

THE TRIPPING MECHANISM OF FLOWERS AFFECTS POLLEN TRANSFER DYNAMICS

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Abstract—Insect pollinators affect pollen transfer dynamics, with consequences for pollen movement and the genetic structure of plant populations. Pollen transfer dynamics has not been previously examined in flowers with a tripping mechanism. Here we examine whether pollen accumulated on a bee's body increases with the number of *Medicago sativa* L. flowers tripped by *Bombus impatiens* Cresson during a foraging bout, while controlling for bee body size and number of visited flowers. In a second experiment, we determine whether the number of revisits to a tripped flower increases pollen deposition onto the stigmas. We set up three *M. sativa* plants with a controlled number of racemes in a greenhouse room, and followed individual bees as they foraged, recording each plant, raceme, and flower visited. For pollen accumulated, we collected bees at the end of their foraging bout and counted pollen grains on their body. For pollen deposition, we collected flowers with between 0 and 6 revisits and counted the pollen grains on the stigmas. The number of pollen grains on a bee's body increased with the number of flowers tripped in a foraging bout, but was not affected by the number of flowers visited or the size of individual bees. The number of pollen grains deposited on a stigma did not increase with the number of revisits to a tripped flower. This latter result contrasts with plants without a tripping mechanism where the number of visits increases pollen deposition and seed set. Tripping affects pollen transfer dynamics and we discuss how its effect may vary with the mode of tripping.

Keywords—Bumble bee, flower visits, pollen deposition, pollen grains on the bee body, *Medicago sativa*, tripped flowers.

INTRODUCTION

Pollinators are the main vectors transferring pollen from flower to flower for a majority of flowering plant species (Ollerton et al. 2011). As a result, pollinators affect not only pollen transfer dynamics, but also pollen dispersal, and plant reproductive success of most flowering plants (Adler & Irwin 2006; Richards et al. 2009; Ratto et al. 2018). Increasing pollinator abundance can increase fruit and seed set (Thomson 2019), and in agriculture, insect pollinators such as honey bees, bumble bees, and leafcutting bees are often managed to ensure sufficient crop pollination and

production. Even in plants that are capable of self-pollination, pollinating insects can increase seed set (Lang & Danka 2001; Abrol 2007) and fruit production (Klein et al. 2003). Pollen transfer dynamics affects how far pollen and the genes they carry are moved (Castellanos et al. 2003; Santa-Martinez et al. 2021; Fragoso & Brunet 2023), and these processes influence the genetic structure of plant populations (Slatkin 1987). In agriculture, these processes also affect the potential for escape of genetically engineered cultivar genes (Kershen & McHughen 2005). Given these implications, it is important to understand how pollinators affect pollen transfer dynamics of plant species.

Pollen transfer by insects is a dynamic process. Bees may actively forage for pollen or passively accumulate it as they collect nectar, or pollen may be collected incidentally onto non-target areas of the body during active pollen collecting (Portman et al. 2019). Interestingly, the foraging behavior of a pollinator can affect its pollen transfer dynamics. Wilson and Thomson (1991) observed pollen-collecting bees deposited fewer pollen grains on stigmas relative to nectar-collecting bees. Similarly, honey bees foraging on *Impatiens capensis* deposited more pollen on stigmas when foraging for nectar than for pollen (Young et al. 2007). In general, the amount of pollen on a pollinator's body that is available for pollination tends to increase as pollinators visit successive flowers and to decrease as pollinators deposit pollen onto stigmas, groom pollen into their corbiculae, or simply lose pollen when it falls off their body (Harder & Wilson 1998). However, there is little correlation between the amount of pollen carried on a pollinator's body and the number of pollen grains deposited on the stigmas of the plant species they are visiting (Adler & Irwin 2006). A variety of processes may help explain this lack of correlation (Minaar et al. 2019).

A small proportion, less than 1% of the pollen removed from a flower by an apid bee, will reach a stigma (Thomson & Goodell 2001). As a bee forages, pollen accumulates in layers on different parts of its body, and the pollen grains in the different layers have different probabilities of reaching a stigma (Harder & Wilson 1998). When a bee grooms, pollen that has accumulated onto the bee's body is moved into the corbiculae, and this pollen becomes unavailable for pollination (Harder 1990). The pollen in the pollen sacs is returned to the hive to feed the developing larvae (Rasheed & Harder 2003). Pollen can also fall off the bee's body during grooming (Castellanos et al. 2003; Tong & Huang 2018). The amount of pollen that is accumulated onto a bee's body and available for pollination is thus highly variable (Harder & Wilson 1998) and can differ between bee species visiting the same plant species (Adler & Irwin 2006).

Pollen deposition can similarly vary greatly between plant species (Snow & Roubik 1987) and among flowers of the same plant species (Thomson 1986). The amount of pollen deposited onto the

stigmas of a plant species by a pollinator is affected by different factors (Thomson & Goodell 2001; Adler & Irwin 2006). For example, in two Panamanian buzz pollinated tree species, *Cochlospermum vitifolium* and *Cassia reticulata*, the amount of pollen deposited per visit by different pollinators was highly variable between the two plant species, but not affected by pollinator size or abundance (Snow & Roubik 1987). However, a meta-analysis of pollen deposition by 127 different pollinator species found an effect of pollinator size, with larger pollinators depositing more pollen per visit than smaller pollinators (Foldesi et al. 2020). Morphological differences between pollinator species do not always explain the variation in pollen deposition on the same plant species (Richards et al. 2009), although flower morphology, and landing approach and orientation of bumble bees have been found to affect the amount of pollen deposited on stigmas (Thomson & Plowright 1980). In a perennial herb, *Delphinium nelsonii*, pollen deposition by bumble bees was found to be extremely variable on flowers visited in succession likely due to the positioning of the pollinator in relation to the stigma (Waser 1988). The high degree of variation in pollen quality, shape, and adhesion also increases variability in pollination systems (Thomson et al. 1986).

While pollen accumulated on a bee's body and pollen deposition on stigmas have been examined in different systems (Harder & Wilson 1998; Adler & Irwin 2006), less is known about these patterns in flowers with a tripping mechanism. Such flowers are common in the plant families Fabaceae and Lamiaceae. In these plant species, the flower remains closed until a pollinator or other force exerts pressure onto the keel petals of the flower, which releases the sexual column (anthers and stigmas) (Larkin & Graumann 1954). During the tripping process, the stigma brushes the pollinator's body as the anthers simultaneously deliver pollen onto the pollinator. Differences in tripping rates among pollinator species can affect the distance traveled by pollen (pollen dispersal), and subsequent gene flow, with bee species with higher tripping rates moving pollen shorter distances relative to bee species with lower tripping rates therefore limiting gene flow (Santa-Martinez et al. 2021; Fragoso & Brunet 2023). However, less is known about the fundamental

processes of pollen transfer dynamics, such as pollen accumulation on a bee's body and pollen deposition on stigmas, for plants with a tripping mechanism.

In this study, we investigate two fundamental aspects of pollen transfer dynamics for a bumble bee species visiting flowers with a tripping mechanism. In a first experiment, we examine whether the number of pollen grains on the body of the common eastern bumble bee *Bombus impatiens* increases as it visits and trips more *Medicago sativa* (Fabaceae) flowers. We concentrate on pollen grains most likely to become available for pollination, that is, on parts of the bee's body that come in contact with the alfalfa's stigmas. We control for the size of a bee, and distinguish between visits to a flower and tripping of a flower. In a second experiment, we determine whether the number of pollen grains deposited on stigmas of alfalfa flowers increases with greater number of revisits to a tripped flower. Unlike plants with open flowers, in plants with a tripping mechanism like *M. sativa*, it is unclear whether revisits to tripped flowers would increase the number of pollen grains on the stigma. Pollen transfer dynamics influences plant reproductive success, pollen dispersal, and gene flow, and it is thus important to examine whether its mechanisms differ in a plant species with a tripping mechanism such as *M. sativa*.

MATERIALS AND METHODS

STUDY SYSTEM

Medicago sativa L., known as alfalfa or lucerne, is a perennial plant in the Fabaceae family grown throughout the world as a forage crop. Flowers are organized in racemes containing up to 40 flowers that can range in color from white to dark purple, but are fairly similar within a plant (McGregor 1976). Each flower has a sexual column including ten stamens and the stigma, which is enclosed by two keel petals and must be released in a process called tripping for pollination to be successful. Tripping occurs when a pollinator applies pressure to the keel, which opens the flower and releases the reproductive organs. When released, the sexual column strikes and dusts the pollinator with pollen, while previously acquired pollen on the bee's body comes in contact with the stigma (McGregor 1976). Tripping ruptures the stigmatic

membrane which releases the moisture needed for pollen grains to germinate (Armstrong & White 1935).

Bombus impatiens Cresson, known as the common eastern bumble bee, is a eusocial generalist species widespread across eastern North America. This bee species commonly visits alfalfa flowers (Brunet & Stewart 2010; Boyer et al. 2020) and is the most common wild bumble bee species in Wisconsin. For the experiments described here, commercial *B. impatiens* hives were purchased from Koppert Biological Systems, Inc. (Howell, MI). This bumble bee species tripped $50.67\% \pm 0.03$ (mean \pm SE) of alfalfa flowers visited in a field setting (Brunet et al. 2019a), and a similar proportion (51.6%) in a greenhouse setting (Santa-Martinez et al. 2021).

EXPERIMENTAL SETUP

A bumble bee hive containing roughly 75-100 workers was used in these experiments. The hive was placed inside a $2.0 \times 2.0 \times 1.8$ m cage built with metal poles and a fine mesh cover. Flowering alfalfa plants were placed inside the cage to train bees to forage on alfalfa. At least 24 hours prior to a trial, all bees were caught and returned to the hive, and the hive entrance was closed. At the beginning of each trial, three flowering alfalfa plants were placed in a line perpendicular to the hive entrance. Because these plants could each have over 500 flowers and we needed to either follow bees visiting and tripping up to 12 flowers, or examine flowers revisited zero to six times, we limited the number of racemes available to the bees, as indicated below, and covered the rest of the flowers with fine mesh fabric so they were unavailable. Plants were replaced as needed when they lacked sufficient numbers of racemes and flowers per raceme.

At the beginning of each experimental run, four or five bumble bees were released from the hive and we waited for one of the bees to start foraging. If none of the bees foraged within five minutes of being released, they were collected and new bees were released. When a bee began to forage, the other bees were removed from the cage and only the foraging bee was observed. These other bees were typically flying or crawling at the corners of the cage and were removed by a separate observer.

EXPERIMENT 1: POLLEN GRAINS ACCUMULATED ON THE BEE'S BODY

For this experiment, we used five to ten flowering racemes distributed as evenly as possible over the three plants which were equidistant and approximately one foot apart. Tripped, damaged, or unopened flowers within a raceme were removed prior to each trial and the number of open flowers per raceme was kept at five or very close to it. The number of racemes used in the experiment provided enough flowers to obtain up to 12 tripped flowers in a foraging bout and permitted the observer to follow individual bees.

Bee observations

For each foraging bout, we recorded whether a flower was “visited” (flower was visited but not tripped) or “tripped” (flower was visited and tripped) by a bee. Bees were allowed to forage until they tripped a predetermined number of flowers, between zero and twelve, at which point they were collected in clean 50 mL centrifuge tubes. A new bee was used for each foraging bout. One to six foraging bouts were completed each day for 15 days.

Because we observed some pollen grains on bees that visited but did not trip any flowers (Table 1), we captured eight bees as they exited the hive. We counted the number of pollen grains on their bodies to test whether bees leaving the hive carried some pollen grains on their bodies, as this could explain the presence of pollen grains on bees that had visited but not tripped any flowers during their foraging bout.

Bee handling and body size measurement

Each centrifuge tube with a bee was immediately placed in a cooler filled with ice packs. Once the bee became immobile, it was placed under a dissecting microscope and pollen was removed from the wings, and the dorsal side of the abdomen and thorax, using a PELCO vacuum pick-up system with a 20-gauge needle tip (Ted Pella Inc., product number 520-1-220, Redding, CA). Pollen was removed from these parts of the bee's body because they have previously been identified as not coming in contact with the alfalfa stigma (Koch et al. 2017), which was confirmed via observations prior to beginning the experiments. The corbiculae were also

removed as pollen in the corbiculae is not available for pollination (Thomson 1986).

After completing these steps, we removed the right forewing and saved it on paper using clear tape. The paper was scanned and the resulting image was uploaded into the software program ImageJ, where the marginal cell length of each wing was measured (Abramoff et al. 2004). As the length of the marginal cell correlates with the body size of bees (Nooten & Rehan 2020), marginal cell length, or “wing size”, was used as an estimate of body size. After removing its right wing, the bee was returned to its original centrifuge tube and stored in a -20°C freezer until the pollen grains remaining on the bee's body could be counted using a particle counter (Z Series Coulter Counter, Beckman Coulter Life Sciences, Indianapolis, IN).

Pollen grain count

Bees were placed in a plastic vial Coulter cup containing 10mL of 1% filtered NaCl solution and sonicated for seven minutes in a Branson 2510 Ultrasonic Cleaner (Brandon Ultrasonics Corp., Danbury, CT). After sonication, bees were removed from the solution, examined under a microscope to ensure all pollen grains were removed, and returned to the freezer.

The pollen solution was stirred for three minutes using a magnetic stirrer bar. We added 1 mL of the pollen solution to each of three Coulter counter vials containing 10 mL of 1% filtered NaCl solution to create three samples for counting. A blank was run before each set of samples to control for non-pollen particles. Each sample was stirred for three minutes using a magnetic stirrer bar before counting, then immediately transferred to the particle counter. The outside of the particle counter aperture tube was rinsed with 1% NaCl solution between samples. The particle counter was set to count particles sized 17 μm – 50 μm to encompass the range of alfalfa pollen grain sizes (Lehman et al. 1969; Tondini et al. 1993). To determine the number of pollen grains collected on each bee body the three sample counts were averaged, the count value of the blank was subtracted, and the result was multiplied by 220 to account for the total volume of the pollen solution.

Data Analysis

To determine the impact of the number of flowers tripped in a foraging bout (independent

variable) on the number of pollen grains present on the bee body (dependent variable), we first performed an analysis of covariance with body size and total flowers visited as covariates in the model, using proc ANCOVA in SAS 9.4 (SAS Institute 2016). Because the two covariates were not statistically significant (see results section - experiment 1), we performed a regression analysis between the number of pollen grains present on the bee body and the number of flowers tripped in a foraging bout using proc REG in SAS 9.4 (SAS Institute 2016). Pollen counts were log transformed prior to analysis to improve the model's residuals.

To compare the number of pollen grains on bees exiting the hive with the number of pollen grains on bees that visited but did not trip any flowers, we performed a Welch two sample t-test using the t-test function in the stats package in R version 4.0.3 (R Core Team 2020).

EXPERIMENT 2: POLLEN GRAINS DEPOSITED ON STIGMAS

For this experiment, we used five to seven racemes on each of the three plants and trimmed each raceme to have five open and untripped flowers. Each plant was labeled, each raceme on a plant was marked with different-colored yarn tied around the peduncle, and each flower within a raceme was marked with different-colored dots of fabric paint. This process was repeated at the beginning of each trial. Our experience with this system indicated that these markings did not affect bee foraging behavior. The goal was to obtain flowers that had received between zero and six revisits.

Bee observations

For each trial, and each flower visited by a bee, we recorded the plant number, raceme yarn color, and flower paint color, and noted whether a flower was tripped during that bee visit. Bees were allowed to forage no longer than 20 minutes during experimental runs to permit stigmas to be isolated and placed on slides with fuchsin jelly within a 45-minute time period (see stigma collection and pollen grain count section). If the bee did not return to the hive within 20 minutes, it was captured and placed back in the hive. This 20-minute time period was sufficient for bees to visit many flowers and revisit individual flowers several times. Data from bees placed back in the hive were included in the analyses.

Stigma collection and pollen grain count

After each experimental run we counted the number of times each flower in a foraging bout had been revisited (visited after being tripped). A flower that was tripped by a bee but not revisited was categorized as zero revisit. After each run, we typically collected one randomly selected flower per available category, for each category between 0 and 6 revisits. We obtained 43 trials over 14 days, and between 10 - 30 flowers per revisit category. Flowers were dissected upon collection and stigmas removed under a dissecting microscope. Each stigma was placed on a glass slide with melted fuchsin jelly (Kearns & Inouye 1993) within 45 minutes of the start of a trial to prevent pollen tube growth. Dissection tools were cleaned with 75% ethanol between each stigma to prevent contamination. After samples were returned to the laboratory, the number of pollen grains on each slide was counted under a light microscope at 10 x magnification. Two separate observers counted each slide and their pollen grain counts were averaged.

Data Analysis

To determine the impact of the number of revisits on the number of pollen grains deposited on the stigma, we used a linear mixed-effect model using proc Mixed in SAS 9.4 (SAS Institute 2016). Revisit to a flower was the fixed effect in the model (independent variable), foraging bout was the random effect, and the number of pollen grains deposited on the stigma was the dependent variable. Pollen counts were log transformed prior to analysis to improve the model's residuals.

RESULTS

EXPERIMENT 1: POLLEN GRAINS ACCUMULATED ON THE BEE'S BODY

We counted pollen grains on the body of 60 bees in this experiment (Appendix 1 - Pollen on bee's body). We grouped bees that had tripped the same number of flowers per foraging bout, a number which ranged between zero and twelve, and obtained between one to ten bees per category (number of tripped flowers in a foraging bout) (Table 1). For each category, we calculated the average wing size (cm) of the bees, the average total number of flowers visited by a bee during a foraging bout, and the average total number of

Table 1. The number of tripped flowers per foraging bout, the sample size (N) is the number of bees examined, mean wing size as an estimate of body size, mean number of flowers visited in a foraging bout, and mean number of pollen grains on the bee body.

Number of tripped flowers	Number of bees (N)	Wing size (cm)		Number of flowers visited		Number of pollen grains on a bee	
		mean	SE	mean	SE	mean	SE
0	9	0.256	0.006	5.6	1.3	1,678.5	245.8
1	5	0.245	0.010	4.4	1.5	1,789.3	415.2
2	10	0.254	0.006	6.7	1.9	5,082.0	862.0
3	6	0.269	0.011	16.5	2.9	3,935.6	758.0
4	5	0.241	0.006	20.4	4.9	6,218.7	1,436.4
5	5	0.255	0.013	17.6	2.7	6,262.7	2,597.0
6	5	0.222	0.005	22.4	2.0	8,829.3	2,058.0
7	3	0.255	0.015	24.0	3.0	7,296.7	3,263.3
8	2	0.258	0.007	31.3	13.3	7,553.3	887.1
9	1	0.285	N/A	31.0	N/A	12,613.3	N/A
10	4	0.266	0.006	22.5	2.4	14,483.3	1,434.1
11	1	0.264	N/A	29.0	N/A	7,480.0	N/A
12	4	0.260	0.011	22.5	2.7	12,430.0	3,564.3

pollen grains on the bee's body at the end of a foraging bout (Table 1).

The covariance analysis indicated no effects of wing size or total number of flowers visited in a foraging bout on the number of pollen grains on the bee's body (Table 2); it was only affected by the number of flowers tripped in a foraging bout (Table 2). When we performed a regression analysis over all foraging bouts, the number of pollen grains on a bee's body (Y) increased by an average of 954 grains (slope) for each tripped flower (X), $Y = 954.29 X + 1968.42$, $F_{1,58} = 61.8$, $P < 0.001$ (Fig. 1). This model explained 51.6% of the variance in the number of pollen grains on the bee's body. The intercept was 1968.42 which is close to the number of pollen grains found on bees exiting the hive (1650 ± 359.5) (mean \pm SE). In addition, the number of pollen grains on the bees exiting the hive (Appendix 1 - Pollen on bee leaving the hive) was similar to the number of pollen grains on bees that visited but did not trip any flowers during a foraging bout (1678.5 ± 245.8 grains) ($t_{12,7} = -0.066$, $P = 0.95$). Therefore, visiting flowers without tripping them did not modify the amount of pollen on a bee's body.

EXPERIMENT 2: POLLEN GRAINS DEPOSITED ON STIGMAS

We collected 196 stigmas over 43 trials (Appendix 1- Pollen grains on stigma), and obtained at least ten stigmas per treatment group (zero to six revisits) (Table 3). The number of revisits had no effect on the number of pollen grains deposited on the stigma ($F_{6,147} = 1.03$; $P = 0.41$) (Fig. 2). We observed variation among foraging bouts (run effect $P = 0.03$), and 104.03 ± 6.01 (mean \pm SE) pollen grains were deposited on a stigma overall (all number of revisits) ($N = 196$).

Table 2. The impact of the number of tripped flowers (numtrip) in a foraging bout on the number of pollen grains on a bee's body when wing size of the bee and the total number of flowers visited during a foraging bout (numvisits) are used as covariates in the model using an analysis of covariance.

Factor	df	MS	F	Pr > F
wingsize	1	158082.8	0.01	0.91
numvisits	1	11080885.5	0.92	0.34
numtrip	12	52485179.2	4.34	0.0001

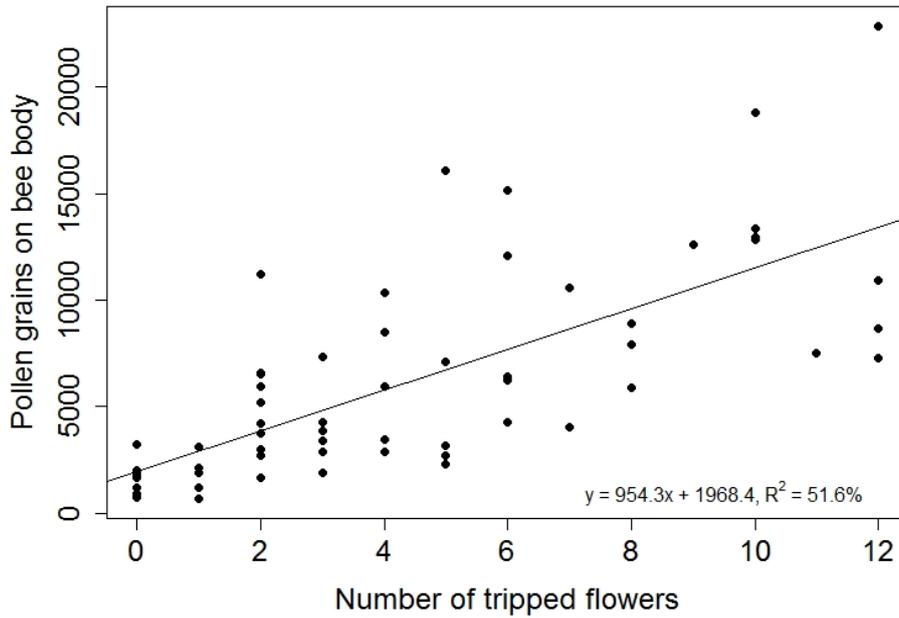


Figure 1. The number of pollen grains on a bee's body increases with the number of tripped flowers per foraging bout.

Table 3. The number of pollen grains (mean ± SE) deposited on stigmas of *Medicago sativa* flowers after different numbers of revisits to a flower. N, the number of stigmas, is the sample size.

Number of revisits	Number of stigmas (N)	Mean pollen grains	SE
0	34	114.9	18.1
1	36	86.8	9.1
2	37	137.5	17.1
3	31	95.1	14.4
4	28	97.0	15.2
5	19	91.1	12.9
6	11	80.0	14.5

DISCUSSION

This study investigates pollen transfer dynamics in *B. impatiens* visiting *M. sativa* flowers. It examines the relationships between the number of flowers tripped by a bee during a foraging bout and the number of pollen grains accumulated onto the bee's body; and between the number of revisits to a tripped flower and the number of pollen grains deposited on the stigma. The number of pollen grains accumulated on the bumble bee's body increased when more flowers were tripped in a foraging bout. Revisits to tripped flowers by bumble bees did not, however, augment the number of pollen grains deposited on the stigmas.

When a bee visits and trips a flower, it accumulates more pollen grains on its body on

average relative to the number of pollen grains it deposits on a stigma. If a bee were to deposit as much pollen on the stigmas as it accumulates from a flower during a visit, then we would not expect an increase in the number of pollen grains on the bee's body when more flowers are tripped during a foraging bout. In this study, prior to counting pollen grains on the bee's body, we removed pollen grains from areas known not to come into contact with the stigmas (Koch et al. 2017), and from the corbiculae as that pollen does not play a role in pollination (Harder & Wilson 1998). This procedure was followed to increase the likelihood that the pollen grains counted on the body of the bees represent grains likely to get deposited on stigmas and be involved in the pollination process. Even when concentrating on pollen grains most

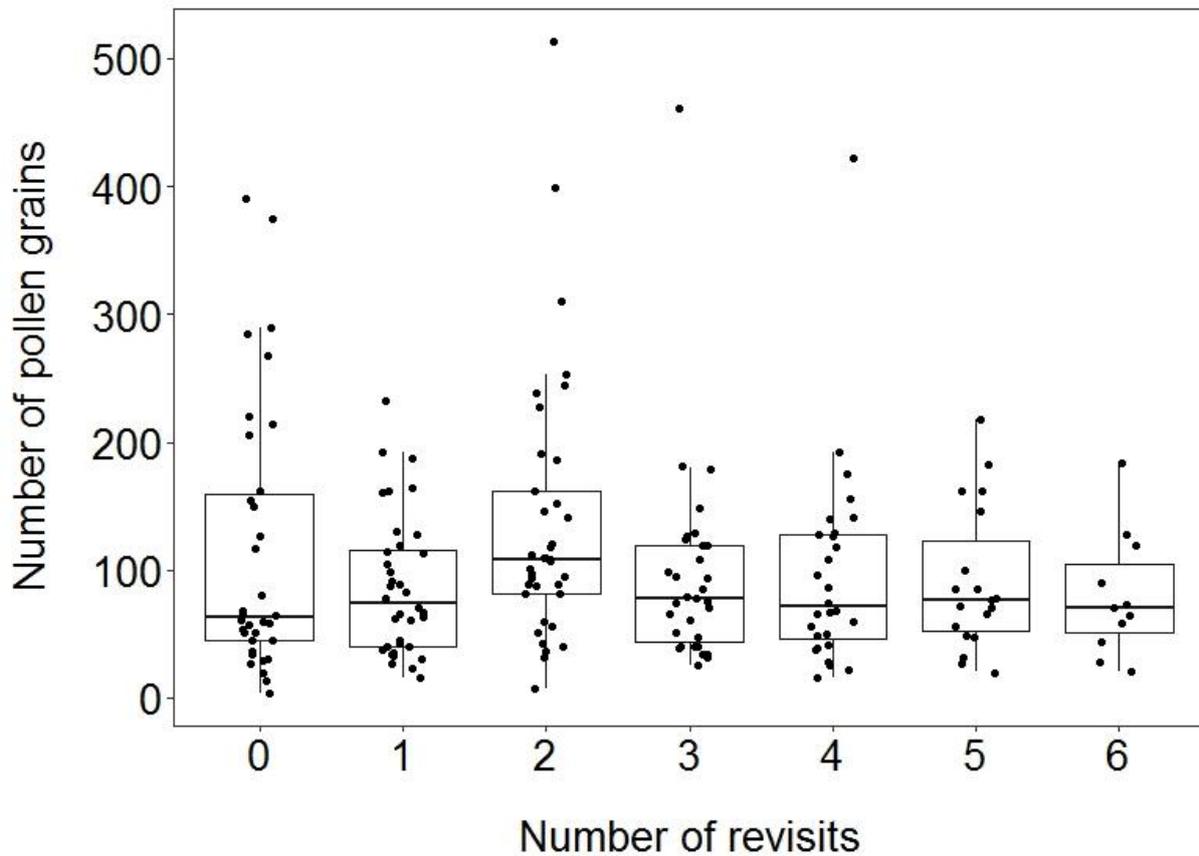


Figure 2. The number of pollen grains deposited on stigmas of *Medicago sativa* flowers following between zero and six revisits to a flower by bumble bees. Median and percentiles (25-75%) and individual data points are presented.

likely to be involved in pollination, a bee accumulated on average 954 pollen grains on its body for each flower tripped, while depositing 104.03 ± 6.01 (mean \pm SE) pollen grains on a stigma. Such a pattern of pollen accumulation could lead to a greater number of pollen grains being deposited on flowers tripped later rather than early in a foraging bout, although a study in a different system found no correlation between the amount of pollen on a bee's body and the number of pollen grains deposited on stigmas (Adler & Irwin 2006). It also suggests that a bee will return to the hive with pollen accumulated on its body, and we did find pollen on the bee's body as it exited the hive.

It would be of interest to determine if the pollen present on the bee's body as the bee exits the hive is viable and if it gets deposited on stigmas, at least for flowers tripped early in a foraging bout. In many plant species, the time since removal from the flower is an important factor affecting pollen viability (Luna et al. 2001; Wang et al. 2004; Prasad et al. 2011; Brunet et al. 2019b). More specifically,

in alfalfa 30% of the pollen was viable after two to three hours, while no viable pollen remained after either 6.1 hours (Brunet et al. 2019b) or 8 hours (Lin 1967). Boyle et al. (2017) let honey bees forage on a glyphosate resistant (GR) alfalfa field prior to moving the hive to a field of conventional alfalfa, and found no GR seeds set in the conventional field when the bees were not released for eight hours. This is expected based on the pollen longevity of alfalfa (Brunet et al. 2019b; Lin 1967). Thus, whether pollen remains viable when a bee exits a hive will partly depend on how long the bee remains inside the hive between foraging bouts. It will also be affected by the temperature inside the hive because temperatures of 40°C and higher, but not between 25° and 37°C, negatively impact pollen viability in alfalfa (Wang et al. 2004; Ge et al. 2011; Rang et al. 2011).

Similar to Richards et al. (2009) who observed significant variation in the number of pollen grains deposited on the stigma with consequences for seed production, we observed significant variation in the number of pollen grains accumulated on a

bee's body with each tripped flower (Fig. 1). The number of pollen grains on the bee's body exiting the hive was similar to the number of grains following a foraging bout where no flowers were tripped. Because bees visited, on average, 5.6 flowers in foraging bouts where no flowers were tripped, these results suggest a lack of pollen accumulation on the bee's body, or significant loss of pollen from the bee, when a bee visits flowers without tripping them. These results also indicate that pollen grains are deposited on the stigma when the flower is tripped.

The amount of pollen deposited on the stigmas did not increase when bumble bees revisited already tripped alfalfa flowers. This contrasts with plants without a tripping mechanism, where the number of visits to a flower often increases pollen deposition and seed set (Engel & Irwin 2003; Karron et al. 2006; Abrol 2007; Stavert et al. 2020). In buzz-pollinated species, pollen deposition also increases with the number of visits to flowers, although in visits to *Pedicularis chamissonis* the greatest amount of pollen was deposited during the first visit (Kawai & Kudo 2009). In alfalfa, Bohart (1957) suggested that the entire receptive surface of the stigma is tightly pressed to the standard petal following tripping of a flower. Thus, when bumble bees revisit a tripped flower, they may be able to collect nectar and pollen, while the deposition of additional pollen onto the stigma may be limited.

Alfalfa flowers remain open after they are tripped, but in some plant species within the Fabaceae family, such as *Trifolium* species, the flower closes again after being tripped and must be tripped by a bee at each revisit (J. Brunet pers. obs.). We predict an increase in the number of pollen grains deposited on the stigmas with more pollinator revisits in such a system. In addition, we predict that the staggering of anther dehiscence would be strongly advantageous for flowers with repeated tripping (Harder & Thomson 1989). With staggered anther dehiscence, fresh viable pollen would be released at each tripping of the flower (Harder & Thomson 1989). This contrasts with a situation where all anthers are dehisced at the first tripping of a flower, and pollen viability strongly affects the success of any pollen grains released and deposited on the stigma in future tripping of the flower. Pollen viability decreases with time and

will be affected by the time elapsed between successive tripping of a flower by a bee (Brunet et al. 2019b). Pollen viability affects the probability of setting a mature seed. Future studies should quantify the relationship between the number of revisits to a flower and the number of pollen grains deposited on stigmas and seed set, and determine whether staggered anther dehiscence is common in systems where a flower closes after being tripped, and is tripped each time a bee revisits the flower.

Tripped flowers that remain open may serve as an honest signal for bees by indicating a previous visit. Bumble bees may learn to associate the visual cue of a tripped flower with decreased reward and thus avoid revisits (Marden 1984). In a similar fashion, scent marks left by bumble bees after a visit have been suggested as a type of honest signal (reviewed in Brunet & Minahan 2023), and have been hypothesized to discourage revisits to those flowers and to limit revisitations (Goulson et al. 1998). However, bumble bees frequently revisited tripped flowers in the experiment. One reason may be because tripped flowers still provide nectar, and possibly pollen to the bees, even though the bee does not deposit pollen on the stigma during the revisit. The foraging behavior of bees is affected by the amount of pollen and nectar provided by flowers (Harder 1990). While bumble bees can visually estimate whether pollen is present in a flower, they cannot visually determine the quantity of pollen present in a flower (Brunet et al. 2015) and they must probe a flower to determine if and how much nectar is available (Cresswell 1990; Cresswell 1999; Harder 1990; Irwin & Brody 1999). Bumble bees spend more time foraging on more rewarding flowers, and such flowers get revisited more often (Harder 1990). Future studies should examine pollen availability and nectar production in already tripped flowers, and estimate visit duration to these flowers relative to flowers tripped during the visit by a bee.

This study highlighted an interesting difference between pollen deposition and number of visits to a flower for flowers with a tripping mechanism where the flower remains open after being tripped, relative to flowers without a tripping mechanism. Results also indicated how the number of tripped flowers, rather than the number of visited flowers, affected the number of grains accumulated on a

bee's body. We expect similar patterns for different bee species, and for different plant species with a similar tripping mechanism. For flowers with a tripping mechanism where the flower closes after being tripped, we predict an increase in pollen grains deposited on stigmas with increasing number of revisits, and we predict a steeper slope for this relationship when anther dehiscence is staggered. The tripping mechanism studied here affected pollen transfer dynamics. Multiple visits to a flower did not increase pollen deposition, hence future seed set, and provided no benefit to the plant. This contrasts to plants without a tripping mechanism where multiple visits to a flower increases pollen deposition and seed set. Future studies should determine to what extent bees benefit by getting pollen or nectar during flower revisits.

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AUTHOR CONTRIBUTION

Concept and design MEDM, FPF & JB, data collection MEDM, CS, A-ML, GK & FPF, data analysis MEDM & JB, writing MDM & JB, edits and approval for publication MDM, JB, CDS, A-ML, GK & FPF.

DISCLOSURE STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data associated with this article are available as supplementary data in the online version of this article, see below.

APPENDICES

Additional supporting information may be found in the online version of this article:

Appendix 1. Data files for pollen accumulation and pollen deposition

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