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The Influence of Pollinator Behavior on Patterns of Pollen Dispersal and its Implications for Floral Evolution

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ABSTRACT

Despite a storied history of pollination research, little information exists on how the unique characteristics of individual pollinators effect patterns of pollen dispersal. As these patterns of pollen dispersal inform our understanding of floral evolution, filling in this knowledge gap is imperative. I attempted to do so by analyzing two different outcomes of pollen dispersal: **1**) pollen carryover, and **2**) geitonogamy (i.e., intra-plant movements). For the pollen carryover, I analyzed the results of an experimental study in which bats in flight cages removed pollen from the male flower of a *Burmeistera glabrata* plant, and then visited 10 female flowers of *B. glabrata*. The goal was to determine the proportions of pollen grains deposited by the bats on each female flower and how grooming in-between visits affected these proportions. In general, bat carryover-curves were similar to those of published work with bees, with the greatest proportion of pollen deposited on the first couple female flowers followed by a steep decline in the proportion reaching subsequent flowers. Grooming had a significant effect on the proportion of grains deposited. However, when the sexes were analyzed separately, significance only remained for female bats. Furthermore, I found females groom more than males. This suggests that increased grooming allows females to consume more pollen, an important way to increase nutrient uptake before and during pregnancy. Patterns of pollen dispersal may fluctuate annually based on the reproductive stage of female bats.

I also analyzed results from a Missouri insect visitation study of *Asclepias* species to measure the potential for geitonogamous movements. Overall, *Bombus sp.* and *Apis mellifera*, were the most abundant visitors, with Halictidae sp., dominating at *A.tuberosa*. Visitors were more likely to move within the plant than to leave, increasing the possibility for geitonogamous pollen transfer. I discussed results within the context of how the unique traits and behaviors of each *Bombus*, *Apis*, and Halictidae may affect the rates of geitonogamy.

Findings from both studies highlight the need for caution in generalizing patterns of pollen dispersal between pollination systems. Continued studies into pollinator behaviors will better inform our understanding of floral evolution.

I. GENERAL INTRODUCTION

The final destinations of pollen grains significantly influence plant fitness¹⁻³. For animal-pollinated plants, the shape of the distribution of these pollen destinations are strongly impacted by pollinator behavior^{1,4,5}. It should therefore come as no surprise that pollination biologists have long been interested in how the critical link between pollinator behavior and pollen movement impacts plant fitness and consequently drives floral evolution. My two-part study is grounded in such interest.

More specifically, this study helps to advance knowledge on how the link between pollinator behavior and pollen movement influences **1)** pollen carry-over curves, and **2)** geitonogamy, or the transfer of pollen between flowers of the same plant. I first study how grooming by bats affects the pollen distribution on a series of flowers, thereby measuring the effect this behavior has on male plant fitness. Second, I analyze the movement patterns on visitors to numerous *Asclepias* species and how such patterns can mediate rates of geitonogamy, and by extension, both male and female plant fitness. Results have implications for how pollinators can drive floral adaptation.

My thesis is organized into three chapters. In the body of this introductory chapter, I provide background about what we know about pollen grain distribution and pollinator behaviors necessary to put the two studies in context. The second chapter covers the study of bat grooming and its impact of pollen carryover. The third chapter covers analysis of a long-term observation study of animal visitors to *Asclepias* species

and the findings' implications for geitonogamy. Finally, I conclude with broader implications and recommendations for further research.

Patterns of Pollen Dispersal and Their Effects on Male Function Fitness

Arguably the most direct consequence of pollen dispersal is its effect on male function in plants (male fitness). Male fitness is a relative quantification; a male is more fit if its genes are passed on at higher rate than other males. Put differently, males who sire more viable individuals relative to other males within the same population have a higher fitness^{6,7}.

The implications for pollen grain fates on male fitness are therefore relatively straightforward. Each pollen grain can be given a probability for its likelihood to sire offspring based on the grain's destination. A grain landing on the stigma of a compatible female is clearly more likely to sire offspring than one that does not¹. Each grain's probability score is summed to produce the probable number of offspring a given male will sire. This number can then be compared to a theoretical optimum number for a given male, under its given ecological and population constraints, could sire. The figure calculated is invariably less than the optimum number, and pollen grains which contribute to this difference are referred to as "wasted".⁸ As such, the number of grains wasted by a given male, relative to those wasted by all other males, is a first indication of that male's fitness.

For grains failing to reach a stigma, the cost to male fitness is obvious as these grains have no chance of contributing to the next generation¹. Even if the grain reaches a stigma, the contribution to the next generation is situationally dependent. For instance, to

self-incompatible plants, as opposed to self-compatible plants, fertilization of a plant's ovules by its own pollen is not possible.^{6,7,9} Alternatively, for self-compatible plants, the number of viable offspring pollen grains can sire when transferred within-plant (geitonogamy) or within-flower (autogamy) varies depending on the severity of inbreeding depression^{2,10}. Therefore, the cost a geitonogamously, or autogamously-transferred grain incurs to male fitness is usually species specific.

Even grains reaching compatible females are not guaranteed to fertilize ovules. The timing of a grain's arrival is non-trivial. First, a grain deposited before a female is receptive, or after all ovules are fertilized, will likely not contribute at all to siring^{11,12}. Second, pollen deposited before the grain's arrival may block the pathway for that grain to reach an ovule^{3,13,14}. This is even true if the early-deposited grains are incapable of fertilizing the female¹⁵. Third, the size of a male's pollen deposition on a given stigma can readily reach diminishing returns¹, given that flowers have an upper limit on the number of grains needed to ensure complete female fertilization^{1,16-18}. This number is often species-specific, depending on the number of ovules and female choice, but this implies that after some number, each subsequent pollen grain deposited is less likely to contribute to the next generation¹. After hitting this level of diminishing returns, the grains are essentially better off, from the male's point of view, being deposited on another stigma. Hence, excess grains can likewise be thought of as wasted.

It therefore becomes clear the role pollinators play in determining the probability score of a pollen grain successfully siring a seed. Any behavior or physiology limiting the ability of a pollinator to transfer a grain to the grain's optimal destination (i.e., the location which maximizes the grain's probability of siring offspring) contributes to

wasting. The magnitude of the cost that pollinator wasting has on male fitness will likewise determine the strength of selection on male function for floral adaptations aimed to minimize pollination inefficiency^{1,8,19}.

How Pollinator Behavior Effects Patterns of Pollen Distribution

In the most straightforward case of pollen wasting, a pollinator may fail to deliver grains to a stigma. This may happen for a myriad of reasons. First, pollinators may fail to pick up all the grains from a male. However, studies show that often pollinators only rarely pick up less than all pollen grains an anther presents^{20,21}. Second, pollen may be lost from pollinators' bodies. Pollen may be shaken off when the pollinator makes contact with objects in the environment. Similarly, certain pollinators are known to groom excessively, shedding and even consuming pollen^{5,8,22}. Corbiculate bees have adapted specialized baskets in which to groom pollen¹. This pollen is a major protein component for larvae^{23,24}. Likewise, studies on non-volant mammals show excessive grooming, with high pollen loads in feces⁵.

Pollinators can also influence pollen dispersal patterns due to their tendencies to visit many plant species²⁵⁻²⁷. Even so-called specialist pollinators may be capable of receiving and depositing pollen from a wide array of plant species^{25,26}. As such, there is a strong potential that unfaithful pollinators may transfer pollen to a heterospecific stigma. Again, these pollen grains cannot contribute to the next generation, thus decreasing male

fitness. Likewise, in visiting numerous flowers, the order in which pollen is placed onto the pollinator is particularly important^{8,13}. If a male deposits its pollen as the first layer onto a pollinator, all subsequent layers from other plants may (if placed on the same location) bury the first layer^{6,8}. Buried pollen may be inaccessible to a receptive female unless the layers on top are first removed¹³. Furthermore, in relative male fitness terms, a conspecific covering pollen layer would be more costly than a heterospecific covering layer.

Collectively, the nuances of pollinator behaviors thereby can strongly impart fitness costs on male function in plants. It should follow that this drives selection for adaptations to optimize pollen transfer and minimize wasting. Indeed, with the increased attention to the differences in male and female functions, studies are finding strong selection through male function for certain floral adaptations^{1,17,19}. These adaptations seek to either modify pollinator behavior or minimize inefficiencies.

Plant Adaptations and Strategies for Maximizing Male Fitness

Certain floral adaptations evolve to exploit characteristics of the pollinator in ways that modify the pollinators' behaviors. First, a male flower that could attract pollinators at a far greater rate than other conspecific males would hold a significant competitive advantage²⁸. Indeed, floral display and rewards have long been known to influence attraction²⁸. For example, pollinators were more attracted to, and visited more often, yellow variants of *Raphanus raphanistrum* than white variants^{28,29}. Both variants had similar female function due to limiting resources depressing the number of seeds a plant can produce. However, yellow variants were far more likely to sire offspring than

males, indicating male fitness conferred selection on the pigmentation. Similarly, rewards such as nectar encourage pollinators to return, and therefore variation in the quality and quantity of the nectar can lead to variation in male competitive ability³⁰.

However, sometimes there are pollinator inefficiencies not surmountable by behavior modifications alone. Plant species infidelity and geitonogamy both appear more prevalent than previously realized^{10,31,32}. Grooming and pollen consuming behaviors also appear rather inflexible^{4,5,22}. Instead, flowers can evolve means to minimize the cost of these pollinator inefficiencies. For instance, floral structures of certain *Palicourea* species scrape heterospecific pollen off from the incoming pollinator.³³

The timing and quantity of pollen production can also be augmented to adjust for pollinator behavior. Pollen presentation theory argues that for many pollinators, the ratio of pollen grains received to deposited (RD ratio) increases with a growing pollen load¹. For instance, the higher the pollen load on bees, the more vigorously the bees groom, leading to a greater proportion of wasted grains^{1,22}. Likewise, hummingbirds also appear to shed pollen grains beyond a certain load, implying a limit to the number of grains they can effectively transfer⁸. The result is that flowers utilizing pollinators high RD ratios have more quickly saturating male fitness curves²⁷. Selection should therefore favor these males to release pollen in smaller, discrete loads, parceled out over time²⁷. Conversely, pollinators who have smaller RD ratios should drive selection for plants to release more pollen in each dose. However, visitation frequency of the pollinator may further influence selection. For example, because hummingbirds are often low visitors, plants pollinated by hummingbirds may be better served releasing pollen in greater

loads²⁷. There should, therefore, exist an optimum number of grains a male releases at any one time, dependent on its pollinator's visitation rate and RD ratio.

The causal link from pollinator behavior to patterns of pollen distribution and finally to effects on male fitness is evident in the literature. Unfortunately, experimental, and observational studies of how particular behaviors in different pollinator species contribute to this link are scarcer. My study aims to bridge that gap.

Fitness Costs of Geitonogamy

Patterns of pollen dispersal also dictate rate of self-fertilization and inbreeding. In the case of geitonogamy (movement of pollen between flowers within the same plant), such consequences are significant for plant fitness and population genetics. Why does geitonogamy pose potential fitness costs to plants? For self-incompatible plants, the costs are relatively obvious. Male function suffers because pollen that could sire seeds on other plants is instead wasted by remaining on the parent plant^{10,32}. Even when they do not lead to geitonogamy, within-plant pollinator movements can still lead to wasted pollen. Certain floral parts³³ also are known to remove pollen from competitors and could thus do so incidentally to pollen from a previously visited anther within the same plant. Alternatively, certain pollinators become increasingly less efficient (i.e., they exhibit a decreasing proportion of received grains that are deposited on compatible females) as pollen loads increase¹. For example, bees are notorious groomers and pollen consumers, but are especially stimulated at heavy pollen loads¹. These pollinators, by

visiting multiple males on the same plant before departing, may carry a heavy enough load that inefficiency poses costs to male fitness.

Female function of self-incompatible plants similarly suffers costs of geitonogamy. Pollen deposited on females from males of the same plant does not fertilize any ovules^{10,34}. Fewer ovules fertilized leads to reduced seed set, and by extension fewer potential offspring³⁵. This problem is further exacerbated when geitonogamous pollen causes abortion of the flower, preventing any further out-cross fertilization^{10,31}. Alternatively, pollen from the same plant may clog the stigmas, physically preventing compatible pollen from reaching the ovules³².

A further cost of geitonogamous movements to both females and males is that of nectar depletion. Nectar can be expensive for the flowers to produce and losing potentially large quantities could strain plant resources². Similarly, if nectar depletion deters would-be “good” pollinators (e.g. those bringing out-crossed conspecific pollen), both male and female functions would suffer due to lower effective visitation³⁶.

In self-compatible plants, male and female functions also suffer but to varying degrees. In this case, males can potentially sire offspring with females from the same plant^{10,32}. The fitness of the offspring, however, is largely dependent on the species evolutionary history and the severity of inbreeding depression. For males, if out-crossing leads to better, or more, offspring, geitonogamy would still reduce fitness³⁷. Likewise, geitonogamy can lead to a depressed seed or fruit set, and thus females may suffer costs of fertilization by males on the same plant³⁵. Pollen from males on the same plant may also arrive faster than out-crossed pollen (by virtue of proximity), preventing the latter from reaching the ovules in time.

Prevalence of Geitonogamy

Despite the potential costs, geitonogamy is quite prevalent in hermaphroditic plants. For example, a study on scarlet gilia (*Ipomopsis aggregata*) indicated that geitonogamy accounted for up to 50% of seed set³⁵. Pollen retention (not leaving the plant because of geitonogamous movements) was 50% in cranesbill (*Geranium caespitosum*) plants with few flowers but increased up to 95% for plants with about 100 flowers³⁸. In nectar producing orchids, geitonogamous movements of pollinaria ranged from 22% to over 50% of all movements². In *Asclepias* species, geitonogamy rates are extremely high. It was estimated that for large clonal populations of *A.syrriaca*, up to 97% of pollinations are from selfing (combination of geitonogamy and autogamy), and 37% of inserted pollinia came from within the umbel³⁹. In *Asclepias* species, geitonogamy commonly results in reduced seed set and fruit abortion^{31,39}.

Benefit of Geitonogamous Movements from a Pollinator's Perspective

From the pollinator's perspective, geitonogamous movements make sense. Foraging can be an expensive gamble as the demands of seeking new flowers are resource intensive, and the potential exists that the pollinator will either fail to find new plants or come into conflict with other pollinators⁴⁰. Indeed, most pollinators, especially vertebrates, have little margin-of-error when it comes to energy replacement^{40,41}. Therefore, it is beneficial for the pollinator to not leave a plant before the maximum number of resources is extracted. Yet it is in the best interest of the plant to have the pollinator leave soon after receiving pollen so as to minimize the chance of geitonogamy^{2,41,42}. This sets up a conflict of interest between plant and pollinator.

The reluctance for pollinator departure is further exacerbated by the paradox of attractiveness^{42,43}. Showy floral displays and high-quality rewards are known to attract, and keep the attention of, pollinators⁴². Additionally, clonal growth in which large numbers of stem are located in close proximity can also promote pollinator attraction and retention⁴⁴. This is especially advantageous for plants in strong competition from co-occurring plant species for pollinators. Floral attraction therefore increases pollinator visits, but also incentivizes pollinator retention, a behavior which increases the potential for geitonogamy.

Plant Adaptations and Strategies to Minimize the Cost of Geitonogamy

For the above reasons, geitonogamy is an important selective pressure on floral and life-history traits for numerous angiosperm species. For instance, it has been posited that the evolution of dioecy may be a consequence of the high cost of geitonogamous movements for self-incompatible flowers¹⁰. Pollen landing on stigmas within the plant contribute nothing to the next generation and therefore separation of male and female flowers would potentially minimize said cost. However, pollen may still be moved within the male plant, resulting in wasting.

Dichogamy, or sequential hermaphroditism, also has been implicated in minimizing geitonogamy. Sequential hermaphroditism within the same flower clearly prevents selfing^{10,45}. However, in order to minimize geitonogamy, all the flowers in the entire plant, or inflorescence, would need to synchronize male and female phases^{10,46}. A few studies report at least partial degrees of synchronization. For example, 87% of flowers in a given umbel of *Butomus umbellatus* were found to be unisex⁴⁷. Similar studies report flowers on the same inflorescences, (particularly umbel inflorescences),

likewise synchronizing between male and female phases⁴⁸⁻⁵⁰. Complete synchronization across the whole plant is much rarer but may not be necessary if pollinators minimize distances traveled within the same plant⁴⁷. Furthermore, complete synchronization may limit mating opportunities, incurring costs greater than the benefit of minimizing geitonogamy¹⁰. Therefore, even if pollinators travel between inflorescences on the same plant, synchronicity within a given inflorescence help to minimize geitonogamy.

Another adaptation to limit geitonogamy is the physical separation of the male and female functions. With reciprocal heterostyly, flowers on one plant can have long styles and short anthers, and the patterns is reversed on another plant⁵¹. Insects that forage at certain heights would be more likely to move pollen from a given anther to its same-sized stigma, and therefore less likely to transfer pollen geitonogamously.

Plants with vertical racemes likewise have a unique adaptation for minimizing geitonogamy. In general, insects tend to move upwards, first visiting the lower flowers and finishing at the top of the inflorescence^{52,53}. These plants often have protandrous flowers in which those lower on the raceme develop first and those on the top, last. In this manner, an insect that forages vertically is likely to make contact with the older, and therefore female-phase, flowers on the bottom, dropping off out-crossed pollen, before removing pollen from the top male-phase flowers and departing. The nectar content is higher in sugar and nectar volume is greater in the older flowers, an adaptation for strengthening insect vertical orientation⁵³. This system elegantly reduces the probability that females will receive pollen from the males on the same flower while still maintaining both sexes simultaneously available on a given plant. Such an adaptation, however, is

not advantageous for plants whose pollinators (e.g. hummingbirds) do not display the vertical orientation behavior⁵⁴.

In certain circumstances however, geitonogamy may present a less-than-perfect solution to pollen limitation. Certain plant species have insufficient interactions with their pollinators^{1,55}. This is often true for plants occurring at low densities or whose pollinators are temporally ephemeral²⁵. In such cases pollen limitation can lead to reduced seed and fruit set³¹. Conversely, facultative selfers may increase rates of autogamy⁵⁶. Therefore, either total seed set is greatly limited or suffers heavy fitness costs through inbreeding⁵⁵. If geitonogamy can increase seed set but maintain cost of inbreeding smaller than that of autogamy's, selection may favor pollinator retention as geitonogamy over autogamy should be selected. However, if pollinator abundance is temporal, the benefit of geitonogamy is no longer straightforward. A year in which low pollinator abundance necessitates increases in geitonogamy and therein pollinator retention, may be followed by a year of high pollinator abundance where pollen is no longer limited²⁵. Such a life history may instead suit generalist plant species who are less impacted by population crashes of any one pollinator.

Summary

Studies of both male fitness and of geitonogamy reveal similar insights into floral evolution. A single plant and its individual pollinator exist in a larger and more complex network of relationships. In such, a pollinator's role in driving floral evolution is derived

from the interaction between the pollinator's behavior, both the pollinator's and the plant's life histories and physiologies, and the ecological community in which the relationship is found. It is therefore exceedingly difficult to rely on generalizations of pollinator behavior to predict floral evolution. Studies on the unique interplay of a pollinator's behavior and the flowers it visits can improve our predictive abilities. In the next two chapters, I aim to do this through the analysis of two systems in which the nuances of a pollinator's behavior may have specific consequences for the evolution of floral traits.

II. BAT CARRYOVER STUDY

Introduction

The discussion in Chapter 1 implies a pollinator's behavior is of high consequence to floral evolution. Unfortunately, this is still a growing field and the implications of the nuances of behaviors on male fitness are still lacking for many pollinator groups. One such group, nectar-feeding bats, is of great importance in the tropics⁸. Though relatively young evolutionary compared to other pollinator groups, bats pollinate a wide range of plant families, especially in the New World Tropics^{8,57,58}. Importantly, bat-pollinated flowers evolved mostly from hummingbird-pollinated species, concomitant with an increase in pollen production^{57,59}. Subsequent studies have demonstrated bats to be highly effective at transferring large pollen loads, implying the consequence selection through male fitness of flowers was producing more pollen⁸.

However, fewer studies have looked at the behaviors of bats between floral visits, and how these behaviors influence pollination efficiency. Those that have suggested bat physiology and behavior is well suited to high pollen removal and deposition⁸. Like many similar pollinators, bats are known to carry pollen from numerous species on their bodies at one time¹⁵. While heterospecific pollen transfer can happen, the large bodies of bats allow for individual plant species to place pollen on unique patches across the bats^{33,60}. However, a less studied behavior in bats, grooming, may be of consequence to male fitness of the flowers they pollinate. Previous research shows that bats can feed on pollen grains^{8,57}. Furthermore, they are frequent groomers, removing parasites and cleaning themselves. If, like with bees, grooming does influence pollen removal, this

should select against bat-pollinated flowers presenting high pollen loads available at one time⁶¹. Furthermore, this can be a direct effect, i.e., grooming for the sake of pollen removal and consumption, or an indirect effect, i.e., removal of pollen as a byproduct of grooming for other purposes. Does selection from grooming limit the extent to which opposing selection can drive adaptations for increased pollen presentation? Quantifying the effect of grooming on pollen transfer would elicit a better understanding of the myriad of selection forces acting on male fitness leading to the optimum pollen load a male should present.

One way to study this is a controlled pollen carryover-curve analysis^{3,13,62}. In such an experiment, a pollinator is given access to a male of a given plant species to receive pollen, and then allowed to visit a subsequent number of females of the same plant species. Past studies have found that, in general, the most pollen is deposited on the first few flowers, with fewer and fewer grains reaching the latter flowers^{2,3,37,62}. This is most pronounced in experiments on bees, where the curves were found to be sharply decelerating^{1,3,22,63}. It was found that while bees do pick up a lot of pollen, grooming and pollen consumption mean relatively few of those grains make it to females, and the greater the time allowed for grooming (i.e. the farther in the sequence of flowers), the greater the proportional loss^{2,3,22}. Conversely, hummingbirds tend to have as sharply decelerating curves, instead more evenly spreading the distribution across all stigmas^{8,13}. The researchers believe that hummingbirds are less efficient receivers and depositors but, because of a lack of inter-flower wasting behaviors, tend to not lose as many grains as other pollinators. This may also explain why some hummingbird-pollinated species can survive in rare, patchy abundances; by not being efficient enough to deposit pollen all on

the first couple of flowers, hummingbirds are more likely, by pure chance, to carry pollen to plants further away⁵⁹.

Such a carryover study is not readily available for bats. Given that bats appear very proficient at receiving and depositing grains, but also groom, bat behavior may present a nice case of how pollen distribution can lead to selection for floral adaptations. We present bats with a male flower followed by 10 sequential female flowers, quantifying pollen deposition on each. Type (i.e., feeding, grooming, and flying) and duration of behavior was also measured between and during flower visits. We hypothesize that: **1)** Carryover curves in bats will be steeply deaccelerating in a similar fashion to those of bees; and **2)** There will be a negative correlation between time spent grooming and the proportion of pollen deposited on a given stigma.

Methods and Materials

The Experiment

The study was conducted by Diana Gamba (D.G.), Camilo Calderón-Acevedo (C.CA.), and Rossana Maguiña (R.M) in Wildsumaco, a private cloud forest reserve on the slopes of Volcano Sumaco in Ecuador. Three bat species, *Anoura caudifer*, *A.cultrata*, and *Lonchophylla robusta* (Phyllostomidae) were used. These bats are the three most abundant nectar-feeding bats at the site^{58,64}. Nineteen individuals were caught (8 *A.caudifer*, 5 *A.cultrata*, and 6 *L.robusta*) and kept inside separate cages for safeguarding. The bats were fed, when not part of the experiment, a concentration of sugar water similar to the nectar concentration of the plants they feed from.

This study took place in 2m X 2m flight tents. The focal plant species was *Burmeistera glabrata* (Campanulaceae), a hemi-epiphytic or free-standing shrub⁶⁴. This is an ideal species for this experiment as the flowers are well adapted to bat pollination⁶⁴. Furthermore, the flowers are protandrous, with the anthers fused into a tube and pollen gradually exposed as the style pushes through the tube⁶⁵. Such a system prevents pollen competition between the donor male and the visited flowers, as only the initial flower has pollen. Finally, the flowers are solitary, and it is therefore easier to isolate a single flower for study purposes.

The study flowers were cut at the pedicel and placed in a microcentrifuge tube filled with water for floral preservation. This flower-tube (hereto referred to as the flower) was held in place by a metal wire designed to angle the flower to represent natural positions. For each trial, one male flower was presented followed by a series of 10 female flowers, with the time recorded after the bat visited each one. For each female flower, double-sided tape was placed on the stigmas to collect pollen deposited. The tape was removed after the bat visited the flower, placed on a microscope slide, and covered with single-sided tape for later analysis in the lab.

Three to six trials were conducted per bat. Using Jwatcher v1.0, we recorded when bats switched between the following behaviors: feeding, grooming, staying still, and flying. This was done for each individual trial of 10 female flowers.

Univariate Analyses

One-Way ANOVA analyses with Tukey Post Hoc Tests were used to determine any significant differences between the species of bats in the proportion of total time

spent grooming and total pollen deposited per trial. This was replicated for comparison between sex per species.

Curve and Slope Estimation

To characterize the carryover curve, I analyzed proportion of pollen deposited per flower per trial. I used proportion instead of raw number of grains deposited because the former allowed for comparison between trials which varied in the starting number of grains a bat initially received. I averaged the proportions across all trials and all individuals of a species. To estimate the slope for each curve, I used the analysis function “Curve Estimation”, in SPSS Statistics 25 (IBM). Curve Estimation produces regression statistics for up to 11 different curve models for a given plotted data set. I estimated the equation for linear, power, inverse, and exponential functions. These specific functions were chosen based both on a visual examination of the data and on previous studies examining carryover curves^{62,66}. The explanatory powers (R^2), significance levels, and slopes for each equation for each curve were compared. Of those that were statistically significant, I chose the function corresponding to the equation which best explained the data (i.e., had the largest R^2).

Regression Analyses

I ran regression analyses to determine the correlation between grooming and deposition rates, using the “Linear Regression” Analysis in SPSS 25 (IBM). Note that linear regression was the best fit, and that non-linear parameterizations failed to explain the data any better.

I pooled all data over all trials, individuals, sexes, and species. Each linear regression consisted of a dependent variable an independent variable as well as random factors. These random factors were Flower Position, Bat Time spent Flying, Feeding, and Perching, Bat Individual, Sex, and Species. The following analyses were chosen as the best possibilities for detecting patterns (note that the analyses are in order in which they were conducted):

1. Time spent grooming by proportion deposited at each flower: Here the main independent variable was the time, in seconds, spent grooming in between Flower_x and Flower_{x-1}, and the dependent variable was the proportion of the total number of grains deposited at Flower_x. I could not use raw number of grains deposited because results would not be comparable between trials.

2. Time spent grooming by ratio of deposition at Flower_{x-1}:Flower_x: The independent variable was the same as in the previous model. However, in this case, the dependent variable was the amount deposited at Flower_{x-1} divided by the amount deposited at Flower_x. The random factors remained the same.

3. Time spent grooming by cumulative proportion deposited after grooming: Again, the independent variable remained the same and I controlled for the same variables. If grooming occurs before Flower_x, we may see an influence in deposition at Flower_x but also at all subsequent flowers. If this is the case, and if grooming before Flower_x increases, then the proportion deposited from Flower_x to Flower₁₀ compared to the proportion deposited at Flower₁ through Flower_{x-1} should decrease. Therefore, the dependent variable was the proportion of the total

amount of pollen deposit on Flowers_x through Flower₁₀. Again, the random factors remained the same.

Note that sex as a random factor was significant (see **Results**). I therefore re-ran the analysis with only female bat data and again with only male bat data.

4. Time spent grooming over each trial: For pollinators such as bees, grooming is exponentially stimulated by greater pollen loads. If this is the case for bats, we should see the majority of grooming taking place before the first few flowers, as this is when the bat has the highest pollen load. Therefore, I ran a final regression analysis in which the independent variable was the flower position, and the dependent variable was time grooming in-between Flower_x and Flower_{x-1}. The random factors were the same but without flower position.

Results

Univariate Descriptive Statistics

On average, among all individuals (19 individuals, 77 total trials, blocked by individual), bats transferred 1200.8 pollen grains in total to the 10 flowers. On average, *A.cultrata* (5 individuals) deposited 1756.3 grains, as compared to 1038.3 and 699.0 grains for *A.caudifer* (8 individuals) and *L.robusta* (6 individuals) respectively (**Figure 1**). A One-Way Anova Analysis with a Tukey Post Hoc Test revealed that *A.cultrata*'s average deposition was significantly greater than that of *L.robusta* (One Way Analysis; $F=4.002$, $P=0.039$, Tukey Post Hoc; $P=.033$, $df=18$). No significant differences for average deposition were found between sexes of the same species.

Of the 19 bat individuals (trials per individual averaged), the greatest proportion of pollen was deposited on the first female flower 84.2% (16 individuals).

Similar to the overall average, *A.caudifer* transferred the greatest proportion pollen 87.5% (7/8 individuals) of the time on the first female. For *A.cultrata*, all 100% of the five bat individuals transferred the greatest proportion of pollen on the first female flower. Finally for *L.robusta*, 66.7% (4/6 individuals) transferred the greatest proportion of pollen on the first female flower.

Likewise, 62.5% (5/8 individuals) of *A.caudifer* deposited more than 50.0% of pollen on the first two female flowers. For *A.cultrata*, 40.0% (2/5 individuals), and for *L.robusta*, 100% (6/6 individuals) bats deposited more than 50.0% of pollen on the first two female flowers

On average, *A.cultrata* spent the most time grooming (70.0 seconds), followed by *A.caudifer* (31.3 seconds), and last by *L.robusta* (19.5 seconds) (**Figure 2.**). However, a One-Way Anova Analysis with a Tukey Post Hoc revealed that average grooming for *A.cultrata* was not significantly greater than that for *A.caudifer* (One Way Analysis; $F=1.49$, $p=0.256$, $df=18$, Tukey Post Hoc; $P=0.390$), and *L.robusta* (One Way Analysis; $F=1.49$, $p=0.256$, $df=18$, Tukey Post Hoc; $P=0.252$).

On average, per trial, females of all species groomed more than males. Using a Non-Parametric Independent Samples analysis, I found this difference to be significant when combining all three bat species (Mann-Whitney, $U=64203.0$, $N=750$, $p=0.037$). I then analyzed this separately for each species and found a significant result for *A.cultrata*

(Mann-Whitney $U=4391.0$, $N=210$, $p=.022$), and nearly significant for *L.robusta* (Mann-Whitney $U=5457$, $N=221$, $p=0.073$).

Curve Estimation

For each species, I plotted proportion of total deposition with flower position across all trials and all individuals (**Figure 3A-C., Table 1.**). I estimate the equation for a linear, inverse, power, and exponential curve using the “Curve Estimation” analysis in SPSS. For each species, the regression model “Inverse” ($y=b_0+(b_1/x)$) best fit the data. These equations were relatively good at explaining the data with $R^2=0.32$ for *A.caudifer*, $R^2=0.56$ for *A.caudifer*, and $R^2=0.56$ for *L.robusta*. For *A.cultrata*, each subsequent flower received, on average, only half the pollen deposited on the previous flower. For *A.caudifer* and *L.robusta* the amount received by each subsequent female flower was roughly 1/3rd the amount deposited on the previous flower.

Linear Regressions

I ran four distinct regression analyses (**Table 2.**). As noted, linear explained the data better than non-linear for all cases. In, the first two regression analyses, coefficients for grooming were not significant. However, in the third regression, the coefficient for grooming by cumulative proportion deposited was significant (Linear Regression Analysis, $t=2.081$, $p=0.038$, unstandardized $B=-0.001$). This implies that for roughly every one second of grooming between Flower_x and Flower_{x-1}, the average cumulative proportion deposited from Flower_x through Flower₁₀ increases by 0.1%. However, there

was also a significant effect of sex on cumulative deposition. The significance between grooming and cumulative deposition disappeared when data included only males. However, with the same analysis for just females, grooming was significantly correlated with cumulative proportion deposited (Linear Regression Analysis, $t=3.216$, $p=0.002$, Unstandardized $B=-0.003$). This means that for roughly every one second of grooming between Flower_x and Flower_{x-1}, the average cumulative proportion deposited from Flower_x through Flower₁₀ decreases by 0.3%.

I ran one final regression with flower position as the independent variable and grooming time between Flower_x and Flower_{x-1} as a proportion of total time spent grooming (**Figure 4**). The assumption was that if pollen was especially important in triggering grooming, the highest proportion of grooming would occur when the pollen load was highest, i.e. the first few flowers. However, the coefficient for flower position was not significant.

Discussion

Overall, the results indicate that: **1)** grooming influenced deposition rates; and **2)** the carryover curves most commonly fit the pattern deaccelerating curves. The first finding was supported by the regression for cumulative deposition after grooming and not by the regression for rates of deposition on an individual flower after grooming. Interestingly, while this pattern was for the overall data, when we analyzed male and female data separately, results were different. There was a significant relation between cumulative deposition and grooming for female data, but not for male data. Furthermore, the ratio between deposition on a flower relative to the previous flower did not differ

between pairwise comparisons across the whole set of 10 flowers. Finally, while there were differences in number of grains deposited for different species, there were no significant differences between steepness of curve, nor proportion deposited at each position.

Based on prior research, it is not entirely surprising that we found an effect of grooming on bat pollination. Bats have been observed grooming themselves extensively, even removing pollen and eating it⁸, thus the effect of grooming on pollen transfer is not unexpected. In bees, such grooming behavior results in few grains deposited^{4,22}. Similarly, non-volant mammals that hoard pollen deposited significantly less pollen as a function of time in between plant visitations⁵. Between plant visits, these mammals groomed pollen from their fur, consuming the grains or storing them for later. On the other hand, while hummingbirds do groom, and they rarely they have been known to eat pollen, and grooming does not seem to influence deposition rates⁸. Presumably, pollen placed on bills would be away from oft-groomed areas of the birds⁸.

Pollen grooming for bats makes sense for a few reasons. First, a high nectar diet is usually lacking in amino acids or proteins, while pollen is high in these compounds⁶⁷. Pollen supplementation would be a rather efficient way to increase protein intake⁶⁸. Studies have shown physiological adaptations, especially for vertebrate pollinators, which improve nutrient uptake from pollen^{68,69}. For instance, although nectarivorous and frugivorous bats process pollen in their guts at roughly the same rates, the former are able to extract higher levels of nutrients from the pollen⁷⁰. Such high extraction efficiency in *Anoura geoffroyi* and *Leptonycteris curasoae* outstripped those of other important vertebrate pollinators, including hummingbirds and pteropodid nectarivores⁷⁰.

Although there was an overall interaction between cumulative deposition and grooming, when female and male data were analyzed independently, this correlation was only significant for females. Furthermore, for all three species females groomed longer than males. Indeed *L.robusta* females groomed, on average, for about 21.5 seconds per trial, compared to only 4.0 seconds for males. In *A.cultrata*, females groomed, on average, for 91.0 seconds, compared to 54.0 seconds for males. Likewise, female *A.caudifer* individuals groomed, on average, 54.0 seconds, compared to only 27.0 seconds for males. I take these results to suggest differences in nutritional needs between the sexes. Females need to increase nitrogen assimilation during the period before pregnancy, during pregnancy, and during lactation^{71,72}. The study was done in June, about a month before the start of peak pregnancy times in *Anoura* species⁷². If females are needing to acquire higher-than-average levels of nitrogen to support both themselves and their offspring, increased grooming may increase pollen availability. That the results are non-significant for just the males is also interesting. This does not mean that males do not groom to consume pollen. Rather, the males' nitrogen requirements may be low enough that the natural variation in grooming and deposition between the trials obscures the relatively lower amount of grooming specifically for pollen consumption that males do.

Further studies can monitor pollen consumption differences between males and females in nectarivorous bat species, especially in the months leading up to, during, and right after pregnancy. Additional tests can determine the surplus nitrogen requirements needed for pregnancy. Likewise, it would be interesting to see if females in general, or at

least during pregnancy, are physiologically adapted to extract nutrients from pollen at greater rates than males.

If there is a significant difference in the pollen grooming rate of reproductive females compared to males and non-reproductive females, this could have interesting implications for fitness of male function in plants. For a certain fraction of the year, increased grooming may temporarily increase pollen wasting. If the costs of pollen wasting to male function fitness are great enough, it may prompt selection in plants to avoid pollen production during peak reproductive periods in bats. Could plants therefore respond by shifting peak pollen production times to not coincide with peak reproduction times in bats?

Returning to our results, the significance of the effect of grooming on deposition may also appear in the general shapes of the carryover curves. First, the curves most often fit power equations, steeply deaccelerating and with the majority of pollen deposited on the first two flowers. These curves are more like those of non-volant mammals and bees than those of hummingbirds^{8,13,62,73}. The sharp deacceleration could partially be explained by cumulative “grooming opportunities” between sequential flower visits as pollen is continually removed.

However, the carryover curve results may also suggest an influence of how efficient bats are at transferring pollen. In studies on bee carryover curves, it was posited that, despite significant pollen loss to due to grooming, bees still transferred a large proportion of post-grooming available pollen (pollen still remaining on the insect after grooming) to the stigmas¹. Bats are more effective at transferring pollen (that is not lost to grooming) than hummingbirds⁸. Therefore, bats and bees may effectively transfer a

high proportion of pollen onto the first few flowers, leaving little for remaining flowers. Conversely, hummingbirds may transfer proportionately less pollen on the first couple of flowers, leaving more for the late-sequence flowers.

Consequently, one resolution to this paradox of “too efficient” pollen transfer, i.e. that carryover is limited, may be to increase pollen availability at any one time. Studies support this idea. In comparing sister species pollinated by hummingbirds or bats (Muchhala and Thomson, 2010) found the bat species to produce much greater amounts of pollen than do the hummingbird species⁸. Blossom inflorescences, a common trait in bat-pollinated species, are clear examples of the high level of available pollen⁷⁴. Furthermore, throughout the angiosperm phylogeny, evolutionary shifts from hummingbird to bat pollination are quite frequent, and outnumber reverse shifts^{8,57}. Hummingbird to bat shifts coincide with increased pollen production. As a result, although hummingbirds deposit a greater proportion of grains on the final females, the actual numbers reaching these flowers are smaller than those reaching similarly-positioned bat pollinated flowers^{8,13}. This could imply that the male benefits from increased pollen production in bat-pollinated flowers by extending the number of females its pollen reaches, essentially breaking through the “barrier” imposed by higher bat transfer efficiency. Greater deposition at each female also increases the chance of fertilization.

Interestingly, selection from bat transfer efficacy for increased pollen availability would likely oppose selection from grooming. Any pollen lost is considered wasted and cannot contribute to male fitness^{1,8}. If grooming, or any other wasting behavior, is too extensive, the cost to male fitness might be strong enough for selection regulating pollen

production. In this case, selection should favor flowers that limit the amount of pollen available at any one time¹. A smaller load would initially imply less chance for carryover, a negative consequence for male fitness. Yet, by the same principle, if the small load is more quickly offloaded, there is less time for grooming or other wasting behaviors to contribute to pollen loss. This is particularly true if high loads stimulate a disproportionately higher amount of grooming, and therefore a higher proportion of pollen loss. Studies have found clear examples of this with bee-pollinated plants¹. Buzz-pollination is an extreme example of that, with poricidal anthers greatly limiting the amount of pollen available at any one time^{19,75}. Indeed, bees may therefore transfer pollen to fewer sequential females, but the plants may suffer comparably less pollen loss. The same principle should apply to bats who excessively groom. Nevertheless, as previously illustrated, switches to bat pollination correlate with increased pollen availability. It therefore appears selection from transfer efficacy is stronger than selection imposed by grooming.

To verify bat grooming and carryover patterns, and to quantify the strengths of the disparate selection regimes, further studies should control for the amount of pollen the bat initially receives and measure remaining amount after each grooming event. This would allow more precise calculation of as a function of initial amount of pollen removed as well as a more precise estimate of pollen loss due to grooming. Furthermore, controlling for the initial amount of pollen would limit variation between trials, giving a stronger true signal. Importantly, we could also model selection under different adaptive regimes. For example, males presenting small loads of pollen may fail to reach latter-positioned females as a consequence of the bat's high transfer efficiency, and or grooming

behaviors. Finally, by incrementally increasing the initial pollen amount per trial, we could directly quantify how pollen presentation load effects additional components of pollen transfer. For instance, contact forces (e.g. electrostatic forces, surface friction), pollinator traits (e.g. body surface area size and fur vs. feathers), and stigma traits (i.e. surface area and surface friction) regulate the amount of pollen a bat can pick up at from an anther, or a stigma can pick up from a bat, at any given time⁷⁶. By increasing pollen availability, we can therefore measure the physiological reception and deposition capacity. Such an analysis may help to predict bat pollination efficacy of other plant species.

Overall, the results of our study suggest that grooming does correlate with a decrease in pollen deposition. Although this was only statistically significant for female bats, I suggest that with further studies controlling for some of the variation between trials, grooming by males will also correlate with the rate of deposition. Nevertheless, the difference in sexes potentially leads to insight in the nutritional requirements throughout the mother bat's reproductive stage. Furthermore, we showed that the carryover curve for bats was steeply deaccelerating, with most individual trial curves fitting a power equation. These results have important implications for male fitness, consequences of which can lead to adaptations regulating pollen production.

III. ASCLEPIAS GEITONOGAMY STUDY

Geitonogamy, or the movement of pollen from male to female parts within the same plant^{2,10}, can contribute severe fitness costs to both male and female function. This leads to a paradox in the relationship between plants and pollinators: a highly attractive plant benefits from the increase in pollinator recruitment but likewise may suffer the costs of pollinators less likely to leave the plant⁷⁷. For a plant pollinated by a diverse array of pollinators (a generalist), there may exist a gradient with different pollinators contributing differently to geitonogamous movements. In this case certain pollinators may be more beneficial and others conditionally parasitic to the plant²⁵. To better understand the role individual pollinators play in pollination and floral evolution, it is imperative to quantify each's tendency for geitonogamy.

Despite decades of research on geitonogamy, questions remain to be answered. Namely, for generalist plant species, do the different pollinating groups differ in their rates of geitonogamy, and therefore in the selection strength they impose on the plants? If so, is the variance in selection strength large enough to make specialization advantageous for the plant? Pollinator groups differ in their abilities to effectively transfer pollen from the flower to stigmas of other individuals^{25,26}. Plants may specialize on certain groups if the conditions are right and the benefit of strengthening the symbiosis between the plant and a very effective pollinator group, and/or the benefit of reducing access to inefficient pollinators is (are) high enough²⁴. Efficacy, therefore, is not merely the transfer rate of pollen but must also include the pollen grains' destination. An insect that can effectively remove and deposit large quantities of pollen would be considered inefficient if it transferred a high proportion of the grains to the same flower or same

plant^{25,78}. Analyzing the degree to which certain pollinators make geitonogamous moves offers greater insight into pollinator efficacy.

Asclepias represents an ideal system to address these questions^{31,31,78}. This is a genus of over 100 herbaceous and perennial species⁷⁹. They are found throughout the Missouri region, and are important sources of both food and toxic defenses for the iconic and endangered Monarch Butterfly (Nymphalidae, *Danaus plexippus*)^{79,80}. This makes asclepiads important keystone species in many prairie ecosystems⁸⁰.

As generalist species, asclepiads can be visited by 100 different pollinator species^{81,82}. Importantly, asclepiad flowers and their pollination are some of the most complicated of angiosperms^{83,84}. Each flower has five petals reflexed backwards that reveal a corona⁸⁵. The corona is made of five hoods paired with five horns, all of which surround and protect the gynostegium^{86,87}. The gynostegium is formed by the fusion of five anthers around a stigma^{79,86,87}. However, these anthers are fused only at their tops and bottoms allowing for splits along the middle in which insects can slip inside^{79,86}. There the slits lead to the stigmatic chamber which houses the pollinia (pollen masses). Two pollinia are connected by a corpusculum and translator arms, forming a winged shape^{79,87}. When the appendage of an insect slips into the slits, the base of the pollinia pair (corpusculum) mechanically attaches to the insect⁸⁷⁻⁸⁹. The process for deposition is reversed, with the insect's appendage carrying the pollinia slipping into it takes per species can correlate with the average time that species' pollinator spends on average per visit. another stigmatic chamber and the pollinia effectively slotting in akin to a lock-and-key method^{85,90}.

Studies have demonstrated multiple adaptations unique to the pollinia system thought to limit geitonogamy. For instance, upon removal, pollinia often become bent, distorted, or swollen⁹¹. These deformations take time to reverse, allowing for pollinators to leave the plant before geitonogamy occurs. Nectar quality, including the toxins synonymous with the *Asclepias* group, has also been implicated in promoting visitor departures⁹².

Before the pollinia revert back to their original shapes, they cannot be inserted. This is thought to be a mechanism limiting the chances of self-pollination through autogamy or geitonogamy⁸³.

However geitonogamy is still very common amongst asclepiads³¹. Additionally, the cost of geitonogamy can be greater, than ‘traditional’ flowers, as asclepiad flowers can only receive one pollinium per slit in the stigmatic chamber (see materials and methods for more detail). Indeed, numerous studies show high rates of geitonogamy, with consequences to seed and fruit set, and offspring fitness^{31,78}. Certain *Asclepias* species are also clonal, essentially extending the range for pollinators to transfer pollen to genetically related stems^{44,78}.

Despite the longstanding use of *Asclepias* species to test hypotheses regarding geitonogamy, many species still lack information as to which visitors contribute most to geitonogamy. Knowing this is especially important for conservation planning. Likewise, exotic pollinators, such as *Apis mellifera*, change the pollinator community, leading to temporal changes in efficacy and thereby selection gradients⁸¹. Therefore, I was interested in the following: Which pollinators visits three *Asclepias* species the most, and

of those which is more likely to contribute to geitonogamy? To do this I tested the following hypotheses: **1)** Honeybees (*Apis* species) and bumblebees (*Bombus* species) will be the most common visitors; and **2)** *Apis* will make more within-plant movements than *Bombus*. The second hypothesis relates to findings from previous studies demonstrating *Apis* has not evolved with the *Asclepias* system and therefore is less skilled at extracting nectar. Therefore, I predict *Apis* will potentially need to visit more flowers per plant to obtain the same amount of nectar. Finally, I also tested if the number of within-plant movements, number of movements per visit, and time spent per visit are each positively correlated with inflorescence size. Finally, I also compared pollinator group visitation rate by year, as certain winters were unseasonably dry, which can negatively impact ground nesting bees.

Methods and Materials

Data Collection

Data on pollination were collected by Dr. Kyra Krakos of Maryville University and members of her lab during the months of June and July from 2012 to 2020. For this particular project, focal plants had been tagged and marked prior to observations. An observer was first tasked with recording data, time of day, location, weather, temperature, number of inflorescences, and display size. From there, that individual observed one focal plant for 20 minutes at a time. He or she recorded who visited (to genus or family), how long it remained, and where it went next (same plant, same species, other species, or left). In certain cases, where the visitor was presumed to be either an incidental or non-pollinating visitor, the recorder did not specify any taxonomic level (i.e., a bird visitor was simply labeled as “bird”). From here on I refer to where a visitor went next as a

movement. Furthermore, “left” refers to the visitor leaving the observable area and thus the visitor’s destination remains unknown. Because of the difficulty in doing so, observers could only differentiate one individual from another in certain cases. In such cases, I could make further analyses regarding visitation duration and number of within-plant visits per foraging bout. Similarly, stigmatic contact and pollen receipt/deposition was also difficult to observe and therefore we are unsure if when visitors actually effected pollination.

Descriptive Statistics

The first analyses were developing descriptive statistics to reveal any potential patterns in the data and to warrant further examinations. Using the recorded data for each plant species, I was able to tabulate the following.

1) The total number of visitor groups to the plants. All visitors to each focal plant were recorded and grouped according to taxonomic divisions. Some groups, such as Halictids, were only differentiated at the family level while others, such as *Apis*, were identified to the genus. Other groups, such as ants and birds, that are considered non-pollinator or incidental visitors, were divided no further. Finally, unidentified visitors were clumped in an “Other” group.

2) The total proportion of decisions made at each plant across all visitors. Decisions were condensed into three categories: same plant (“Same”), different conspecific (“Different”), or leave (“Leave”). Recordings of a heterospecific plant species as the destination were incredibly rare and thus categorized as “Leave”. The

result was a summed number, and proportion, of total decisions for each plant species across all visitor groups.

The next step was to break down the decisions by visitor group. Again, the result was a summed number, and proportion, of total decisions by each visitor group for each plant species. The results were displayed in a table and a clustered bar graph.

Furthermore, while no analytical statistics were run, I chose to focus all subsequent analyses on the three visitor groups that contributed the most to the total number of decisions made. These groups were Halictidae, *Apis*, and *Bombus*.

Difference in Movements Between the Main Three

After I established the three visitor groups contributing the most to total movements, I analyzed significant differences between these groups and the types of movements being made. From here on I combined “Leave” and “Different” movements into one category labelled “Away” in order to focus analyses on geitonogamy, or the proportion of within-plant movements relative to all other movements. From there, I ran a Chi-Square analysis to establish any significant differences in the proportion of geitonogamous movements between the three pollinator groups at each plant species. I added Bonferroni adjustments to tease apart which group(s) was(were) responsible for any significant results.

Furthermore, I was also interested in between-plant species differences in each pollinator group’s decisions. I thus carried out Non-Parametric Binomial analyses to test

if, for example, the proportion of *Bombus*' geitonogamous movements at *A.tuberosa* differed from those at *A.syriaca* (nine Non-Parametric Binomial analyses, one per insect group per plant species).

Presence Proportion for the Big Three

As the number of visitors was impossible to track, visitation rate (number of visitors per a given time period) was not possible. Instead, we used the proportion present as an indicator for visitation rate, and as described as follows. To quantify the proportion present, I summed the number of observations for each plant species in which a given visitor group was present at least once and divided by the total number of observations for that plant species. Similar to the methods for part 3, I first conducted a Chi-Square analysis with a Bonferroni adjustment to determine if the visitation rates of the three groups significantly differed from each other at each plant species, and/or if any groups differed in visitation rates between species.

We wanted to test whether the presence proportion of any of the main three pollinator groups significantly changed following the winter droughts. I used a Chi-Square analysis with a Bonferroni to measure each of the three groups' presence proportion per species per year.

Presence Proportion in Relation to Environmental Variables

For each of the three insect groups, I was interested in whether there was an influence of environmental variables on the presence proportion. Insect visitation rates, including those for *Bombus*, *Apis*, and Halictidae, are often subject to environmental conditions.

To see if there were relationships between environmental variables and presence

proportion, I carried out the following Generalized Linear Probit Models with the following parameters. The dependent variable was the presence or absence of an insect group for a given observation (same as prior presence proportion analyses). For each insect group all data across the three plant species were compiled together. The independent variables were: Plant Species, and Temperature, Weather, and Time of day during each focal observation. I reduced weather to the following categories: Sunny, Cloudy, Partly Cloudy, Windy, Rainy. Note that when data was taken at night, and the observer noted it was “starry” out, I counted this as “sunny” and when the observer noted “clouds”, I marked this as cloudy. I did this because there is an obvious correlation between night and temperature, and between night and time. Instead, the weather variable is particularly concerned with the presence/absence of rain and clouds. I further included Temperature*Time and Temperature*Weather as interaction effects. Using the Generalized Linear Models-Probit function in SPSS 25, I first ran analyses for each insect group across all three plant species including the interaction effect variables. If an interaction variable was not significant, I would re-run the analyses, removing the variable.

Length and Number of Geitonogamous Movements per Visit

For some cases, the recorders kept track of individual visitors. This allowed me to tabulate a complete visitation sequence, including average time spent per flower, number of within-plant movements made, and average total time spent per visitation. Data was only sufficient enough at *A.syrriaca*, and *A.tuberosa*. Furthermore, I was only particularly interested in differences between *Apis* and *Bombus* as these appeared the two

most important pollinators and flower handling and plant visitation times are both important in quantifying pollinator success.

I ran Independent Sample T-Test analyses comparing either average time at each flower, average total time per visit or average number of movements per visit between the two insect groups for each plant species.

Next, I ran linear regression analyses, with flower display size as the independent variable and either total time per visit or number of movements per visit as the dependent variable, controlling for visitor group and plant species.

Results

For a summary of all significant findings, refer to **Table 3**.

The number of visiting groups were as follows: *A.tuberosa* 12; *A.syriaca* 14 (including “Other”); *A.purpurascens* 2; *A.sullivantii* 4; *A.viridis* 5. *Apis* and Halictidae were recorded at all species except *A.viridis* and *A. purpurascens*, respectively.

Therefore, and given that *A.viridis* and *A.purpurascens* had low sample sizes, statistical analyses following focused only on *A.syriaca*, *A.tuberosa*, and *A.sullivantii*. Indeed *A. purpurascens* had the fewest observations, with only butterflies and apids visiting.

Total Movement

The number of total movements (defined as all movements after first arriving at a focal inflorescence) was summed up for each pollinator group at each of the three *Asclepias* species (**Table 3**). Note that no analytical statistics were run and instead data

were displayed on a simple bar graph and table, including only those visitors making 10 or more movements. Movements were categorized as within-plant (“Same”), between plants of the same species (“Different”), and departures from (“Left”).

In general, halictids, apids, and bombids accounted for greatest number of total movements (**Figure 5.**). For example, at *A.tuberosa*, these three pollinator groups made 91.7% (922 total) of 1005 total movements. Of these 922 total movements, halictids made 60.5% (608), apids 21.3% (214), and bombids 10.0% (100) movements.

Similarly, the above three groups made up 79.9% (428) of the 536 total movements recorded at *A.syriaca*. Of these 428 total movements, *Bombus* accounted for 60.1% (257), *Apis* for 32.0% (137), and Halictidae for 07.9% (34). As a note, despite the moth group having more total movements (35) than the Halictidae group (34), further statistical analyses continued to use Halictidae as well as *Bombus* and *Apis*.

Movements by the Big 3 Visitor Groups

Statistical analyses were conducted with only the Halictid, *Apis*, and *Bombus* groups considered.

Each of the three pollinator groups made significantly more “Same” than “Away” movements (**Figure 6.**). *Apis* made significantly more “Same” than “Away” movements at each of *A.syriaca* (Non-Parametric Binomial Test, Proportion Same=0.69, N=137, $p<0.001$), *A.tuberosa* (Non-Parametric Binomial Test, Proportion Same=0.76, N=214, $p<0.001$), and *A.sullivantii* (Non-Parametric Binomial Test, Proportion Same=0.69, N=473, $p<0.001$). *Bombus* made more “Same” than “Away” movements at *A.syriaca* (Non-Parametric Binomial Test, Proportion Same=0.65, N=257, $p<0.001$), and

A.sullivantii (Non-Parametric Binomial Test, Proportion Same=0.61, N=152, p=0.007).

Finally, Halictidae made significantly more “Same” than “Away” movements only at

A.tuberosa (Non-Parametric Binomial Test, Proportion Same=0.67, N=608, p<0.001).

None of the three visitor groups differed significantly in the proportion of movement types between the different plant species. Restated, the proportion of geitonogamous movements made by each of the three groups did not differ between *Asclepias* species.

However, at *A.tuberosa*, *Bombus* made a significantly smaller proportion of geitonogamous movements than did *Halictidae* (Pearson Chi-Square with Bonferroni correction, $X^2=5.724$, p=0.017) and did *Apis* (Pearson Chi-Square with Bonferroni correction, $X^2=13.680$, p<0.001). No such significance was found at either *A.syriaca* or *A.sullivantii*.

Proportion Present by the Big 3 Visitor Groups

Proportion present calculated as the number of focal observations (per plant species) where one of the visitor groups was recorded, divided by all focal observations for said plant species (**Figure 6**).

Apis's proportion present significantly differed between plant species (Pearson Chi-Square = 48.69, p < 0.01). Of the 30 focal observations of *A.sullivantii*, *Apis*'s was present 63.3% (11 total) of the time. This proportion was significantly different than *Apis*'s visitation rate at *A.syriaca* (13.5%, 17 out of 126) (Pearson Chi-Square with Bonferroni correction, $X^2=33.909$, p,0.001), and at *A.tuberosa* (13.2%, 30 out of 228) (Pearson Chi-Square with Bonferroni correction, $X^2=43.383$, p<0.001).

Likewise, Halictidae visitation rate significantly differed between the plant species (Pearson Chi-Square = 32.430, $p < 0.01$). Halictidae visited *A.tuberosa* at a significantly greater rate (36.0%, 82 out of 228) than it did at *A.syriaca* (9.5%, 12 out of 126) (Pearson Chi-Square with Bonferroni correction, $X^2=7.655$, $p=0.006$), and at *A.sullivantii* (16.7%, 5 out of 30) (Pearson Chi-Square with Bonferroni correction, $X^2=42.417$, $p,0.001$).

Finally, *Bombus* also significantly differed in its visitation rates between species (Pearson Chi-Square = 64.104, $p < 0.01$). *Bombus* visited *A.tuberosa* (11.4%, 26 out of 228) at a rate significantly smaller than at *A.syriaca* (37.3%, 47 out of 126) (Pearson Chi-Square with Bonferroni correction, $X^2=33.252$, $p<0.001$), and at *A.sullivantii* (60.0%, 18 out of 30) (Pearson Chi-Square with Bonferroni correction, $X^2=44.261$, $p<0.001$). The rate at *A.sullivantii* was greater than that at *A.syriaca* (Pearson Chi-Square with Bonferroni correction, $X^2=5.136$, $p=0.023$). However, this was not significant when using Bonferroni corrections ($p>0.017$).

These differences can also be seen from the perspective of the plants. For instance, at *A.tuberosa*, Halictidae visited at a much greater rate (36.0%, 82 out of 228) than did *Bombus* (13.2%, 30 out of 228), (Pearson Chi-Square with Bonferroni correction, $X^2=57.421$, $p<0.001$), or *Apis* (11.4%, 26 out of 228) (Pearson Chi-Square with Bonferroni correction, $X^2=50.223$, $p<0.001$). This was reversed at *A.sullivantii* (Pearson Chi-Square = 16.34, $p < 0.01$), where both *Apis*'s visitation rate (63.3%, 19 out of 30) (Pearson Chi-Square with Bonferroni correction, $X^2=13.611$, $p<0.001$) and *Bombus*' (60.0%, 18 out of 30) (Pearson Chi-Square with Bonferroni correction, $X^2=11.915$, $p=0.001$) were significantly greater than Halictidae's (16.7%, 5 out of 30).

Finally, at *A.syriaca*, *Bombus* visited at a significantly higher rate (Pearson Chi-Square = 29.20, $p < 0.01$) (36.7%, 47 out of 126), compared to Halictidae at 9.5% (12 out of 126) (Pearson Chi-Square with Bonferroni correction, $X^2=27.110$, $p<0.001$), and *Apis* at 13.5% (17 out of 126) (Pearson Chi-Square with Bonferroni correction, $X^2=18.850$, $p<0.001$).

Visitation by the Big 3 Visitor Groups by Year

The next step was to break up presence proportions between years, with the hope of establishing patterns of fluctuation in pollinator presence or absence. Specifically, we were interested in whether rates of visitation per year correlated with the drought winters.

Nevertheless, my analysis did show that neither *Apis* nor Halictidae differed significantly in visitation rates between years. The only significant result was a change in *Bombus* presence at *A.tuberosa* (Pearson Chi-Square = 20.65, $p < 0.01$). Indeed, *Bombus* was not present at any focal observations in 2016 (0 out of 84) or 2017 (0 out of 5) but was present at 16.3% (16 out of 98) in 2015 and 24.4% (10 out of 41) in 2020. No correlation was found between drought winters and significant population changes.

Time Spent Per Flower and Visitation Between Apis and Bombus

For many focal observations, we were able to complete individual visitations for a given insect, thereby allowing us to calculate average time spent at a flower, average time spent per visitation to a plant, and average number of movements per visit. There were enough data to compare *Apis* and *Bombus* at *A.syriaca* and *A.tuberosa*.

At *A.tuberosa*, *Apis* spent an average of 6.0 seconds per flower, significantly greater than the average 3.0 seconds *Bombus* spent per flower (Independent Samples T-

Test, $F=14.73$, $p<0.001$, $df=57$). At *A.syrriaca*, *Apis* also spent, on average, more time per flower (4.16 seconds) compared to *Bombus*, which spent on average 3.4 seconds per flower. However, this was not significantly different (Independent Samples T-Test, $F=1.938$, $p=0.166$, $df=158$)

Similarly, *Apis* spent, on average, more time per visit at *A.tuberosa* (10.2 seconds) than did *Bombus* (8.0 seconds) but this difference was not significant (Independent Samples T-Test, $F=2.847$, $p=0.097$, $df=57$). The same was true at *A.syrriaca*, where *Apis* spent an average of 14.1 seconds per visit compared to *Bombus* (8.4 seconds), (Independent Samples T-Test, $F=1.735$, $p=0.190$, $df=158$).

Finally, there were no patterns in the average number of movements per visit between the two insect groups. Furthermore, there were no significant correlations between drought years and presence proportion for any of the insect groups across the three plant species.

Presence Proportion in Relation to Environmental Variables

In our GLM-Probit analyses, the presence proportion of all three insect groups was correlated with environmental variables. For *Bombus*, the presence proportion was significantly correlated with the Time and Temp variables (GLM-Probit, Omnibus Test, $X^2=124.77$, $df=9$, $p<0.001$). The same variables also correlated with the presence proportion for Halictidae (GLM-Probit, Omnibus Test, $X^2=67.72$, $df=9$, $p<0.001$). Conversely, the presence proportion for *Apis*, was significantly correlated with Time and nearly Weather (GLM-Probit, Omnibus Test, $X^2=79.43$, $df=9$, $p<0.001$). Note that in all

three analyses, no interaction effects were significant and therefore removed from the final analyses.

Discussion

In general, the results support the idea that geitonogamous movements are common among visitors to *Asclepias* species. Members of the three main visitor groups--halictids, bombids, and apids--all were more likely to move to another inflorescence of the same plant than leave the plant. Proportions of geitonogamous movements ranged from either 55% (Halictidae at *A.sullivantii* and *Bombus* at *A.tuberosa*) on the low end to 76% (*Apis* at *A.tuberosa*). Furthermore, at *A.syriaca* and *A.sullivantii* no proportions of geitonogamous movements between each insect group were significantly different. However, at *A.tuberosa* the proportion of geitonogamous movements of Halictidae (67%) and of *Apis* (76%) were significantly greater than that of *Bombus* (55%). Results of each one of the pollinator groups has implications for the effect of geitonogamy on *Asclepias* species.

Our results identify *Bombus* species as the most common visitor to *A.syriaca* and *A.viridis*. At *A.sullivantii*, the two most common visitors were *Apis* and *Bombus*. Although halictids dominated presence proportion at *A.tuberosa*, discounting them as non-pollinators (discussed later), *Bombus*' presence proportion was only a short second to that of *Apis* at *A.tuberosa*. Many studies have also demonstrated *Bombus* species as one of, if not the most, prevalent pollinators of *Asclepias* species^{78,81,82,88,89}, and definitely when excluding non-native pollinators such as *Apis mellifera*. Howard and Barrows (2014) showed that *B.griecollis* was the most common visitor to *A.syriaca*, surpassing even *A.mellifera*⁷⁸.

Our visitation results for *Bombus* are not surprising. Yet consistent with past research, these results do not imply that *Bombus* moves the most *Asclepias* pollinia. For instance, Ivey et al. (2003) found that, of six insect species, mean rates of pollinium removals were 5th lowest for *Bombus*⁹³. A study by Howard and Barrows (2014) mirrored these results, showing *Bombus* to insert and remove pollinia rates lower than those of *A.mellifera* and lepidopterans⁷⁸.

However, especially when considering geitonogamous movement, removal/insertion rates and ratios are not always predictive of pollinator efficiency. For example, Howard and Barrows (2014) also found that, despite *A.mellifera* removing and inserting pollinia at greater rates, there were no statistical differences in the importance of *A.mellifera* and *Bombus* as pollinators to *A.syriaca*. This is predominantly because the visitation rate of *Bombus* far exceeded that of *A.mellifera*. Furthermore, although *A.mellifera* out-crossed a greater quantity of pollen, it also contributed greatly to self-pollination through geitonogamy. Likewise, Ivey et al. (2003) found that *Bombus* was the least likely to contribute to geitonogamous pollination⁹³. In these and other studies, it appears that when considering probability of moving pollinia within flowers, visitor abundance, and efficacy in removal and insertion of pollinia, *Bombus* is usually the most or second most important pollinator^{81,88,94}. This means that even though we found *Bombus* made significantly more within-plant movements than either “leave” or “different plant” movements, the insect group’s overall impact on geitonogamy might be less than other that of other groups.

Researchers have offered a few reasons why lower removal/insertion rates and ratios may benefit the plants more. First, a pollinator greatly adroit at inserting pollinia

will quickly exhaust its pollinia load possibly before it is satiated enough to leave the plant⁸⁸. In this case, the pollinator may be more likely to pick up new pollinia from that plant and insert them before leaving⁸⁸. Conversely, a pollinator like *Bombus*, although not carrying as many pollinia, may transfer less geitonogamously. This also is supported by *Bombus*'s generally low time per visit and their skillful handling of the flowers⁹³. *Bombus* have also been known to keenly avoid slipping their legs into the stigmatic slits, thus avoiding picking up pollinia⁸⁸. Initially, this may sound detrimental to the plant. However, pollinia are high-payoff gambles for the plants^{95,96}. Fertilization of each ovule requires only one successfully inserted pollinia, minimizing the cost of inefficiency^{87,90,94}. Therefore, because of their quick-lasting and rather inefficient visits, *Bombus* may have a lowered risk of geitonogamous pollination while still providing sufficient rates of cross fertilization. Moreover, *Bombus*' inefficiencies in pollen movement are further compensated for by their high abundance^{88,93,94}.

Apis provides an interesting contrast to *Bombus*. Assuming Halictidae as a nectar robber (discussed lower and because it was never found to carry pollinia), *Apis* was always the second or most prevalent pollinator at each *Asclepias* species. Moreover, separate insect-capture experiments revealed *Apis* to carry, on average, the greatest number of pollinia (K. Krakos, unpublished data). Our findings are supported by previous studies highlighting *Apis* as one of the most important pollinators of *A. incarnata*⁹³, *A. exaltata*^{89,97}, *A. syriaca*^{39,78}, *A. meadii*⁸⁹, and *A. tuberosa*⁸¹. In many of these cases, *Apis* both carried the greatest number of pollinia and had the greatest removal/insertion rates and ratios.

However, pollinia loads and insertion and removal rates again fail to convey the whole story. First, Howard and Barrows (2014) found that despite being the most important pollinator of *A.syriaca* in regards to removal and insertion rate, *Apis*'s importance was not significantly greater than that of *Bombus* when geitonogamy rate was factored in⁷⁸. Likewise, Ivey et al. (2003) found *Apis* as the second most efficient pollen vector in *A.incarnata* but also the second greatest contributor to geitonogamy⁹³. These studies suggest that indeed *Apis* transfers pollinia within the same plant at greater rates than other important pollinators such as *Bombus*.

A few factors may explain *Apis*'s large contribution to geitonogamy. First, *Apis* tends to spend a longer time at the plant per visit^{78,81}. Although not statistically significant, we did find some evidence that *Apis* spent longer than *Bombus* at *A.tuberosa* and *A.syriaca*. Furthermore, unlike *Bombus*, *Apis* always made significantly more “Same” movements than “Away” movements at *A.tuberosa*, *A.syriaca*, and *A.sullivantii*. Other studies have found that unlike the native pollinators such as *Bombus* that appear very efficient at extracting nectar, the exotic *Apis mellifera* requires more time^{78,81}. This suggests an interesting dynamic; *Apis* is an efficient remover and inserter of pollinia but not skilled at handling the flowers. *Apis* arrives at the plant, quickly exhausting its imported pollinia load. *Apis* then quickly picks up more pollinia, and, because it needs more time at the plant to reach satiation, is more likely than native pollinators to insert that pollinia in a flower of the same plant before leaving. To support this, Howard and Barrows (2014) also found that as inflorescence size increases, so too does the rate of geitonogamy for *Apis* but not *Bombus*⁷⁸. Furthermore *Apis*, compared to *Bombus*, is more attracted to larger displays, such as the large clonal size of *A.syriaca*. The result is

that *Apis* stays very local, potentially not only moving pollinia within-plant but also frequently between genetically identical stems.

One final aspect of the peculiar pollination system of *Asclepias* species may further support the notion that the contribution to geitonogamy from *Apis* is far greater than that of *Bombus*. Upon removal from the stigmatic slit, the pollinia often becomes deformed, requiring a certain amount of time to deflate or unbend in order to be correctly inserted^{98,99}. This time delay, combined with relatively short *Bombus* visits, helps reduce the risk of geitonogamy by *Bombus* visitors. Visits to sequential male flowers can lead to sequential layering of pollen, ostensibly preventing pollen from the first male from reaching a stigma¹⁰⁰. Layering is harder with pollinia. Conversely, unlike with lightweight pollen grains, too many pollinia significantly weigh down insects, either trapping them on the plant or slowing down their dispersal rates⁷⁸. *Apis*, carrying a greater load than *Bombus*, become less efficient at removing/inserting. A pollinator like *Bombus* is less likely to be overburdened, and as such is less inclined to stay at the plant (thereby increasing the probability of geitonogamy) or become immobilized. I therefore suggest that any apparent inefficiency of *Bombus* compared to *Apis* is more a result of measuring only rates of insertions and removals without considering other aspects of pollinator behavior.

The higher presence (visitation rate) and greater number of total movements (both same and away) of Halictidae's at *A.tuberosa* is also of note. In separate measurements of pollinia loads on visitors to *Asclepias* species, no Halictidae was ever caught with pollinia. Previous studies on *Asclepias* species, including *A.tuberosa*, also showed that while halictids were frequent visitors, they rarely carried pollinia⁸¹. Therefore, it seems

more likely that halictid bees act as nectar robbers, especially on *A.tuberosa*. There are a few reasons why halictid bees may prefer *A.tuberosa* over other *Asclepias* species. First it appears that the flowers of certain *Asclepias* species are too large for smaller-bodied bees, such as Halictids, to access nectar⁸¹. However, *A.tuberosa* has nectar contained within narrow tubes, a characteristic of butterfly pollination but nonetheless may allow for small-bodied insects to still access the nectar⁸¹. Such constriction of the nectar-containing vessels is known to prevent nectar robbing from larger sized bees, reducing competition for smaller insects¹⁰¹. This appears not to be the case with *A.tuberosa*, which provides nectar to a wide diversity of insects, including its most efficient pollinators, apids and bombids⁸¹. Instead, *A.tuberosa* is known for its showy floral display, often with many more umbels per stem⁷⁹. Furthermore, they often demonstrate low annual mortality⁷⁹. Both factors suggest that *A.tuberosa* is a steady and high-quality source of nectar. Its large display size may also minimize visitor competition, evidenced by the greater diversity observed in our study.

The final reason that halictid bees may prefer *A.tuberosa* is that, compared to other milkweeds, *A.tuberosa* has the lowest amount of cardenolides and does not produce the milky latex characteristic of the group^{79,102,103}. Previous studies have shown that cardenolides are even present in nectar as a possible deterrent to nectar robbers or herbivores^{102,103}. For example, in one study Monarch butterflies (Nymphalidae, *Danaus plexippus*) and bumblebees were presented with artificial nectar containing cardenolide levels equivalent to those in *Asclepias* species⁹². The butterflies, though not deterred enough to drink the nectar, laid fewer eggs in flowers associated with high-cardenolide level nectar. Bumblebees were not deterred unless allowed to forage for several days,

indicating a high level of tolerance to cardenolides. In *A.tuberosa*, relaxation of these secondary compounds may make it possible for a wider range of species to visit the flowers. This may be especially important for Halictids who, as smaller bodied than Bombids, may be more susceptible to cardenolide toxicity. Certain insect species may have developed higher tolerance than others, such as *Bombus griseocollis* compared to *B.impatiens* and *B.bamcaulatus*¹⁰⁴. Visitors lacking these adaptations may prefer the lower level of cardenolides present at *A.tuberosa*. More research should be done on the susceptibility of Halictids to cardenolides in nectar.

That nectar may contain secondary defense compounds may also influence patterns of geitonogamy. First, as the study on the Monarch butterflies and bumble bees showed, the longer or the more intense the feeding bouts, the more likely the bumblebees were to avoid the plants⁹². If the toxicity in cardenolides is quick acting in a visitor, that insect may be dissuaded to remain at the plant. Likewise, if the insect becomes ill after ingesting a certain level of the defense compound, it may leave the plant after only a few flowers^{92,104}. Interestingly, while *Bombus griseocollis* appears to tolerate cardenolides at higher levels than *B.impatiens* or *B.bimcaulatus* can, the former is quicker to avoid plants with cardenolides at higher levels¹⁰⁴. If a pollinator becomes sick, or can detect its limit before becoming sick, at certain levels of cardenolides, the plant may use defense compounds in nectar to promote visitor departure, minimizing the chance geitonogamy. Likewise, plants whose nectar stores have been depleted will be less likely to attract pollinators⁵³. Therefore, nectar robbers, or conditional parasites, possibly such as Halictids, reduce plant fitness by reducing nectar available for “good” pollinators. Again, plants would benefit from causing nectar robbers to leave as soon as possible. This could

lead to selection for a level of nectar toxicity, encouraging only brief visits from its “good” pollinators and very short visits from its parasites. Indeed, it appears larger-bodied pollinators are the more effective pollinators. In this case the balance of cardenolides might very quickly dissuade the small-bodied nectar robbers but only effect the larger bombids and apids after a certain time spent visiting.

By comparing the visitation rate and movement patterns of apids, bombids, and halictids to each insect group’s natural history, our study offers a reminder that pollination effectiveness is one of relativity. A highly active visitor, such as Halictidae may serve as little more than a nectar robber. Conversely, a highly effective pollen remover and depositor, *Apis*, may carry a detrimental cost through increased geitonogamy. The intermediary pollinator, *Bombus*, in terms of visitation, removing, and depositing, may be the most effective given the correct system. However, we found *Bombus* was absent at *A.tuberosa* for two years, coinciding with an introduction of *Apis* in the area (K. Krakos, personal communication, July 13th, 2021). Therefore, shifting pollinator communities means a concurrent shift in which pollinator group is most effective and which have become conditional parasites or completely absent. This implies that a shift towards specialization may be: **1)** difficult because changes in the pollinator communities mean no long-term directional selection and **2)** problematic if specialization results in dependence on a potentially ephemeral pollinator. In this manner, a plant may be limited in the number of visitors it can exclude from visiting. More likely, the plant may adapt a series of small adaptations for promoting outcrossing and restricting any forms of inbreeding or hybridization. *Asclepias* species appear to support this notion. The lock-and-key and the time-delay in insertion availability of the

pollinia, coupled with the architecture of the gynostegium, and the toxicity of the nectar are all examples of small adaptations that are, in general, effective at reducing geitonogamous movements across an array of visitors.

V. CONCLUSION

Despite the obvious advantages in using animal-vectors as pollinators, plants still take a risk by relenting control of their pollen. As these studies have shown, the risk to both male and female function fitness can be quite severe. My results therefore provide further insight into evolutionary mechanisms. An understanding of the evolutionary mechanisms, in the context of community ecology processes, can enhance conservation practices.

For example, *Asclepias* pollination warns of the costs to alterations in the pollinator community. The introduction of the exotic *Apis mellifera* has been implicated in many instances of changed pollinator regimes^{39,89,93,97}. For *Asclepias* species, I hypothesize that the honeybees may have increased recent historical rates of selfing. Furthermore, the honeybees may competitively exclude other, would-be native pollinators. In turn, *Asclepias* species may suffer increased fitness costs related to inbreeding and geitonogamy. It would be interesting to test whether there is a correlation between the introduction of *Apis mellifera* and a strengthening of inbreeding signatures in *Asclepias* species. If there is, we can further examine whether there is selection to reduce the proclivity for *Apis*'s high contribution to geitonogamy. On a long-term evolutionary scale, one may predict *Asclepias* flowers adapt in response to selection imposed by *Apis*. If, in turn, the flowers' adaptations render them less attractive or rewarding to native pollinators, such as *Bombus*, those native insects may become less effective pollinators. Such a change in the pollinator community is an unfortunately common reality of anthropogenic disturbances and a changing climate^{105,106}. Nevertheless, studying these disturbances can help predict their outcomes and lend strength to mitigation strategies.

One specific example is the decline of the Monarch Butterfly's (Nymphalidae, *Danaus plexippus*) population, which has been, and will continue to be, exacerbated by concurrent declines in *Asclepias* species' populations^{107,108}.

A similar insight can be gleaned from my studies on bat carryover curves. Knowing which pollinators are better able to transfer pollen to multiple subsequent female flowers, is critical for predicting plant species' ability to reproduce. For example, plant species living in low density and isolated populations may differ in their abilities to survive dependent on their pollinators⁵⁹. This is particularly important as anthropogenic disturbances can split plant populations and shift ranges, which can limit the ability of pollinators to transfer pollen between members^{109,110}. Moreover, if female bats do indeed groom more during pregnancy, plants may face temporally fluctuating fitness costs. This may especially be problematic if climate change and anthropogenic disturbances lead to new synchronizations between peak bat gestation periods and peak flower times for plant species. As we face a changing environment, it is therefore essential to continue studying how the nuances of pollinator behavior translates into unique patterns of pollen distribution.

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VII. FIGURES AND TABLES

Figure 1. Average number of grains deposited per trial. For each bat species the following steps were conducted. First, number of pollen grains deposited for each trial for each individual bat were averaged together. Second, each individual's average was averaged across each bat species. Lower case numbers denote statistical difference where $p < 0.05$. The only statistical differences found were average depositions for both male and female *A.caudifer* bats compared to average deposition for both males and females in the other two species. No statistical difference was found between sexes within the same species.

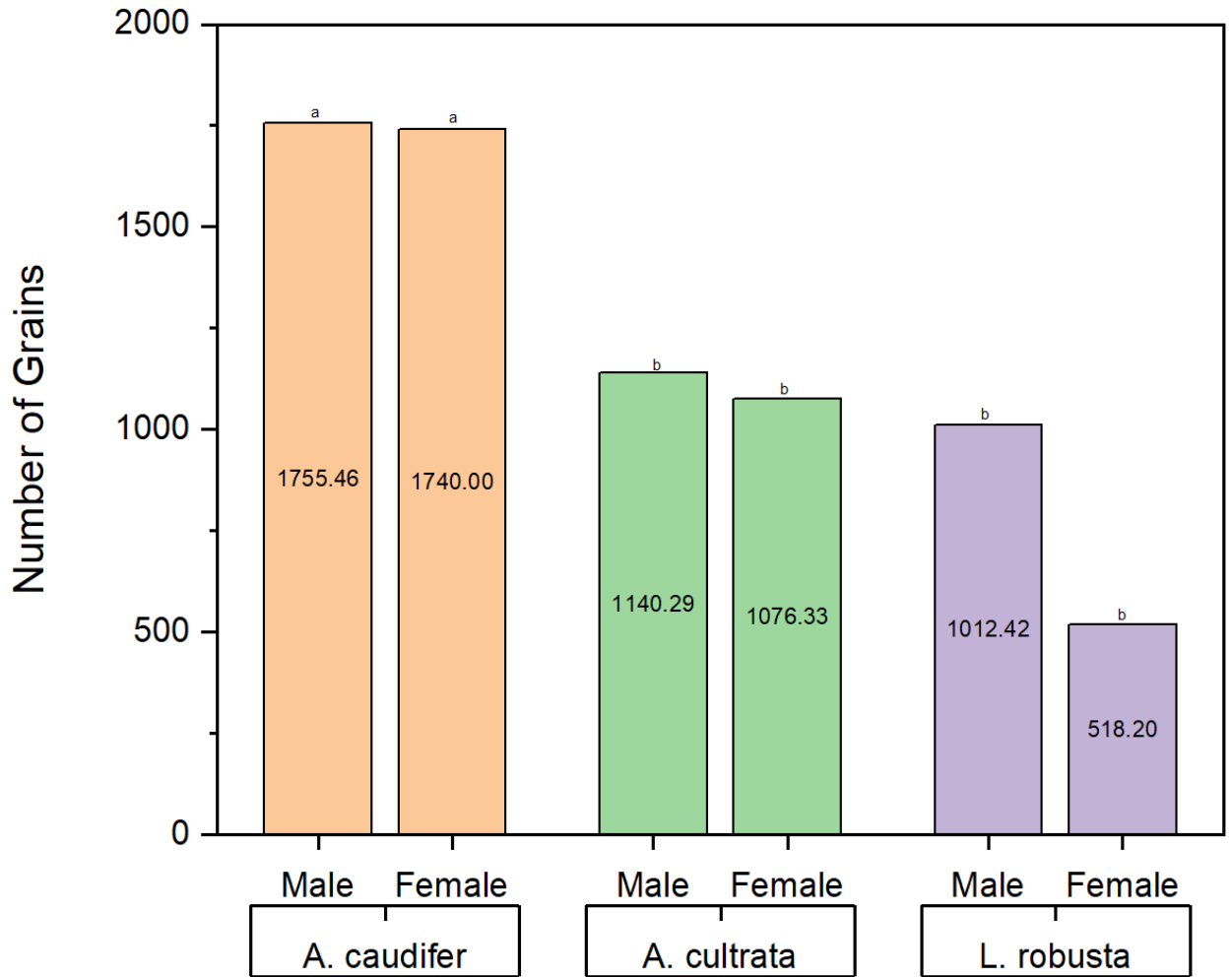


Figure 2. Average time spent grooming between visits to two female flowers. For each bat species the following steps were conducted. First, number of pollen grains for each trial for each individual bat were averaged together. Second, each individual's average was averaged across each bat species.

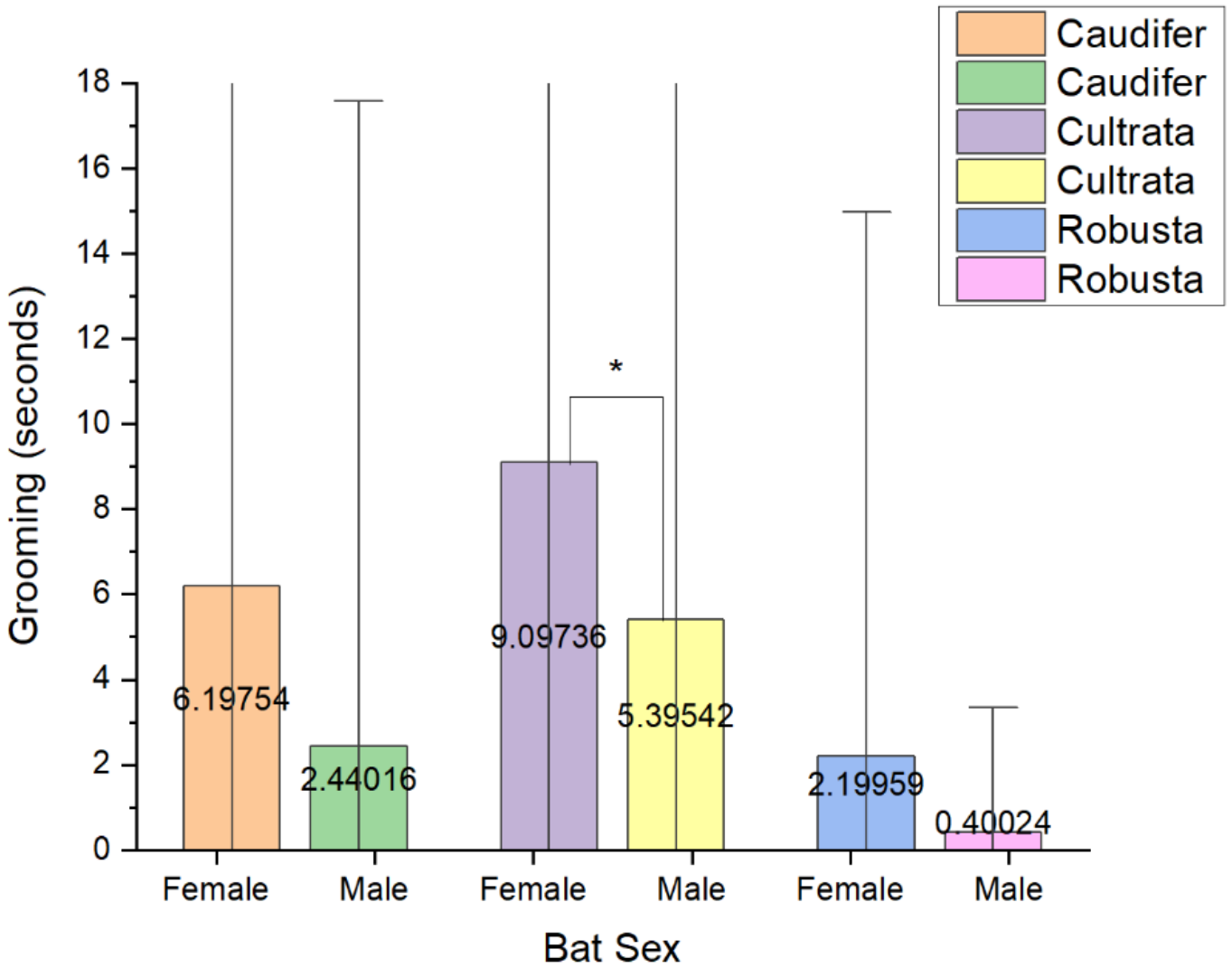


Table 1. Carryover curve estimation for inverse regression model. The number of pollen grains deposited at each flower position for each trial was converted into a proportion of total pollen deposited during that trial. The proportion deposited at each flower position per trial was averaged for each individual. This resulted in 10 data points per bat representing average proportion of pollen deposited at each of the 10 flower positions. Regression plots represent data from each individual of a given bat species. Curve estimations for multiple regression models were computed and the model with the greatest explanator power (R^2) was chosen. In all cases the inverse regression model best fit the data. See **Figure 1A-C** for a graphical representation.

	R^2	F	p-value	Equation
<i>A.caudifer</i>	0.32	155.23	<0.001	$\frac{0.32}{X} + 0.006$
<i>A.cultrata</i>	0.56	263.07	<0.001	$\frac{0.502}{X} - 0.047$
<i>L.robusta</i>	0.56	276.82	<0.001	$\frac{0.370}{X} - 0.008$

Figure 3A. Carryover curve estimation for inverse regression model. Regression plot with fitted inverse equation for the carryover curve for *Anoura caudifer*. See Table 1. for more information.

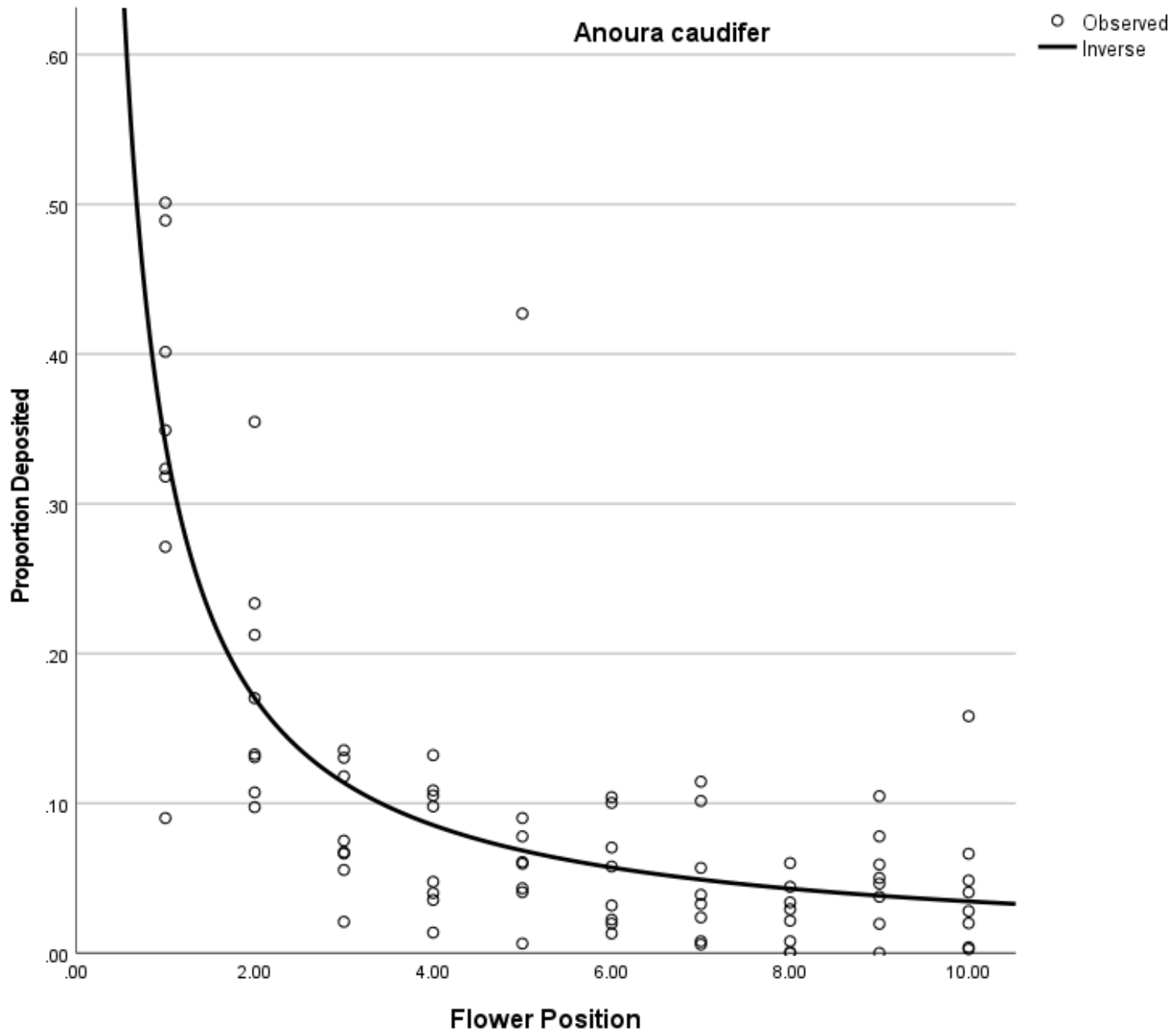


Figure 3B. Carryover curve estimation for inverse regression model. Regression plot with fitted inverse equation for the carryover curve for *Anoura cultrata*. See Table 1. for more information.

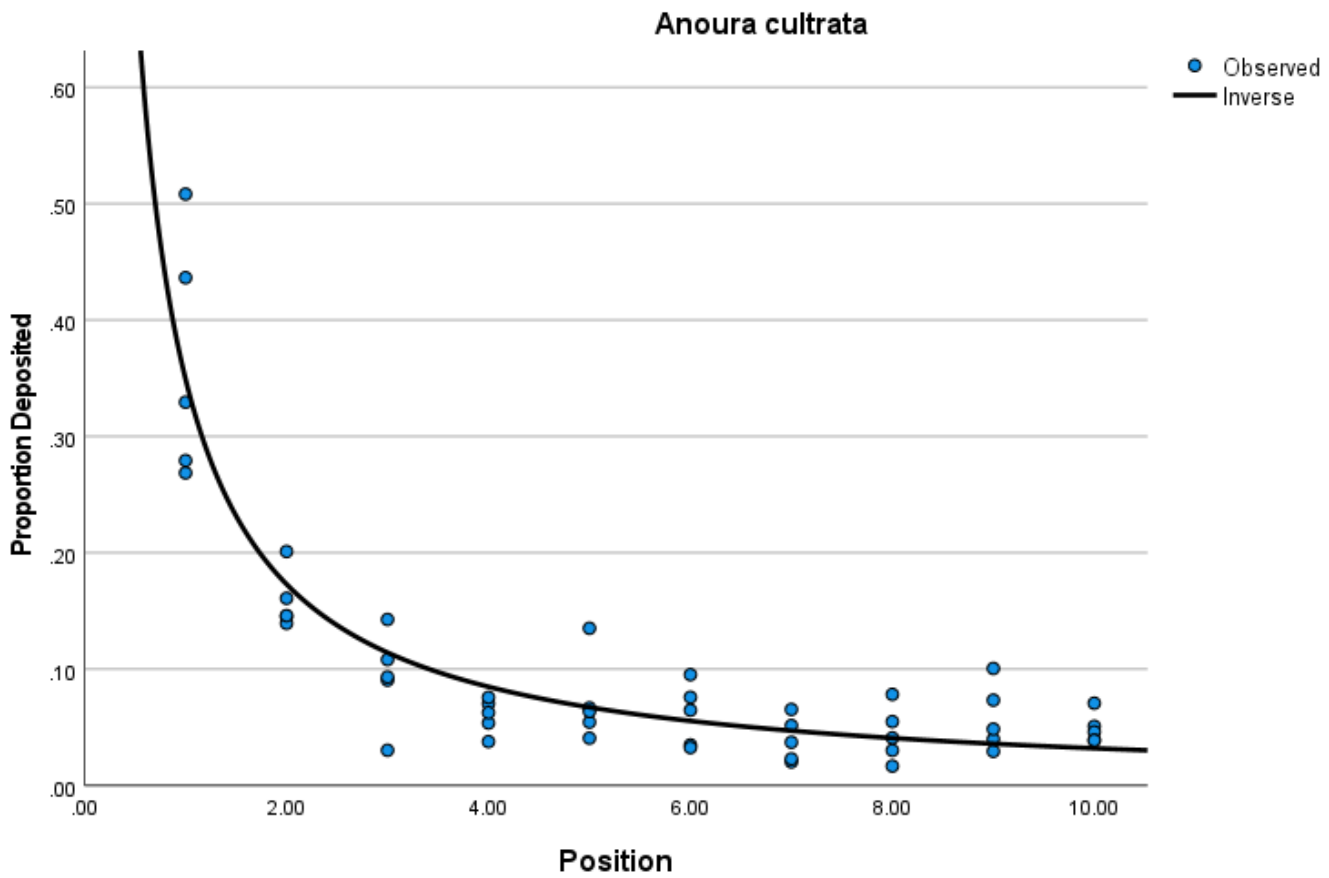


Figure 3C. Carryover curve estimation for inverse regression model. Regression plot with fitted inverse equation for the carryover curve for *Lonchophylla robusta*. See Table 1. for more information.

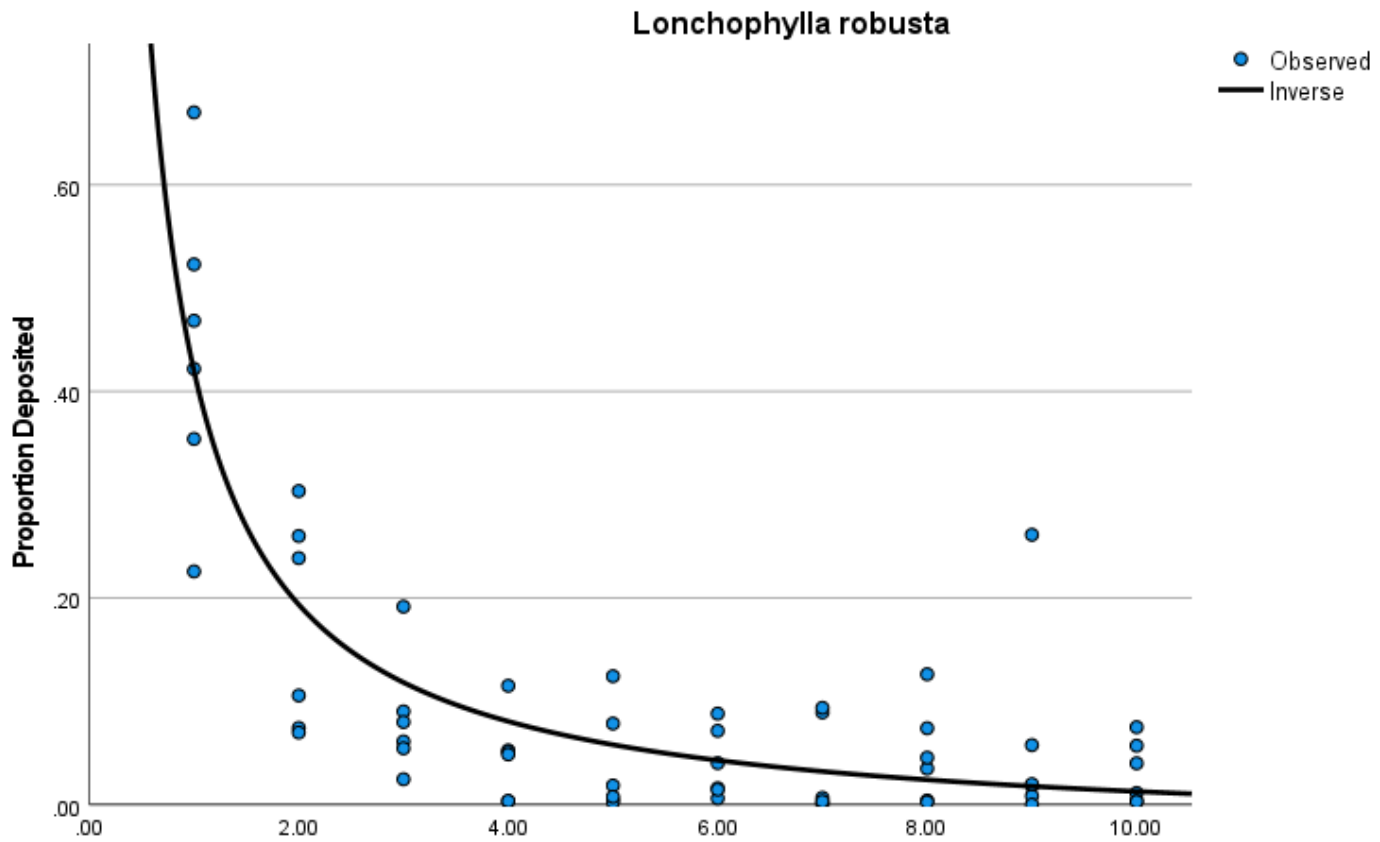


Table 2. Summary of the four linear regression analysis for the influence of grooming on

deposition. Each row is a quick description of the regression analyzed, including (except for Regression 4) the grooming coefficient, statistical significance, and whether any factors were also significant. For Regression 4, the independent variable of interest was flower position.

Regression 3, as the factor “Sex” was significant, analyses were repeated but only for one bat sex at a time.

	Grooming Coefficient (GC)*	t-value	p-value	Other Significant Coefficients of Note
1. Time Spent Grooming by Proportion Deposited at Each Flower	-0.627	-1.541	0.124	N/A
2. Time spent grooming by ratio of deposition at Flower _{x-1} :Flower _x	-0.025	-1.07	0.287	N/A
3. Time spent grooming by cumulative proportion deposited after grooming	-0.001	2.081	0.038	Sex: p<0.001
3A. Time spent grooming by cumulative proportion deposited after grooming MALES ONLY	0.00	0.014	0.989	N/A
3B. Time spent grooming by cumulative proportion deposited after grooming FEMALES ONLY	0.003	3.216	0.002	N/A
4. Time spent grooming by flower position	*In this case the coefficient of interest was that of Flower Position 0.03	0.179	0.858	N/A

Figure 4. Scatterplot of flower position by time spent grooming (milliseconds). Data is the average across all trials of each individual's grooming time before a given female flower position. No separation was made between male and female bats as Regression 4 was non-significant for flower position and for the factor "Sex".

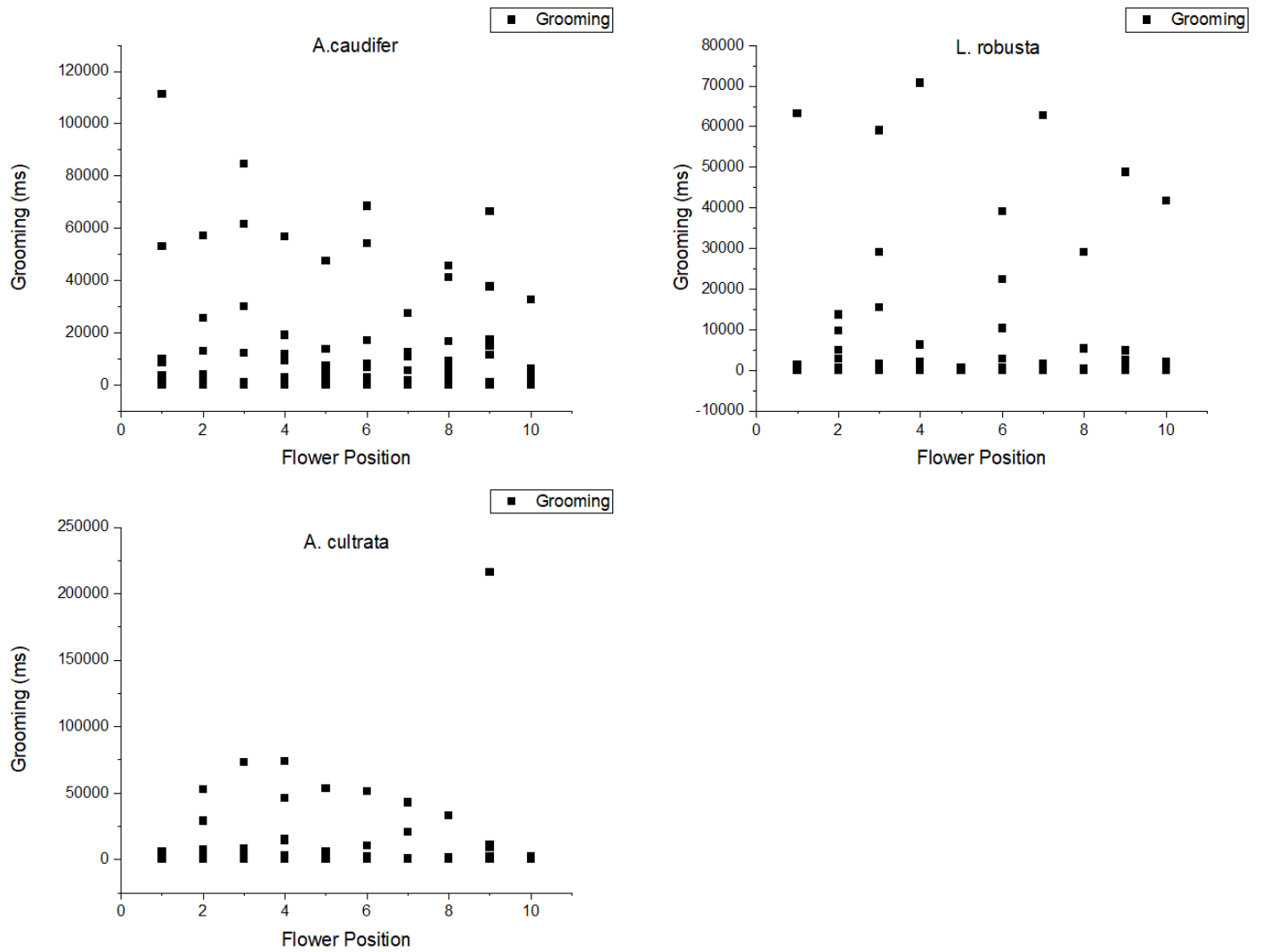


Table 3. Summary table of movements for each recorded visitor at the three *Asclepias*

species. Summation of movements for each visitor across all 20-minute focal-plant observations for each *Asclepias* species. Individuals were identified to the most specific taxonomic level and those that were not identifiable were placed in an “Other” category. “Same” movements refer to movements within the same plant (between flowers or between inflorescences), “Different” movements refer to movements between conspecific plants within the focal observation area, and “Leave” movements refer to movements away from the focal plant and away from the focal

	<i>Same</i>	<i>Different</i>	<i>Leave</i>	<i>Total</i>
				<i>Asclepias tuberosa</i>
Halictidae	409	69	130	608
<i>Apis</i>	162	41	11	214
<i>Bombus</i>	55	35	10	100
Beetle	9	1	3	13
<i>Xylocopa</i>	8	8	2	18
Butterfly	7	8	5	20
Bird	6	0	1	7
Syrphidae	3	3	2	8
Megachilidae	3	2	0	5
Moth	3	0	2	5
<i>Andrena</i>	2	2	1	5
Ant	1	0	1	2
Sum	668	169	168	1005
				<i>Asclepias syriaca</i>
<i>Bombus</i>	166	65	26	257
<i>Apis</i>	95	30	12	137
Moth	15	7	13	35
Halictidae	21	5	8	34
Butterfly	17	9	4	30
<i>Xylocopa</i>	13	9	8	30
Beetle	0	2	2	4
Ant	0	2	2	4
Megachilidae	2	1	0	3
<i>Agapostemon</i>	2	1	0	3
Syrphidae	0	1	1	2
Sum	329	130	76	535
				<i>Asclepias sullivantii</i>
Halictidae	6	0	5	11
<i>Apis</i>	327	72	74	473
<i>Bombus</i>	93	36	23	152
Megachilidae	1	0	1	2
Sum	427	108	103	638
				<i>Asclepias viridis</i>
Halictidae	3	3	4	10
<i>Apis</i>	0	0	0	0
<i>Bombus</i>	10	9	6	25
<i>Xylocopa</i>	2	4	0	6
Beetle	4	4	1	9
Sum	19	20	10	49

Figure 5. Comparison of movements between the three main visitor groups at the three *Asclepias* species. For the purpose of studying geitonogamy, only two types of movement categories were used for statistical analyses. “Same” refers to a movement within the focal plant, and “Away” refers to a movement away from the focal plant, regardless of direction. Lines and asterix represent significant differences with $p < 0.05$.

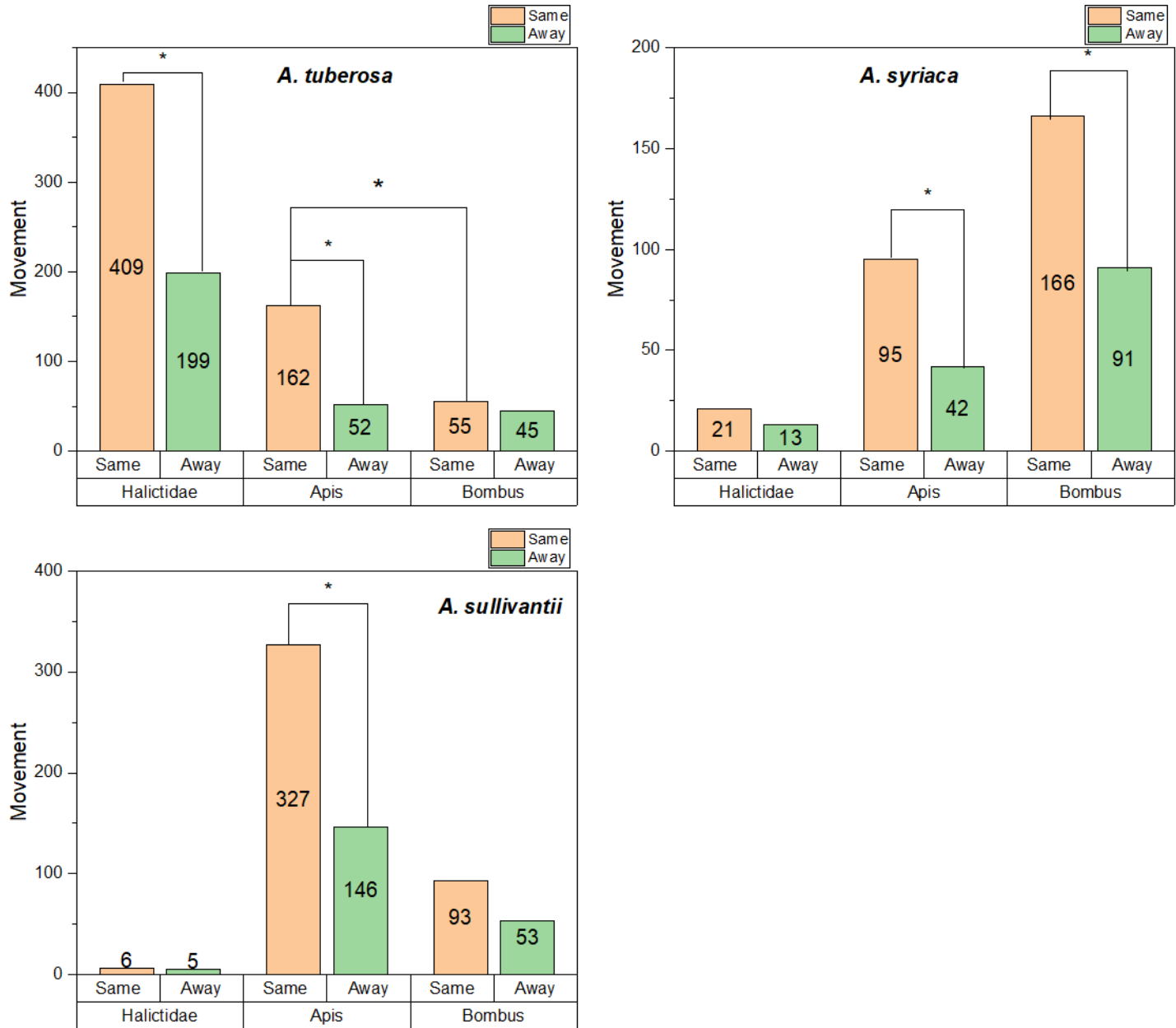


Figure 6. Comparison of proportion present between the three main visitor groups at the three *Asclepias* species. As a substitute for visitation rate proportion present was used.

Proportion present for a given visitor group at a given *Asclepias* species is defined as the numbers of focal plant observations in which at least one individual of said visitor group was recorded divided by the total number of focal plant observations. The first graph (top left) includes all visitor groups and all plant species. Lines and asterix in this graph here represent significant differences *between* visitor groups *within* the same plant species. The remaining three graphs compare proportion present *within* the same visitor group *between* different plant species. Lines and asterix here represent significant differences in the proportion presence of a given visitor group between different plant species. All analyses were significant when $p < 0.05$.

