trials are then interpreted in light of the relative abundance of honey bees and squash bees in both systems in order to estimate pollinator importance.

METHODS

We studied interactions between squash and their bee pollinators over a three-year period (2017-2019) from June through September each year. We worked at two study sites: an open riparian woodland with free-living *C. foetidissima* at the Los Peñasquitos Canyon Preserve (32°55'04.9" N, 117°11'37.4" W) and an experimental plot with hand-planted *C. pepo* at the University of California San Diego Biology Field Station (32°53'07.8" N, 117°13'45.3" W). At the former site, we studied 45 plants along a 5-km stretch of Los Peñasquitos Creek as well as a 1-km stretch of Lopez Creek (a tributary of the former creek). At this site, buffalo gourd, which are perennial, grow either alone or in patches of one to several individuals separated by large expanses lacking this species. Because of the modest number of plants, we kept track of plant identity and studied the same individuals in successive years. For the annual *C. pepo*, plants were germinated from seeds (Honey Bear F1 acorn squash seed from Johnny's Selected Seeds$^{©}$) planted directly in the ground in a grid with 1.2 m spacing between plants. Plants were irrigated throughout the summer. In addition to single-visit and multiple-visit trials, we monitored a set of flowers of each *Cucurbita* species to obtain measures of relative pollinator abundance, and open-pollinated fruit and seed set.

Our two study sites are within 5 km of one another and support populations of native squash bees (*Eucera* (*Peponapis*) *pruinosa*, *Eucera* (*Xenoglossa*) *strenua*) and the non-native, western honey bee (*Apis mellifera*). Trials conducted on *C. pepo* focus on female *E. pruinosa*, but for *C. foetidissima* we also consider female *E. strenua.* Comparisons of honey bees and squash bees primarily focused on female squash bees given that their behavior differs from that of males, which visit flowers primarily in search of females (Cane et al. 2011). Moreover, our fieldwork was often conducted after the peak of male squash bee activity (see Tepedino 1981) and male bees were sometimes quite uncommon. For both species of *Cucurbita*, we provide data for males in Appendix 3. Feral honey bees are abundant throughout San Diego County (Kono and Kohn 2015, Hung et al. 2018). In addition, 16 managed honey bee colonies were present at the Biology Field Station from 2017 - 2019. Feral honey bees likely accounted for the majority of honey bee visitation at the Los Peñasquitos Canyon Preserve; this field site is located away from agricultural areas where honey bees would be used for pollination.

Open flowers

For both species of *Cucurbita* we monitored a subset of male and female flowers that were left open to pollinators to quantify temporal patterns of visitation, to estimate the relative abundance of bees, and to obtain measures of fruit and seed set. To document patterns of visitation by honey bees and squash bees, we videotaped flowers for approximately 150 min between 0600-0900. The camera (2017: GoPro HERO Session (model C3141326666972) camera ; 2018: Victure Action Camera WIFI 14MP 1080P; 2019: Victure Action Camera 4K WiFi 16MP) was set close enough (20 - 40 cm) to each flower to fill the frame of the video but far enough away such that bee visitation did not seem to be affected. Once a focal flower wilted (i.e., the same afternoon as the video recording), we placed an organza mesh bag (Paper Mart 15 cm x 23 cm) over the flower and tightened the bag with drawstrings below the base of the ovary to exclude additional visitors. Flowers remained bagged for 3 d after opening. Open flowers were checked over a period of several days following senescence to determine whether or not they set fruit. Fruits were harvested either 35 d (*C. foetidissima*) or 50 d (*C. pepo*) after opening. These different durations reflect interspecific differences in fruit maturation (Awdh ba-amer and Bemis

1968, Loy 2004). Seeds were removed from fruits in the lab, dried, counted, and weighed. We used total seed weight to estimate seed set.

Videos were later transcribed to obtain a record of bee visits per unit time, which we used to quantify temporal patterns of visitation and to estimate the relative abundance of honey bees and squash bees. To quantify temporal patterns of visitation, we binned visitation into 30-min intervals between 0600-0900. For videos that encompassed only a portion of a time bin, we extrapolated visitation from the number of visits observed for the portion of the bin for which we have a video record. To estimate relative abundance, we first averaged the number of bee visits per unit time (for each bee species and sex of squash bee) on flowers from within the same plant and used plant-level means as data points. We then divided values for each species and sex of bee by the summed total visitation of honey bees and squash bees (see Park et al. 2016) and averaged these values across individual plants. We pooled years of observation together in these analyses because relative abundance estimates did not significantly differ among years.

Single-visit trials

These trials were performed to measure duration of contact with anthers and pollen removal from male flowers, or duration of stigmatic contact, stigmatic pollen deposition, fruit set, and seed set for female flowers. We first identified male and female flower buds that looked like they would open the following day based on their size and color. On the afternoon prior to these buds opening, we covered each with a wax-lined, paper bag (Seedburo Treated S27 Shoot Pollinating Bag (5 cm x 2.5 cm x 18 cm)) to exclude pollinators (Stoner & Eitzer 2012). On the following day, we arrived at a focal plant within an hour after sunrise, set up a video camera (2017: GoPro HERO Session (model C3141326666972) camera ; 2018: Victure Action Camera

WIFI 14MP 1080P; 2019: Victure Action Camera 4K WiFi 16MP), unbagged the focal flower, and then observed it until either a honey bee or a squash bee entered the flower. Single-visit trials interrupted by additional pollinators were discontinued. For each trial, we recorded the species and sex of the visitor as well as the duration of the visit. Videos provided an additional and more detailed record of single visits. Videos began after pollination bags were removed and were directly compared to visual observations of visitation for each flower. Once a pollinator had left a focal flower, we placed an organza mesh bag over the flower (Kearns & Inouye 1993) and tightened the bag with a drawstring around the petiole. Bees were never observed in bagged flowers.

After the conclusion of each trial, we estimated pollen removal from male flowers, and pollen deposition, fruit set, and seed set for female flowers. To estimate pollen removal, we first collected the pollen that remained after each single visit. We used a steel spatula (0.64 cm wide × 19 cm long) to separate pollen from the anthers and then weighed this pollen mass on a balance. We used the same method to determine the mean mass of pollen on unvisited anthers of male flowers (*C. pepo*: n = 104; *C. foetidissima*: n = 79) from the same plant (Williams and Thomson 2003). Based on these measurements we estimated the amount of pollen removed during each single visit as follows: (total pollen – remaining pollen) / total pollen. We also obtained the duration of time that bees spent in contact with anthers from the video recordings of each visit.

To estimate pollen deposition, fruit set, and seed set for the two species of *Cucurbita*, we employed similar methods. For female flowers of *C. pepo*, we sacrificed individual flowers by removing the entire pistil (i.e., by making an incision under the ovary) immediately after each single-visit trial. For female flowers of *C. foetidissima*, we waited approximately 24 h after single-visit trials before excising stigmas from pistils, leaving ovaries to develop into fruits. This approach allowed to us obtain measures of pollen deposition, fruit set, and seed set for the same flower in this species because 24 h was sufficient time for pollen grains to germinate and fertilize ovules (*personal observation*). For both species of *Cucurbita*, we placed each stigma in a 50-mL centrifuge tube containing 100% ethanol (Winsor et al. 2000) and three drops of basic Fuchsin dye solution (Kearns & Inouye 1993) immediately after removal and then refrigerated all samples. After waiting at least 3 d, we used a dissecting scope to count pollen grains under 50X magnification (Artz & Nault 2011). Flowers allowed to fruit remained bagged for 3 d after single-visit trials. By this time *Cucurbita* flowers had senesced, and further pollination was no longer possible. We measured fruit set and seed set resulting from single pollinator visits using the same methods described for open flowers.

All statistical analyses were run in R 3.3.1 (R Core Team 2016). In our analyses of the results of single-visit pollinator trials, most comparisons included multiple flowers from the same plant, and we thus employ mixed effects models (in the ‘*lme4*’ and ’*lmerTest*’ packages) in which we consider individual plants as a random effect. We used linear mixed models to test whether or not pollinator type (honey bee or female squash bee) affects the following response variables: duration of contact with anthers, proportion of pollen removed, duration of contact with stigmas, pollen deposition, and total seed weight. Except for the proportion of pollen removed, all response variables were log-transformed prior to analysis to improve normality of the residuals. For analyses on *C. pepo* that focused on total seed weight, duration of anther contact, and proportion of pollen removed, we used Welch’s *t*-tests to compare honey bees and squash bees because of modest sample sizes and because each replicate for these analyses represented a different plant. To test whether or not pollinator type (honey bee or female squash bee) and pollen deposition (*C. foetidissima* only) affected fruit set, we used generalized linear mixed models in which we consider individual plants as a random effect. Inspection of Q-Q plots and residual plots indicated that the data met assumptions of mixed effects models.

Pollinator importance

To estimate pollinator importance, we first multiplied mean per visit fruit set by mean per visit seed set for honey bees and female squash bees to obtain a per visit estimate of seed set for each pollinator that accounts for flowers that did not set fruit. For each type of bee, these products were in turn multiplied by relative abundance estimates (averaged across male and female flowers for each *Cucurbita*). To obtain estimates of pollinator importance in terms of seed production, we divided the value for each species by the sum of the values (a measure of total seed production). Note that statistical comparisons of pollinator importance between honey bees and female squash bees are not possible given that there is no way to estimate the error of these values (see also Sahli and Conner 2007, Bruckman and Campbell 2014).

Multiple-visit pollinator trials

To complement single-visit trials, we conducted an additional set of analyses that involved multiple visits by honey bees and squash bees on female and male flowers of *C. pepo*. These trials were performed in the same manner as the single-visit trials described in the previous section, except as follows. We restricted bee visitation to one female flower per plant and randomly assigned that plant to an experimental visitation category that ranged from 0 to 12 bee visits. On the day of each trial we removed the pollination bag and then observed the focal flower until the specified number of bee visits had accumulated for that flower. Each trial was recorded with a Victure Action Camera 4K WiFi 16MP as described in previous section. In some trials we used an aspirator to blow puffs of air at arriving honey bees (prior to them entering the flower) to prevent individuals of this species from monopolizing visitation. Application of this method discouraged honey bee visitation but did not seem to affect visitation by squash bees (see Nabors et al. 2018). Once the designated number of bee visits had occurred for that plant, we placed an organza mesh bag over the focal flower and tightened the bag with drawstrings around the petiole to prevent subsequent visitation by pollinators (Kearns & Inouye 1993). We recorded the number of visits made by honey bees and by female squash bees and measured pollen removal, pollen deposition, fruit set, and seed set as described in the previous section.

To determine how the number of visits from honey bees and female squash bees affected squash reproduction of multiply visited flowers, we performed model selection on sets of models based on multiple logistic regression (fruit set) or multiple regression (proportion of pollen removed, pollen deposition, and seed set). Each full model included the following variables: linear and quadratic terms for the number of visits by honey bees and by female squash bees, and an interspecific interaction term (based on the two linear terms). For each response variable, we compared the full model to models from a set of nine reduced models in which linear terms, quadratic terms, or the interaction term were excluded. We then used Akaike Information Criterion (AIC) to identify the best-supported model within the set of candidate models. We tested the assumptions of 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. Response variables (except for fruit set) were log-transformed prior to analysis to improve normality of the residuals. These analyses include some flowers that were also visited 1-3 times by male *E. pruinosa.* Exclusion of these data points does not qualitatively alter our results.

RESULTS

Patterns of bee visitation

On open *C. foetidissima* flowers, the relative abundance of honey bees exceeded that of squash bees (species and sexes combined) on female flowers (Wilcoxon signed rank test: V = 223, $P < 0.01$, n = 23 plants), but the opposite pattern was evident on male flowers (Wilcoxon signed rank test: V = 133.5, $P < 0.05$, n = 32 plants; Fig. 3.1). On open *C. pepo* flowers, the relative abundance of honey bees exceeded that of squash bees (species and sexes combined) on both female flowers (Wilcoxon signed rank test: V = 120, $P < 0.0001$, n = 15 plants) and male flowers (Wilcoxon signed rank test: V = 171, $P < 0.0001$, n = 18 plants; Fig. 3.1).

Honey bees and squash bees broadly overlapped in the timing of their visits to *Cucurbita* flowers (Fig. 3.2; sample sizes are the same as in the calculations for Fig. 3.1). On female flowers of both species of *Cucurbita*, peak activity of honey bees and female squash bees occurred at approximately the same time of the morning (Fig. 3.2A & 3.2C). This pattern was also evident on male *C. pepo* flowers (Fig. 3.2D), but not on male *C. foetidissima* flowers (Fig. 3.2B) on which honey bee visitation was low overall.

Single-visit trials

In both species of *Cucurbita*, on a per visit basis, female squash bees removed significantly more pollen from the anthers of male flowers and deposited significantly more pollen on the stigmas of female flowers compared to amounts removed and deposited by *A. mellifera* (Fig. 3.3). For pollen removal from male flowers of *C. foetidissima*, honey bees removed less pollen than *E. pruinosa* (linear mixed effects model: $t_{59.65} = 4.97$, $P < 0.0001$) and than *E. strenua* (linear mixed effects model: $t_{57.91} = 2.45$, $P < 0.05$) (Fig. 3.3A). For pollen removal from male flowers on *C. pepo*, honey bees removed less pollen than *E. pruinosa* (linear mixed effects model: $t_{28.88} = 3.71$, $P < 0.001$) (Fig. 3.3B). For pollen deposition the stigmas of female flowers of *C. foetidissima*, honey bees deposited less pollen than *E. pruinosa* (linear mixed effects model: $t_{30.3} = 4.34$, $P < 0.0001$) or *E. strenua* (linear mixed effects model: $t_{29.4} = 2.96$, $P < 0.001$) (Fig. 3.3C). For pollen deposition the stigmas of female flowers of *C. pepo*, honey bees deposited less pollen than *E. pruinosa* (linear mixed effects model: $t_{60.97} = 6.61$, $P < 0.0001$) (Fig. 3.3D).

Statistical differences between honey bees and female squash bees in terms of pollen removal and deposition were associated with pronounced, interspecific behavioral differences. Honey bees typically landed on the corolla and then walked down to the floral nectaries located at the base of the stamen (male flowers) or pistil (female flowers) with only minimal contact with anthers or stigmas. Whereas female squash bees usually landed on either the fused anthers or the stigma and accessed nectaries, or collected pollen when in male flowers, from this position. Accordingly, female squash bees spent significantly longer in contact with anthers and stigmas on a per visit basis compared to the duration of contact observed for honey bees (Table 3.1).

Higher per visit pollen deposition observed for female squash bees compared to honey bees translated into a greater likelihood of fruit set and, higher observed seed set for all comparisons, though some did not reach statistical significance (Figs. 3.4-3.5). For *C. foetidissima*, the likelihood of fruit set fruit was higher for single visits by female *E. strenua* than it was for honey bees (generalized linear mixed model: $z = 2.606$, $P < 0.001$), but the comparison between female *E. pruinosa* and honey bees was not quite statistically significant (generalized linear mixed model: $z = 1.89$, $P = 0.058$) (Fig. 3.4A). For *C. pepo*, the likelihood of fruit set fruit was higher for single visits by female *E. pruinosa* than it was for honey bees (generalized linear mixed model: $z = 3.75$, $P < 0.0001$) (Fig. 3.4B). In addition, single visits by female *E. strenua* resulted in higher seed set compared to single visits by honey bees (linear mixed effects model: $t_{13.1} = 2.31$, $P < 0.05$), but the comparison between *E. pruinosa* and honey bees was not statistically significant (linear mixed effects model: $t_{12.17} = 1.55$, $P > 0.05$) (Fig. 3.5A). For *C. pepo*, the comparison of seed set resulting from single-visits by female *E. pruinosa* and honey bees was not statistically significant (Welch's two-sample *t*-test: $t_{2.58} = 2.70$, $P = 0.07$) (Fig. 3.5B).

Fruit set and seed set of open-pollinated flowers

For both species of *Cucurbita*, the observed fruit set and seed set of flowers open to all pollinators were as high or higher than that observed in single-visit trials (Figs. 3.4-3.5). For buffalo gourd, fruit set of open flowers (Fig. 3.4A) did not differ from that of flowers visited once by female *E. strenua* (generalized linear mixed model: $z = 0.20$, $P = 0.84$) but was higher compared to fruit set of flowers visited once by female *E. pruinosa* ($z = -3.43$, $P < 0.001$) and by honey bees ($z = -4.11$, $P < 0.0001$). Likewise, seed set of fruit produced by buffalo gourd flowers left open to all pollinators (Fig. 3.5A) did not differ from that observed for flowers visited once by female *E. strenua* (linear mixed model: $t_{57.89} = -1.181$, $P = 0.24$) but was higher compared to seed set of fruit produced by flowers visited once by female *E. pruinosa* ($t_{56.13} = -2.83$, $P = 0.0065$) and by honey bees ($t_{49.55} = -3.87$, $P = 0.00032$). For cultivated squash, fruit set of open flowers (Fig. 3.4B) was higher than that of fruit set of flowers visited once by female *E. pruinosa* (generalized linear mixed model: $z = 3.43$, $P < 0.001$) or honey bees ($z = 6.20$, $P < 0.0001$). Seed set of fruit produced by squash flowers left open to all pollinators (Fig. 3.5B) did not differ from that observed on flowers visited once by female *E. pruinosa* (linear mixed model: $t_{58} = 0.35$, $P =$

0.73) but was higher compared to the seed set of fruit produced by flowers visited once by honey bees ($t_{58} = -3.15$, $P = 0.0026$).

Pollinator importance

After accounting for the relative frequencies of visits by honey bees and female squash bees, female squash bees were responsible for the majority of seed production for both species of *Cucurbita*. On *C. foetidissima*, female squash bees were responsible for 33.3% (*E. pruinosa*) and 42.0% (*E. strenua*) of seed production compared to 24.6% for honey bees. On *C. pepo*, female *E. pruinosa* were responsible for 61.8% of seed production compared to 38.2% for honey bees.

Multiple-visit trials

Table 3.2 summarizes best-fit regression models for the multiple-visit trials. No model for seed set is included because none of the multiple regression models for this response variable were significant. For the other three response variables, the linear term for female *E. pruinosa* visitation was significant, whereas only the linear term for fruit set was significant for *A. mellifera*. Moreover, coefficients for pollen removal ($F_{1,96} = 15.18$, $P < 0.001$) and deposition ($F_{1,37} = 6.47$, $P < 0.05$) were significantly higher for female *E. pruinosa* compared to honey bees. The multiple-visit trials further revealed no significant interactions between how honey bee and squash bee visitation affected the variables listed in Table 3.2. The only significant quadratic term in these analyses was a negative term for the relationship between *E. pruinosa* visitation and pollen removal. In this analysis, pollen removal reached a plateau after only a few *E. pruinosa* visits.

DISCUSSION

For both cultivated squash (*Cucurbita pepo*) and wild buffalo gourd (*C. foetidissima*), female squash bees removed more pollen from the anthers of male flowers and deposited more pollen on the stigmas of female flowers per visit than honey bees (Fig. 3.3). Multiple-visit trials on *C. pepo* revealed that these findings were not an artifact of single-visit trials but were more generally evident when honey bees and squash bees simultaneously visit flowers (Table 3.2). Observed disparities in pollen removal and deposition reflect interspecific behavioral differences (Tepedino 1981): female squash bees spent more time per visit in contact with stigmas and anthers than did honey bees when visiting *Cucurbita* flowers (Table 3.1). Interspecific differences in pollen removal and pollen deposition translated into higher per visit likelihoods of fruit set for female flowers of both *Cucurbita* species (Fig. 3.4), though this difference was not apparent in multiple visit trials on *C. pepo* (Table 3.2; coefficients for honey bees and female *E. pruinosa* are not different from one another). Given the low likelihood of fruit set for flowers visited once by honey bees, seed set comparisons were limited by small sample sizes. Nonetheless, these comparisons followed the observed trend of female squash bees out performing honey bees, at least on a per visit basis (Fig. 3.5). Even though honey bees were numerically dominant as floral visitors of *Cucurbita* (Fig. 3.1), their estimated importance in contributing to squash and gourd pollination appears lower when compared to female squash bees.

Based on our single-visit estimates of pollen removal and pollen deposition, female squash bees can be considered high-removal, high-deposition pollinators (Williams & Thomson 2003) compared to low-removal, low-deposition honey bees. During single visits, female *E. pruinosa* remained in contact with anthers for an order of magnitude longer than honey bees

(Table 3.1). Moreover, single visits by female *E. pruinosa* resulted in the removal of more than half of the pollen present in male flowers, on average, for both species of *Cucurbita* (Fig. 3.3), whereas mean values for honey bees were just above zero (Fig. 3.3). Moreover, the significant negative quadratic term for visitation by female *E. pruinosa* in the multiple-visit data set (Table 3.2) illustrates that only a few visits were likely adequate to deplete anthers of pollen. Per visit disparities between honey bees and female squash bees in terms of pollen deposition were also large and consistent across species of *Cucurbita*. Pollen removal and deposition data suggest that female *E. strenua* may be a superior pollinator compared to female *E. pruinosa*; both species deposited similar amounts of pollen but female *E. strenua* removed only about half as much on a per visit basis.

Previous studies on *C. pepo* conducted in agricultural settings (Tepedino 1981, Artz & Nault 2011, Garibaldi et al. 2014) report that honey bees and squash bees are more similar to one another than what we report here in terms of per visit pollen deposition and likelihood of fruit set (Tepedino 1981, Artz & Nault 2011). However, these studies pooled male and female squash bees in their single-visit trials. When examined separately, male squash bees remove less pollen, deposit less pollen, and contribute less to fruit set per visit compared to female squash bees (Cane et al. 2010, Canto-Aguilar et al. 2010, Delgado-Carrillo et al. 2018; Appendix 3). Moreover, at least in temperate areas, the seasonal peak of male squash bee activity occurs earlier in the season compared to the peak in female activity (Tepedino 1981). Thus, studies conducted early in the season that combine male and female squash bees will be disproportionately influenced by male bee behavior compared to studies conducted later in the season. Although Canto-Aguilar et al. (2010) and Delgado-Carrillo et al. (2018) worked with different species of cultivated squash and squash bees than those considered here, their findings are similar to ours with respect to differences between single visits of honey bees and female squash bees in pollen removal, pollen deposition, and fruit set.

In terms of the patterns of significance evident in single-visit comparisons between honey bees and squash bees, the congruence of the results for *C. pepo* and *C. foetidissima* seems somewhat surprising. Buffalo gourd plants were patchily distributed over a large area as is typical of this species (Kohn 1989), whereas cultivated squash were clumped together in one location. As specialists on *Cucurbita*, the behavior of female squash bees (e.g., pollen collection) may be somewhat inflexible with respect to the spatial distribution of floral resources. In the present study, one way in which patterns of bee visitation did conspicuously differ between the *C. pepo* and *C. foetidissima* systems was the higher relative abundance of honey bees (Figs. 3.1-3.2). This disparity may be due to the presence of managed honey bee colonies present at the *C. pepo* site, or it may have been a behavioral response by honey bees to the existence of a clumped patch of rich nectar resources. Honey bees, for example, often preferentially forage on floral resources as a function of local abundance (Hung et al. 2019).

Although the relative abundance of honey bees was higher at the *C. pepo* study site than at the *C. foetidissima* study site, *Apis mellifera* was numerically dominant at both sites (Fig. 3.1). These levels of relative abundance, however, were insufficient to tip pollinator importance estimates in favor of honey bees because female squash bees contributed so much more to pollination and reproduction on a per visit basis compared to *Apis*. Low transfer of pollen to stigmas by honey bees may be due to the fact that they were only occasionally seen foraging for cucurbit pollen in our study. Instead, foraging was directed towards collection of nectar and contact with anthers and stigmas much reduced in comparison to female squash bees who require both cucurbit pollen and nectar for reproduction. At least on *C. pepo*, however, we did occasionally observe honey bees purposefully collecting pollen.

Pollinator importance values suggest that squash bees contributed more to the reproduction of *C. pepo* and *C. foetidissima* than did the much more abundant honey bees. A focus on fruit set and seed set may tell only part of the story, however, in that these metrics ignore potential differences in pollen quality and how this variable may affect offspring fitness. Honey bees, for example, often differ from other pollinators with respect to their greater tendency to visit multiple flowers on the same plant (Goulson 2010, Garibaldi et al. 2014). If that is the case in the *Cucurbita* systems examined here, the consequences of geitonogamy resulting from honey bee pollination could negatively affect offspring performance. Both *C. foetidissima* and the wild form of *C. pepo* (ssp. *texana*) exhibit negative fitness effects from inbreeding (Kohn 1988, Stephenson et al. 2001, 2004, Hayes et al. 2005). Potential problems associated with geitonogamy could be further exacerbated by honey bee visitation for two reasons. First, honey bees often prefer female *Cucurbita* flowers over male flowers (Tepidino 1981; see also Fig. 3.1) and would thus likely transport pollen from a smaller number of male flowers compared to the number of male flowers visited by female squash bees. Second, honey bees deposit less pollen per visit compared to that deposited by female squash bees, and increasing pollen loads positively affect seed set and offspring vigor (through pollen competition) in both *C. pepo* (Winsor et al. 1987) and *C. foetidissima* (Winsor et al. 2000). Future studies should thus focus on comparing the vigor of *Cucurbita* seeds resulting from pollination by honey bees versus squash bees as a complement to comparisons of pollinator importance.

The results of the present study provide additional evidence that honey bees, even when relatively abundant, can contribute little to pollination services in both managed and non-managed systems. In the absence of native pollinators, honey bees can provide essential pollination services in some cases (Dick 2001, Hanna et al. 2013). In the presence of native pollinators, however, contributions to pollination services may be more limited, though perhaps important in years when native pollinators are rare or by complementing the actions of native pollinators (Hoehn et al. 2008). Although we did not address interspecific competition in the present study, the numerical dominance of honey bees on both species of *Cucurbita* involves the removal of nectar and pollen that would likely otherwise be exploited by squash bees. In regions where honey bees, especially feral populations, are abundant, the exploitation of floral resources by *Apis* (Cane and Tepedino 2007) may compound the effects of other factors negatively affecting native pollinators.

Our findings demonstrate that, even when less common than honey bees, as specialist pollinators squash bees are indeed generally more effective than generalist honey bees. Yet the squash bee’s effectiveness as a specialist to cucurbits may depend on which generalist species are also present in the system. For example, Artz and Nault (2011) compared pollinator performance between squash bees (*Eucera pruinosa*) and two generalists, honey bees and the common eastern bumble bee (*Bombus impatiens*). They found that bumble bee visits led to higher pollen deposition, fruit set, and seed weight compared to either honey bees or squash bees (Artz & Nault 2011). Their study, however, did not include *Eucera strenua* bees, which we found to be the superior specialist compared to *E. pruinosa* due to its medium-removal and high-deposition of pollen, at least in *C. foetidissima*. Future studies should aim to compare *E. pruinosa* and *E. strenua* to both honey bee and bumble bee generalists in the native range of *Cucurbita* in order to evaluate pollinator effectiveness of all species.

Compared to previous studies in *Cucurbita*, the results of this study demonstrate that squash bees appear to be more important pollinators than honey bees in squash and gourd systems. Squash bees collected and deposited more pollen per flower visit, which led to increased per-visit fruit and seed set. When taking honey bee abundance into account, squash bees were still responsible for the majority of seed production in both *C. foetidissima* and *C. pepo*. An assessment of multiple visitation by both honey bees and squash bees to flowers corroborated these findings. Therefore, the value of native, specialist squash bees in providing pollination services in both managed and unmanaged systems should not be overlooked in favor of non-native, super-generalist honey bees.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Gambel, Jess; Martinez, Maria; Davids, Jessica; Eastwood, Kellen; Warner, Jeremy; Kohn, Joshua; Holway, David A. The dissertation author was the primary investigator and author of this material.

TABLES

Table 3.1. Honey bees (*Apis mellifera*) and female squash bees (*Eucera pruinosa*, *Eucera strenua*) compared with respect to mean (± SE) per visit duration of contact with anthers and stigmas on flowers of (A) *Cucurbita foetidissima* and (B) *Cucurbita pepo*. See Methods for statistical tests used in each comparison. *P < 0.05, **P < 0.001, ***P < 0.0001

(A) *Cucurbita foetidissima*	*A. mellifera*	*E. pruinosa*	*E. strenua*
length of stigmatic contact (s)	16.47 ± 5.93	64.82 ± 23.72	67.20 ± 29.71
sample size	n = 19 flowers	n = 11 flowers	n = 5 flowers
statistical comparisons	*A.m. vs E.p.* ($t_{28.5}$ = 3.08**)		*A.m. vs E.s.* ($t_{29.4}$ = 3.43**)
length of anther contact (s)	11.55 ± 5.74	147.72 ± 24.62	70.44 ± 14.18
sample size	n = 11 flowers	n = 36 flowers	n = 15 flowers
statistical comparisons	*A.m. vs E.p.* (t_{60} = 9.18***)		*A.m. vs E.s.* (t_{60} = 6.47***)
(B) *Cucurbita pepo*	*A. mellifera*	*E. pruinosa*	
length of stigmatic contact (s)	11.41 ± 2.88	102.85 ± 10.92	
sample size	n = 19 flowers	n = 20 flowers	
statistical comparison	*A.m. vs E.p.* (t_{90} = 10.26***)		
length of anther contact (s)	2.81 ± 0.95	84.75 ± 15.29	
sample size	n = 16 flowers	n = 20 flowers	
statistical comparison	*A.m. vs E.p.* ($t_{32.29}$ 10.25***)		

Table 3.2. Best-fit regression models for three measures of pollinator performance as a function of multiple visits by honey bees (*Apis mellifera*) and squash bees (*Eucera pruinosa*) on male (i) and female (ii-iii) flowers of cultivated squash (*Cucurbita pepo*). $^{*}P < 0.05$, $^{**} P < 0.001$, $^{***} P < 0.0001$

Measure / predictor variables	Model / parameter estimates
(i) estimated pollen removed	$F_{5,96} = 8.94^{***}$, $R^2 = 0.28$
Number of *Apis* visits	0.087 ± 0.068, $t = 1.29$
(Number of *Apis* visits)^2	-0.0012 ± 0.0084, $t = -0.138$
Number of *Eucera* visits	0.39 ± 0.078, $t = 5.023$ ***
(Number of *Eucera* visits)^2	-0.038 ± 0.0093, $t = -4.057$***
No. of *Apis* visits X No. of *Eucera* visits	-0.36 ± 0.019, $t = -1.85$
(ii) pollen deposition	$F_{3,36} = 5.1^{*}$, $R^2 = 0.24$
Number of *Apis* visits	-0.10 ± 0.077, $t = -1.30$
(Number of *Apis* visits)^2	0.027 ± 0.013, $t = 1.99$
Number of *Eucera* visits	0.055 ± 0.018, $t = 3.05$***
(iii) fruit set	$\chi^2_{100} = 30.40^{***}$
Number of *Apis* visits	0.29 ± 0.13, $z = 2.22$*
Number of *Eucera* visits	0.45 ± 0.12, $z = 3.75$***

FIGURES

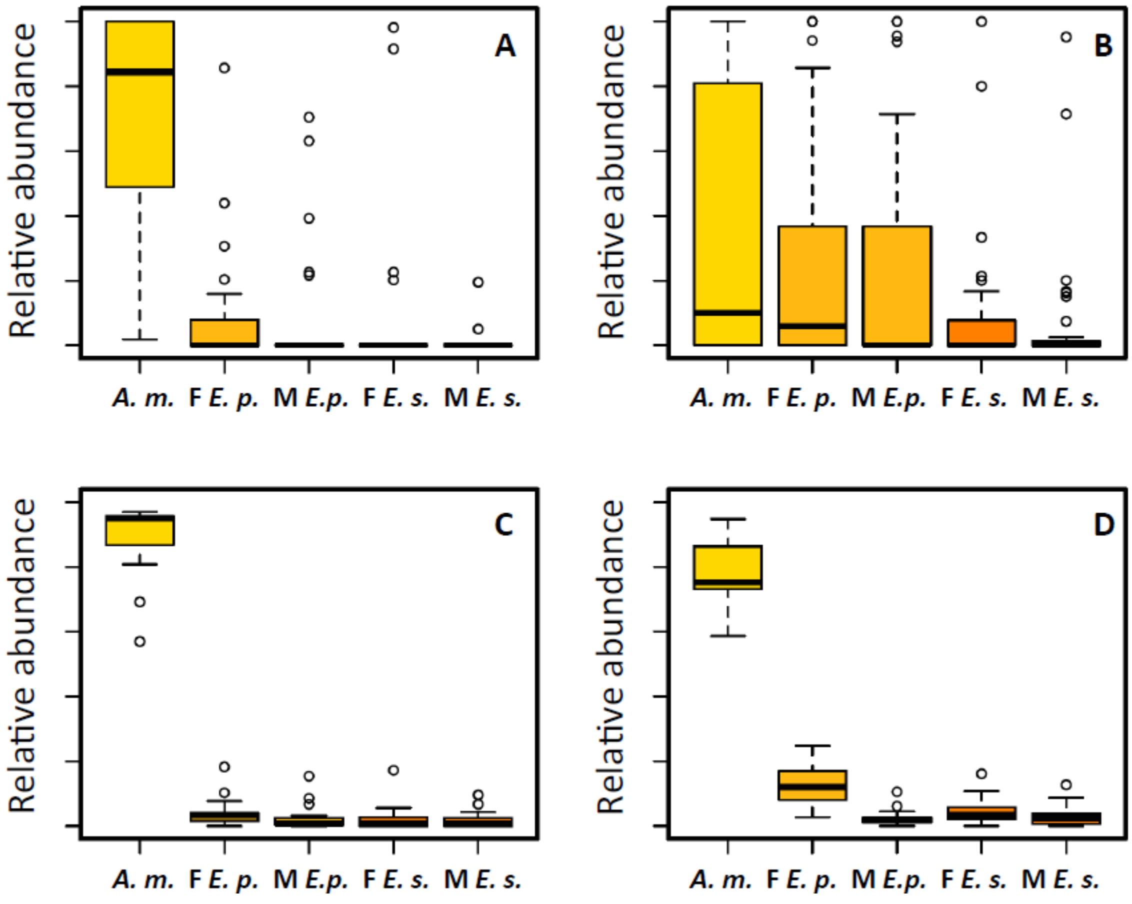

Figure 3.1. Relative abundance of honey bees (*Apis mellifera* (*A. m.*)) and female (F) and male (M) squash bees (*Eucera peponapis* (*E. p.*), *E. strenua* (*E. s.*)) on **(A)** female flowers (n = 23 plants) and **(B)** male flowers (n = 32 plants) of *Curcurbita foetidissima*, and on **(C)** female flowers (n = 15 plants) and **(D)** male flowers (n = 18 plants) of *C. pepo*. Boxes show central 50% of data and median; whiskers show quartiles ± 1.5 x interquartile range, or most extreme values of data, whichever is closest to median. Points indicate extreme values.

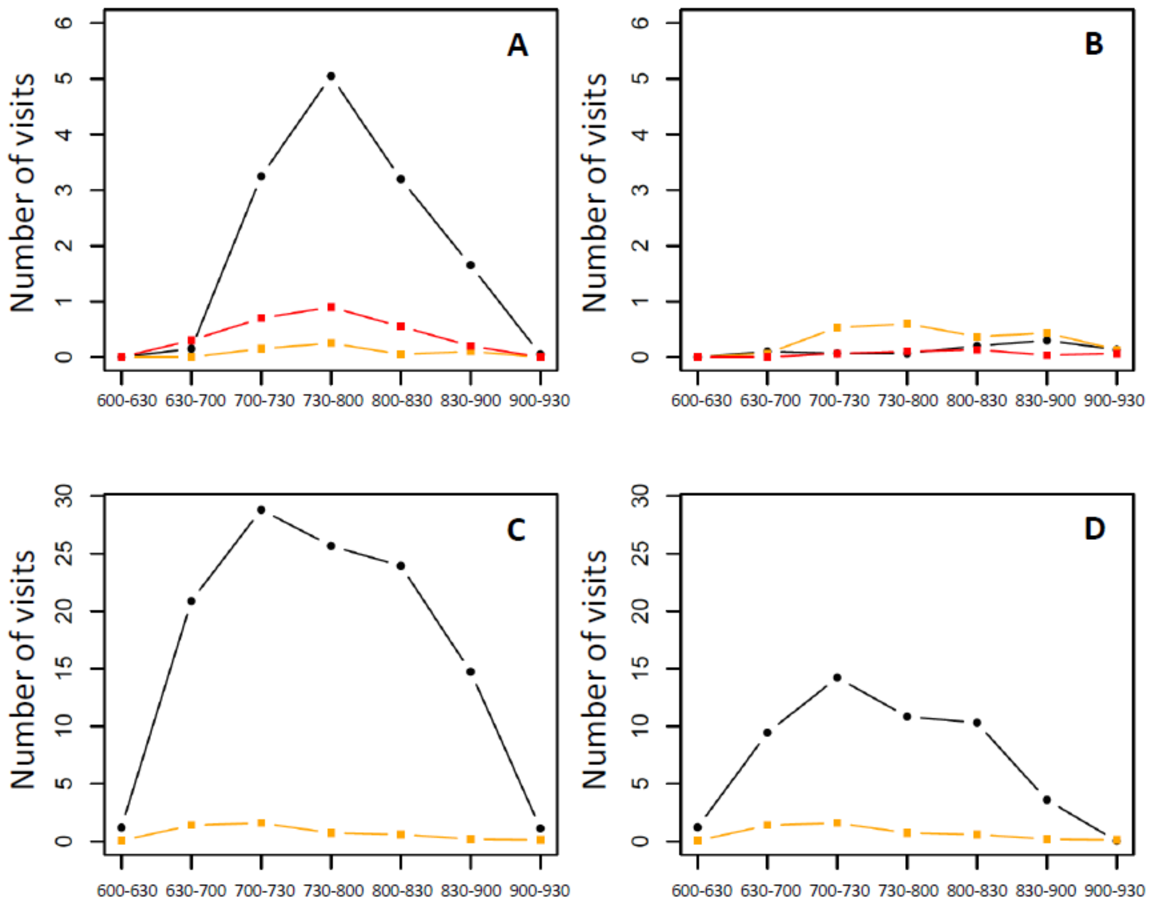

Figure 3.2. Mean numbers of visits in 30-min time intervals (from 0600-0930) by honey bees (*Apis mellifera* (*A. m.*) = black lines) and squash bees (female *Eucera peponapis* = orange lines, female *E. strenua* = red lines) on **(A)** female and **(B)** male flowers of *Curcurbita foetidissima*, and **(C)** female and **(D)** male flowers of *C. pepo*. Sample sizes same as in Fig. 3.1.

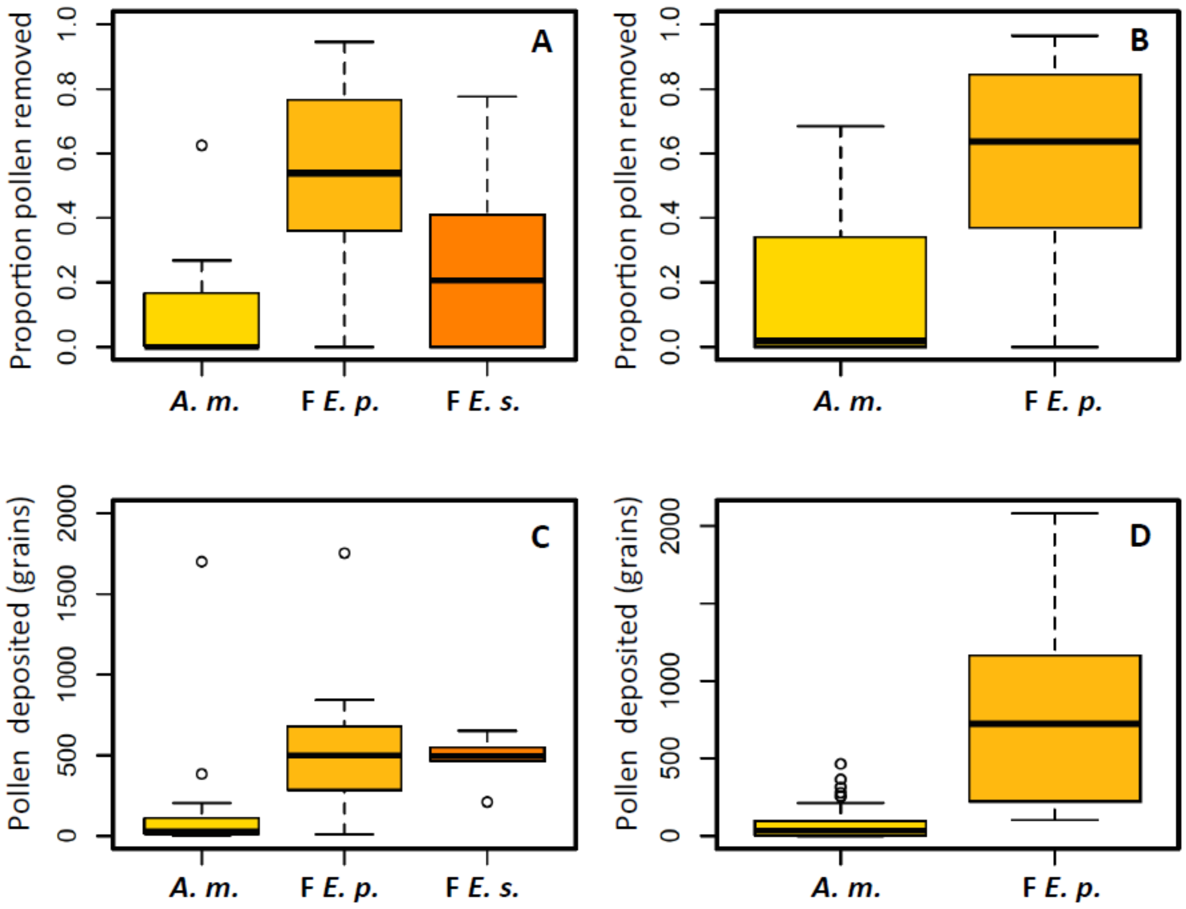

Figure 3.3. (A) Estimated pollen removal (proportion of total) from anthers of male flowers of *Cucurbita foetidissima* for single visits by honey bees (*Apis mellifera* (*A. m.*), n = 11 flowers) and squash bees (female *Eucera peponapis* (F *E. p.*), n = 36 flowers; female *E. strenua* (F *E. s.*), n = 15 flowers). **(B)** Estimated pollen removal (proportion of total) from anthers of male flowers of *C. pepo* for single visits by honey bees (*A. m.*, n = 16 flowers) and squash bees (F *E. p.*, n = 20 flowers). **(C)** Pollen deposition on stigmas of female flowers of *C. foetidissima* for single visits by honey bees (*A. m.*, n = 19 flowers) and squash bees (F *E. p.*, n = 11 flowers, and F *E. s.*, n = 5 flowers). **(D)** Pollen deposition on stigmas of female flowers of *C. pepo* for single visits by honey bees (*A. m.*, n = 46 flowers) and squash bees (F *E. p.*, n = 18 flowers). Box plots set up as in Fig. 3.1.

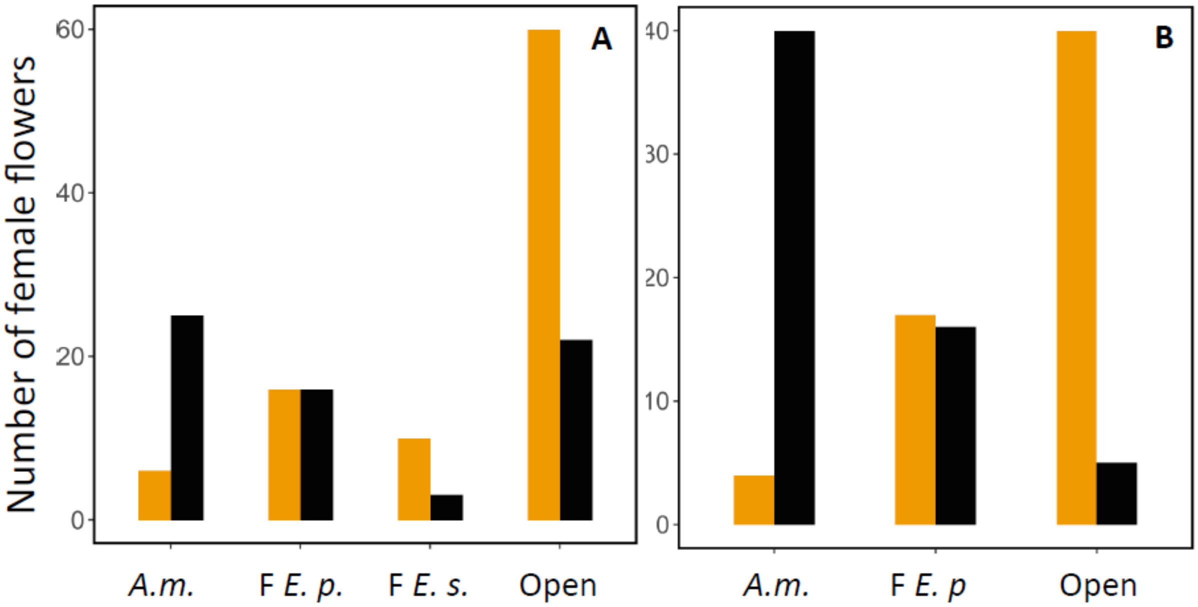

Figure 3.4. Fruit set resulting from single visits by honey bees (*Apis mellifera* = *A. m.*) and squash bees (female *Eucera peponapis* = F *E. p*. and female *E. strenua*; = F *E. s*.) on female flowers of **(A)** *Cucurbita foetidissima* and **(B)** *C. pepo*. Fruit set values for "Open" refer to flowers left open to all pollinators. Orange bars = set fruit, black bars = aborted fruit.

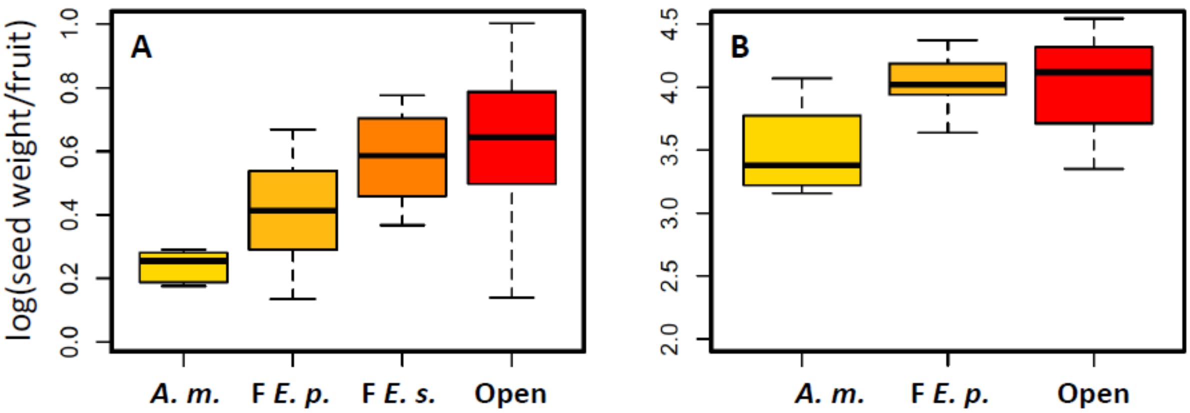

Figure 3.5. Seed set (total seed weight) per fruit resulting from single visits by honey bees (*Apis mellifera* = *A. m.*) and squash bees (female *Eucera peponapis* = F *E. p*. and female *E. strenua*; = F *E. s*.) on female flowers of **(A)** *Cucurbita foetidissima* and **(B)** *C. pepo*. Seed set values for "Open" refer to flowers left open to all pollinators. Box plots set up as in Fig. 3.1.

Appendix 3. Single-visit data for male squash bees

Cucurbita foetidissima
Pollen removal: analysis not performed
Pollen deposition: *Peponapis* F > *Peponapis* M > *Apis*
Seed set: *Peponapis* M ≈ *Peponapis* F > *Apis*
Fruit set: *Peponapis* F > *Peponapis* M ≈ *Apis*

Analyses were not performed for *Cucurbita pepo.*

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