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Bee species visiting *Medicago sativa* differ in pollen deposition curves with consequences for gene flow

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PREMISE: Pollinator foraging behavior can influence pollen dispersal and gene flow. In many plant species a pollinator trips a flower by applying pressure to release its sexual organs. We propose that differences in tripping rate among grooming pollinators could generate distinct pollen deposition curves, the pattern of pollen deposition over successive flowers visited. This study compares the pollen deposition curves of two grooming pollinators, a social bumble bee and a solitary leafcutting bee, with distinct tripping rates on *Medicago sativa* flowers. We predict a steeper deposition curve for pollen moved by leafcutting bees, the pollinator with the higher tripping rate.

METHODS: *Medicago sativa* plants carrying a gene (GUS) whose product is easily detected by staining, were used as pollen donors. After visiting the GUS plants, a bee was released on a linear array of conventional *M. sativa* plants. The number of GUS pollen grains deposited over successive flowers visited or over cumulative distances was examined. Distinct mixed effect Poisson regression models, illustrating different rates of decay in pollen deposition, were fitted to the pollen data for each bee species.

RESULTS: Pollen decay was steeper for leafcutting bees relative to bumble bees for both models of flowers visited and cumulative distance, as predicted by their higher tripping rate.

CONCLUSIONS: This is the first report of a difference in pollen deposition curves between two bee species, both grooming pollinators. Such differences could lead to distinct impacts of bee species on gene flow, genetic differentiation, introgression, and ultimately speciation.

KEY WORDS Bees and adventitious presence; β-glucuronidase (GUS) and pollen dispersal; gene flow by social and solitary bees; gene flow risk; *Medicago sativa* and gene flow; pollen carryover; pollen grains on successive flowers visited; tripping rate and pollen dispersal.

The foraging behavior of pollinators can influence pollen dispersal and gene flow. Pollinators mediate pollen dispersal as they remove pollen from the anthers of a flower and deposit it on the stigma of the next flower they visit (Campbell and Waser, 1989; Thomson and Thomson, 1989). The pattern of pollen deposition, or how pollinators deposit pollen from a specific donor onto the stigmas of successively visited flowers, impacts pollen carryover or the number of flowers that receive pollen from a specific donor, mate diversity, pollen dispersal, and gene flow (Lertzman, 1981; Waser, 1988; Castellanos et al., 2003; Richards et al., 2009; Mitchell et al., 2013). A pollinator that deposits most of the pollen grains from a specific donor onto the first few flowers it visits is expected to have a steeper pollen deposition curve relative to a pollinator that deposits pollen grains more evenly among flowers. Steeper pollen deposition curves translate into shorter pollen carryover, shorter pollen dispersal, and gene flow distances. In agriculture, pollen deposition curves can affect gene flow risk or the probability that a genetically engineered (GE) gene moves to a non-GE field, such as an organic or a conventional field, or to cross-compatible weedy or wild relatives (Ellstrand et al., 2013; Brunet, 2018).

Previous studies of pollen deposition curves have mainly examined single pollinators (Thomson and Plowright, 1980; Lertzman, 1981; Price and Waser, 1982) or compared grooming and non-grooming pollinators (Castellanos et al., 2003; Richards et al., 2009). Pollen deposition curves were originally predicted to follow a model of exponential pollen decay, where a similar proportion of pollen is deposited on each flower visited in succession during a foraging bout (Bateman, 1947). However, in the majority of studies,

longer tails of the pollen distribution than predicted under an exponential model of pollen decay have been reported (Thomson and Plowright, 1980; Lertzman, 1981; Price and Waser, 1982; Morris et al., 1994). Moreover, grooming pollinators like bees were predicted to have steeper pollen deposition curves relative to non-grooming pollinators like hummingbirds and hawkmoths, because grooming removes pollen from the body of a pollinator (Thomson, 1986; Holmquist et al., 2012). This prediction has been supported by experimental results (Thomson, 1986; Waser, 1988; Harder and Wilson, 1998; Castellanos et al., 2003; Richards et al., 2009). Creswell et al. (1995) examined pollen deposition using dyes as pollen analog for honey bees and two bumble bee species foraging on oil-seed rape and found no significant differences among bee species in the rate of decline in deposition across successive flowers visited.

Although no variation in pollen deposition curves has been predicted or observed among grooming pollinators, we propose that bee species with distinct tripping rates will have different pollen deposition curves. Many plant species in the families Fabaceae and Lamiaceae have a tripping mechanism, where the pollinator puts pressure on the flower to release its stigma and anthers. The tripping rate of distinct bee species visiting the same plant species can vary and such differences can influence seed set (Cane, 2002; Brunet and Stewart, 2010; Bauer et al., 2017). We propose that pollinator species with higher tripping rates will have steeper pollen deposition curves, and thus carry pollen and move genes shorter distances relative to pollinators with lower tripping rates (Brunet, 2018; Brunet et al., 2019). In an untripped flower, the anther and stigma are not released following a visit by a pollinator, they remain hidden inside the flower. Therefore, pollen is neither picked up from the anthers nor deposited on the stigmas of untripped flowers by pollinators. As a consequence, more pollen from a given pollen donor remains on the body of the pollinator and the pollen is not displaced by freshly collected pollen leading to a longer tail of the pollen distribution (Harder and Wilson, 1998). A pollinator with a low tripping rate will have many untripped flowers in its foraging bout creating a less steep pollen-deposition curve and longer distances traveled by pollen from the donor flower. The situation shares similarities with emasculated flowers where no pollen can be picked up from the flowers and thus pollen from the donor flower is not displaced also leading to a longer tail of the pollen distribution (Price and Waser, 1982; Morris et al., 1994).

Pollen deposition curves are intrinsically difficult to measure because one must follow a bee visiting flowers in succession and identify the pollen that originated from a specific pollen donor. Methodologies have been developed to simplify the process. For example, inflorescences have been trimmed to a single flower or hand-held flowers have been presented to a pollinator at preset intervals (Waser and Price, 1982; Waser, 1988). Fluorescent dyes have been used as pollen analogs to facilitate identification of the pollen donor and stigmas of recipient flowers have been emasculated to limit interference from pollen from the recipient flowers (Thomson and Plowright, 1980; Lertzman, 1981; Price and Waser, 1982; Waser and Price, 1982; Galen and Plowright, 1985; Thomson et al., 1986; Waser, 1988; Castellanos et al., 2003). However, the methodology used may influence the results (Price and Waser, 1982; Morris et al., 1994, 1995; Harder and Wilson, 1998). For example, the use of dyes as pollen analogs does not always adequately mimic pollen dispersal (Waser and Price, 1982). Moreover, because no pollen is being picked up from emasculated flowers as a pollinator moves from flower to flower, they can modify pollen transfer dynamics leading to longer pollen dispersal tails (Price and Waser, 1982; Morris et al., 1994).

Besides experimental methods, some of the statistical methods used to analyze the pollen deposition data may also increase the tail of the pollen dispersal curve (Price and Waser, 1982; Morris et al., 1994, 1995; Harder and Wilson, 1998). For example, when performing statistical analyses, using the average number of pollen grains deposited on a stigma (for a given flower order) instead of individual data points for each foraging bout can create longer pollen dispersal tails (Harder and Wilson, 1998). Moreover, the stochasticity in pollen deposition created by among individual pollinator variation (different bees of a given species) can result in a longer dispersal tail (Morris et al., 1995; Harder and Wilson, 1998). A different type of stochasticity, the one created by the variation in pollen deposition among flowers within a pollinator visit (foraging bout), has provided mixed results. One model predicted a shorter pollen dispersal tail than expected under an exponential model of pollen decay (Galen and Rotenberry, 1988); a second model indicated a longer dispersal tail (Lertzman and Gass, 1983) while no changes were predicted by a third model (Harder and Wilson, 1998).

The current study uses methodological and statistical approaches that minimize the unintentional creation of longer dispersal tails. Plants transformed to express the β-glucuronidase (GUS) gene in pollen were used as pollen donors and, by doing so, issues associated with the use of fluorescent dyes as pollen analogs or the use of emasculated flowers creating longer pollen dispersal tails were avoided (Price and Waser, 1982; Waser and Price, 1982; Thomson et al., 1986). Second, statistical models were fitted using all individual data points rather than averages to avoid longer dispersal tails created by using averages (Harder and Wilson, 1998). Third, different Poisson distributions with different rates of pollen decay were fitted to the pollen deposition data because different models have been suggested as potential fit for pollen deposition and pollen dispersal curves (Morris et al., 1995; Richards et al., 2009). Fourth, to determine the potential impact of among bee variation, we included a random intercept and/or random slope factors in the models. A random intercept examines the variation in intercept, i.e., variation in the number of GUS pollen grains deposited on the first flower visited, among different bees (runs). It is equivalent to adding a random bee term in the model. A random slope considers variation in slope, i.e., the rate of pollen decay, among bees (runs). Previous studies have fitted a curve to each individual bee run to circumvent the potential bee to bee variation (Richards et al., 2009). Here, we compared mixed models with only the intercept as a random factor to models with both random intercept and random slope. The development of mixed models and increased computer power now permits the use of individual data points for all runs combined and the inclusion of random factors for intercept and slope to consider bee to bee variation.

This study compares the pollen deposition curve of one social bee, a bumble bee, and one solitary bee, a leafcutting bee, visiting *M. sativa* flowers. Because leafcutting bees have a higher tripping rate than bumble bees, at least at higher temperature (Cane, 2002; Pitts-Singer and Cane, 2011; Brunet and Stewart, 2010; Brunet et al., 2019), we predict a steeper pollen deposition curve and shorter pollen dispersal distances for leafcutting bees relative to bumble bees. We also compared different foraging metrics between bee species including the number of unique flowers visited

(no revisits), the tripping rate and total number of GUS pollen deposited over a foraging bout to better link differences in pollen deposition curves to pollen dispersal. We discuss the implications of differences in pollen deposition curves between bee species on gene flow risk in agriculture and on population differentiation and introgression and ultimately speciation of wild plant populations.

MATERIALS AND METHODS

Study species

Medicago sativa L. (Fabaceae) plants used in this study are tetraploid and self-compatible although they set very few seeds in the absence of pollinators. Flowers have five petals with the two lower petals forming a keel. The stigmatic tissue in *M. sativa* is covered by a membrane, the cuticle, which acts as a partial barrier to self-pollen reaching the stigmatic fluid (Kreitner and Sorensen, 1984). When a pollinator applies pressure on the keel, the stamens and pistil are released in a process called tripping. Pollen is deposited on the bee's thorax during the tripping process, much of the stigma's cuticle remains on the petal which allows contact of pollen grains with the stigmatic fluid and thus hydration and germination of pollen grains, and pollen already on the pollinator's body gets deposited on the flower's stigma. We used two distinct pollinators in this experiment. The alfalfa leafcutting bee (*Megachile rotundata* Fabricius, 1793) (Hymenoptera: Megachilidae) is a managed pollinator used in alfalfa seed production fields while the common eastern bumble bee (*Bombus impatiens* Cresson, 1863*)* (Hymenoptera: Apidae), is a wild pollinator of *M. sativa* (Bohart, 1957; Brunet and Stewart, 2010) used by breeders in greenhouses and small experimental plots. We purchased commercially available leaf-lined cocoons of the alfalfa leafcutting bee for this experiment and obtained a bumble bee hive from Koppert Biological Systems (Howell, Michigan, USA).

Generation of *M. sativa* with GUS-marked pollen

To create *M. sativa* plants with the GUS gene expressed in the pollen, the anther specific promoter from the LAT52 gene of tomato fused to a ß-glucuronidase (GUS) gene and NOS terminator, was cloned from the plasmid pLAT52-7 (Twell et al., 1990) as a SalI-EcoRI fragment into the plant transformation vector pBIB-HYG (Becker, 1990) digested with the same enzymes using standard methodologies (Sambrook et al., 1989). The resulting construct was transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Wise et al., 2006) and this *A. tumefaciens* strain was used to transform a highly regenerable clone of Regen-SY alfalfa (Bingham, 1991) using 25 μg/mL hygromycin for selection of transgenic events as described by Samac and Austin-Phillips (2006). Presence of the GUS transgene was confirmed in *M. sativa* plants by PCR using GoTaq Green Master Mix (Promega Corporation, Madison, Wisconsin, USA) using manufacturer-suggested cycling conditions with genomic DNA as template and the primers 5'-CTCGACGGCCTGTGGGCATTCAG-3' (forward) and 5'-CGGCGGGATAGTCTGCCAGTTC-3' (reverse). Pollen was stained with 5-Bromo-4-chloro-3-indoxyl-beta-D-glu curonide (X-gluc; GoldBio, St. Louis, Missouri, USA) essentially as described by Preuss et al. (1994) to confirm expression of the GUS gene and to identify plants with a single gene insertion event (i.e., a plant that had approximately 50% of pollen stain with X-gluc). After one hour of exposure to X-gluc at 37°C, the β-glucuronidase enzyme produces a blue precipitate when it breaks down the β-D-glucuronide substrate. The X-gluc solution contains 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 50 mM sodium phosphate (pH 7.0) and 0.5 mg/ml X-Gluc (from a 20 mg/ml stock solution in dimethylformamide) in dH_2O.

Because the plants were tetraploid and the experiment required all pollen grains from a pollen donor to express the GUS gene, plants were generated that contained three or four copies of the GUS transgene at a single locus. Because the expression of the GUS gene is dominant, all pollen grains from an individual with at least three copies of the GUS gene would express the trait. We first identified a T_0 plant that contained a single locus GUS transgene and crossed it to a small group of wild-type plants to produce a T_1 generation. Individuals of the T_1 generation with a single copy of the GUS gene were intercrossed to produce a T_2 generation, and individuals of the T_2 generation with two copies of the GUS gene (as determined by PCR and pollen staining with X-gluc) were intercrossed to produce a T_3 generation. Individuals from the T_3 generation with all or nearly all their pollen grains staining blue with X-gluc indicative of the presence of three or four copies of the GUS gene, were used in the experiments and seeds are available upon request.

Experimental set up

Experiments were conducted at the Walnut Street greenhouses at the University of Wisconsin in Madison, Wisconsin. Pollen deposition curves were quantified for bumble bees and leafcutting bees visiting *M. sativa* flowers. Prior to running trials, bumble bees were trained to visit *M. sativa* flowers by placing a colony of approximately 50 worker bees in the small cage described in the paragraph below, together with a different set of conventional *M. sativa* plants from the ones used for the trials. Experiments started after the bees actively foraged on *M. sativa* plants. For leafcutting bees, we placed nesting sites (50-cm wide bee boards) in the small cage and released 20 male and 20 female leafcutting bees. Bees were allowed to forage on conventional *M. sativa* plants as was the case for bumble bees. The males were used to maximize mating opportunities as mated females lay eggs and forage to provision their eggs. Female leafcutting bees were used for the pollen deposition curves. One trial was run per day and a bee was not reused following a run.

Plants carrying at least three allelic copies of the GUS gene were used as pollen donors. At the start of a trial, these GUS plants were kept in a 1.52 × 1.52 × 2.13 m mesh cage (small cage) which was connected to and separated from a larger 2.28 × 3.66 × 2.13 m mesh cage by a screen made of bridal veil to prevent unintentional movement of bees between the cages. The larger cage housed an experimental array of 13 potted conventional *M. sativa* plants each separated by 15.2 cm distance. Prior to a trial, the conventional plants in the array (large cage) were trimmed to 5 to 6 racemes per plant to standardize floral display size and each raceme was trimmed to 5 to 6 untripped flowers. Because *M. sativa* flowers remain open following tripping by a pollinator, an untripped flower represented a fresh unvisited flower. In order to individually identify a raceme and flower during a trial, each raceme was marked with a thin thread of a different color and the banner of each flower was marked with a small dot of a specific color using fine-tip felt pens.

Pattern of pollen deposition

A single bumble bee at a time was released from the colony and allowed to forage on the GUS plants placed in the small cage. After a bumble bee visited and tripped ten GUS flowers, the bridal veil separating the small and large cages was lifted to permit the bee to move into the large cage with the 13 conventional *M. sativa* plants. A single bee was tested at any given time in the large cage. When a test bee began foraging on the conventional plants, we recorded each plant, raceme, and flower visited in succession by the bee for the duration of the foraging bout (run). We also noted whether a flower was tripped by the bee and recorded instances of grooming, where a bee cleaned itself by scraping its head or thorax with its fore or middle legs after leaving a flower. A foraging bout began when a bee visited the first flower in the array, irrespective of the plant position within the array, and ended when the bumble bee started flying back towards the hive. Flowers could be revisited during a foraging bout. The distance (cm) between all pairs of consecutively visited flowers were measured at the end of a run. The test bee was captured, put in a vial and kept in a freezer.

A female leafcutting bee, with no pollen on its body and leaving its nesting site, was collected from the small cage and placed in a container with 3 to 5 freshly collected racemes from GUS plants. After the bee visited and tripped ten GUS flowers, the bee was released into the large cage. The methodology was otherwise similar to the one used for bumble bee except the end of a foraging bout was indicated by a bee landing for more than two minutes without grooming. Prior experience with leafcutting bees in the greenhouse indicated that bees were very unlikely to forage again following a two-minute resting period.

Flower collection and staining of pollen grains

We collected tripped flowers an hour following the end of a trial to provide time for pollen to germinate and anchor on the stigma. Each flower was individually placed in a 5 ml centrifuge tube identified with bee species, run number, and order of flower visit. The stigma of each flower was isolated from the other floral parts under a stereomicroscope. After carefully removing the anthers of each visited flower, no pollen grains were visible on the removed floral parts. Each stigma was put back into its respective tube before being stained to identify GUS pollen grains. We first added 100 µl of 90% acetone to each tube before placing the tubes in a vortex for 10 sec and centrifuging for 3 min at 600 rpm. The acetone was drained and the stigmas dried at room temperature for approximately 30 min. We then added 35 µl of the X-Gluc solution (described in the section above) to each tube and incubated the tubes for an hour at 37°C. Finally, we added 100 µl of distilled de-ionized (DDI) water to each tube, placed each stigma together with loose pollen in a separate well of a 96-well plate and gently smashed each stigma using a glass rod to release the pollen grains. We counted all blue pollen grains in each well under a Leica MZ16 dissecting microscope at 10× magnification (Leica, Buffalo Grove, Illinois, USA).

Model selection

We examined the relationships between the number of GUS pollen grains deposited on the stigma of a flower and the order in which a flower was visited during a foraging bout (Flower) or the cumulative distance traveled (Distance). The first flower visited was the origin for distance and distances between pairs of consecutively visited flowers were added incrementally. Mixed effect Poisson regression models were fitted to the combined runs for each bee species, using the 'glmer' function in the lme4 package in R (Bates et al., 2015). In the current context, Poisson regression is a generalization of simple linear regression; for a given value of the independent variable (flower number or distance) the dependent variable (observed count of blue pollen grains) is modeled as a Poisson random variable for which the natural logarithm of the mean (dependent variable) is a linear function of the independent variable. Equivalently, the mean of the Poisson distribution can be expressed as the exponential of a linear function of the independent variable. This is the first form of the model that we fitted. Because different models have been suggested as potential good fit for pollen deposition and pollen dispersal curves (Morris et al., 1995; Richards et al., 2009), we also evaluated two alternative models for which the logarithm of the Poisson mean was not a linear function of the independent variable, but rather exhibited a different rate of decline as a function of the independent variable. Thus, in addition to the first "standard" Poisson model described above, we also fitted a model where: (2) the natural log of the Poisson mean was a linear function of the natural log of the independent variable; and (3) the natural log of the Poisson mean was a linear function of the square root of the independent variable. In the remainder of the manuscript, we abbreviate case (2) as the 'Poisson log' and case (3) as the 'Poisson sqrt' models. The first Poisson model described above is equivalent to an exponential (continuous) or geometric (discrete) model of pollen decay. Models (2) and (3) both allow for possibly different rates of decay in pollen deposition as the independent variable increases but they are not directly equivalent to the model forms explored by Morris et al. (1995) or Richards et al. (2009).

For each of the three Poisson models, we used a mixed effects model with either both random intercept and random slope, or a mixed effects model with only a random intercept. In either case we had fixed effects for the (population mean) intercept and slope. Random slope and random intercept allowed us to determine whether there was substantial variation in slope and/or intercept parameters among bees. Note that a model with intercept as the only random effect is equivalent to a model with only a "bee" random effect. However, we specifically included for consideration a model with both random intercept and random slope because, for pollen deposition curves, the slope of the relationship, which describes the rate of pollen decay, is most relevant. We thus examined six models for flower or distance for each bee species which included the three distinct Poisson-type models and for each Poisson model, the case with either only the intercept or both the slope and the intercept as random effects (Table 1). The six different models were compared using the Akaike Information Criterion (AIC). We selected the model with the lowest AIC as representing the best fit to the data in each case. When evaluating the different models, we considered a difference of 2 in AIC value between models as indicating different fits to the data (Burnham and Anderson, 2002). The figures we present for the best models illustrate the curves constructed by back-transforming the fixed effects of the Poisson linear model into an exponential model.

We first fitted the six different models for each bee species to pollen deposition data that included only the tripped flowers. These are the flowers where pollen grains could be deposited on the stigmas. This data set did not have revisited flowers because flowers are tripped only once and they remain open after being tripped. Second, to determine how untripped flowers affected pollen deposition curves, we fitted the six models to the pollen deposition data that included all visited

TABLE 1. The best Poisson model(s) to describe pollen deposition by bumble bees (Bbee) and leafcutting bees (Lcbee) over successive flowers based on AIC values. The six Poisson models are described in the text and compared here for tripped flowers only and for all flowers visited (tripped and untripped flowers). The best model(s), the model with the lowest AIC value, is in bold. A difference of 2 in AIC value between models indicated different fits to the data. N is the sample size or the total number of racemes visited over all foraging bouts.

	Tripped flowers only		All visited flowers	
Model	**Bbee (N = 330)**	**Lcbee (N = 138)**	**Bbee (N = 784)**	**Lcbee (N = 215)**
Standard Poisson with random intercept	3950.9	461.8	7375.3	665.7
Standard Poisson with random intercept and random slope	**3671.5**	**440.1**	6957.2	636.6
Poisson log with random intercept	3904.1	464.7	7175.1	627.1
Poisson log with random intercept and random slope	3686.2	456.0	**6771.3**	616.3
Poisson sqrt with random intercept	3934.8	457.6	7294.4	632.2
Poisson sqrt with random intercept and random slope	**3671.1**	445.0	6842.1	**614.2**

flowers, both tripped and untripped flowers. For the Flower model, which examines consecutive flowers, the inclusion of untripped flowers increased the number of flowers visited during a foraging bout. For the Distance model, which considers cumulative distances traveled between flowers, adding untripped flowers did not modify the cumulative distances between tripped flowers but added untripped flowers in between tripped flowers. Each untripped flower received a zero pollen count because pollen is neither removed or deposited on untripped flowers. Revisited flowers were present in the data set with all visited flowers and the count of GUS pollen grains was associated to a flower when it was tripped by the bee. Therefore, for revisited flowers, a pollen count of zero was assigned to a flower visited prior to or following tripping of the flower. This procedure was followed because we have no evidence that the number of GUS pollen grains on a stigma increased with revisits (Appendix S1) and there exist no data to suggest pollinators deposit pollen on already tripped *M. sativa* flowers.

Comparing models between bee species

For the cases where a similar Poisson model represented the best model for the two bee species, we used the "glmer' function in the lme4 package in R (Bates et al., 2015), to compare the slope and intercept between the two bee species. For example, the standard Poisson model was the best fit for both bumble bee and leafcutting bee for the Flower model for tripped flowers (Table 1). We added species and the interaction term species × flower as fixed effects to the best Poisson model. A statistically significant 'species' effect indicates differences in intercepts between the two bee species while a statistically significant 'interaction' effect specifies a difference in slopes between bee species.

Differences in foraging metrics between bee species

Several foraging metrics were compared between the two bee species. These metrics included the number of flowers uniquely visited in a foraging bout (no revisits), the proportion of visited flowers that were tripped by the bee (tripping rate) and the total number of GUS pollen grains deposited on stigmas over the entire foraging bout by a bee. In addition, we compared the number of GUS pollen grains deposited on the stigma of the first flower visited in the sequence between bee species. Finally, we examined differences in the number of grooming episodes and the number of flower revisits per foraging bout between the two bee species. These tests were performed using one-way analysis of variance in RStudio version 1.0.136 (RStudio Team, 2017).

RESULTS

Pattern of pollen deposition tripped flowers only

Flower model − bumble bee—We obtained 15 pollen deposition runs for bumble bees with 330 observations (stigmas with blue pollen grain counts). The median number of flowers tripped by bumble bees was 16 per run. Two of the Poisson models with random intercept and random slope (RIRS) had the lowest and very similar AIC values; the AIC value for the Poisson sqrt model was AIC = 3671.1 and for the standard Poisson model AIC = 3671.5 (Table 1). The equations for the sqrt and the standard Poisson models were, respectively, $\log_e$ (mean count GUS pollen grains) = 3.05 – 0.62 sqrt Flower and $\log_e$ (mean count GUS pollen grains) = 2.45 – 0.14 Flower (Table 2). Both best models included RIRS and illustrate a steep decline in the number of GUS pollen grains deposited on stigmas as more flowers are visited in a foraging bout (Fig. 1A). The third model with RIRS was not as good a fit to the data (Table 1, Appendix S2a). All three Poisson type models with RIRS had lower AIC values than the same models with only random intercept (Table 1), indicating that the variation in slope among runs (individual bees) improved the fit of the model to the data.

TABLE 2. Intercepts and slopes with standard errors as fixed effects of the best Poisson regression model for pollen deposition over consecutive flowers or cumulative distance for bumble bees (Bbee) and leafcutting bees (Lcbee), for only tripped or for all visited flowers. The best models always included the random slope and random intercept. The second-best model for leafcutting bees for all flowers visited, the Poisson log, is also included. We use this model to compare with the best bumble bee model for pollen deposition over consecutive flowers (Table 5).

Bee Type	Run Type	Best Model	Intercept	SE	Slope	SE
Flower						
Bumble bee	tripped	Square root	3.05	0.40	−0.62	0.16
Bumble bee	tripped	Standard	2.45	0.30	−0.14	0.05
Leafcutting bee	tripped	Standard	1.21	0.49	−0.30	0.08
Bumble bees	all visited	Log	2.02	0.33	−0.56	0.14
Leafcutting bee	all visited	Square root	1.94	0.65	−1.07	0.25
Leafcutting bee	all visited	Log	1.09	0.42	−1.04	0.20
Distance						
Bumble bee	tripped	Square root	2.40	0.28	−0.06	0.01
Leafcutting bee	tripped	Square root	0.87	0.41	−0.19	0.05
Bumble bee	all visited	Square root	1.67	0.31	−0.08	0.02
Bumble bee	all visited	Log	1.60	0.29	−0.22	0.05
Leafcutting bee	all visited	Log	0.23	0.33	−0.32	0.08

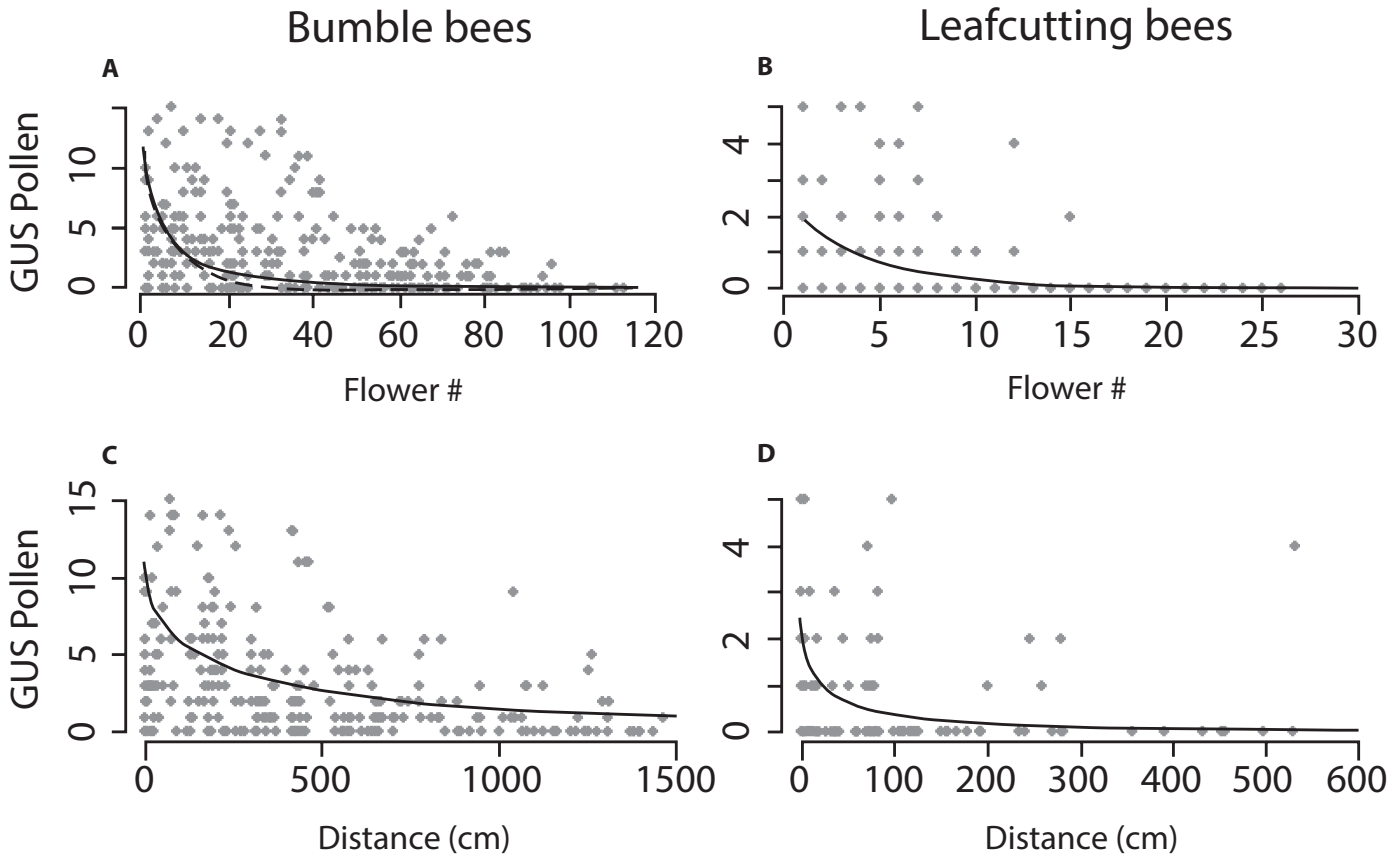

FIGURE 1. The best model for pollen deposition curves for tripped flowers with individual pollen deposition data. The graph illustrates the untransformed number of GUS pollen grains on a stigma and the order in which a tripped flower was visited in succession during a foraging bout (Flower model) or cumulative distance traveled by a bee between tripped flowers (Distance model). For the number of flowers visited in succession (Flower model) for (A) bumble bee, the two best models are Standard Poisson (- -) and Poisson Sqrt (—); (B) leafcutting bee, the Standard Poisson; for the cumulative distance traveled by a bee (Distance model) for (C) bumble bee, Sqrt Poisson; and (D) leafcutting bee, Sqrt Poisson.

The standard errors (SEs) around the intercept and the slope were 0.40 and 0.16, respectively, for the Poisson sqrt model and 0.30 and 0.05 for the standard Poisson model (Table 2). The standard errors represent the accuracy of the intercept and the slope for the fixed effects of the model (population means). The standard deviations (STDs), associated with the random intercept and random slope, were respectively 1.49 and 0.60 for the Poisson sqrt model and 1.11 and 0.17 for the standard Poisson model (Table 3). These standard deviations, associated with the random effects, illustrate the variation among bees (runs) in the number of GUS pollen grains deposited on the first visited flower in the foraging bout (random intercept) and the variation among bees (runs) in the linear decline in the number of pollen grains deposited on stigmas in successively visited flowers (random slope).

Flower model – leafcutting bee—We obtained 138 pollen deposition data points and 13 runs for leafcutting bees. The median number of flowers tripped by leafcutting bees was eight. The standard Poisson regression model with RIRS represented the best model for leafcutting bees for pollen deposition on successively visited and tripped flowers and had an AIC = 440.1 (Table 1). The equation for this model was $\log_e$ (mean count GUS pollen grains) = 1.21 – 0.30 Flower, with SEs of 0.49 and 0.08 for the intercept and slope, respectively (Table 2). The STDs associated with the random intercept and slope effects, were 1.61 and 0.23, respectively (Table 3). There was a steep decline in GUS pollen grains over flowers visited and tripped in succession for the standard Poisson model (Fig. 1B). All three Poisson models with RIRS showed a decline in pollen grains over successive flowers (Appendix S2b), although the standard Poisson model was the best fit to the data (Table 1).

Distance model – bumble bee—Using the same 15 runs and 330 observations as for the Flower model, the best model for distance for bumble bees was the Poisson sqrt model with RIRS and an AIC = 3734.3 (Table 4; Fig. 1C). The equation for the fixed effects for this model was $\log_e$ (mean count GUS pollen grains) = 2.40 – 0.06 sqrt (Distance), where Distance is the cumulative distance traveled to the x^{th} tripped flower visited (Table 2). The SEs for the intercept and slope (fixed effects) were 0.28 and 0.01, respectively (Table 2), while the STDs associated with the intercept and slope (random effects) were, respectively, 1.08 and 0.05 (Table 3). Again, the three top models were the models with RIRS (Table 4, Appendix S2c).

Distance model – leafcutting bee—The best model for pollen deposition with Distance for leafcutting bee was the Poisson sqrt model with RIRS with an AIC value of 436.3 (Table 4). The equation for

TABLE 3. Standard deviation (STD) around the random effects of the intercept and slope in the best models selected. These values reflect the variation in the number of GUS pollen grains deposited on the first flower visited (intercept) or the variation in the decline of the curve (slope) among runs (individual bees). Bbee stands for bumble bees and Lcbee for leafcutting bees. The blank lines indicate that there was only one best model in these cases. For example, the bumble bee Flower model for 'tripped flowers' had two best models (Poisson sqrt and standard Poisson) while the 'all visited flowers' case only had one best model (Poisson log).

Model	Bee Type	Best Model (tripped)	Intercept STD	Slope STD	Best Model (all visited)	InterceptSTD	Slope STD
Flower	Bbee	Square root	1.49	0.60	Log	1.25	0.53
	Bbee	Standard	1.11	0.17			
	Lcbee	Standard	1.61	0.23	Sqrt	2.06	0.73
Distance	Bbee	Square root	1.08	0.05	Sqrt	1.19	0.09
					Log	1.09	0.19
	Lcbee	Square root	1.37	0.15	Log	1.09	0.23

fixed effects was $\log_e$ (mean count GUS pollen grains) = 0.87 – 0.19 sqrt (Distance) (Table 2) and GUS pollen declined with increasing distance traveled (Fig. 1D). The RIRS models were better than the random intercept-only models (Table 4), and GUS pollen declined with increasing distance for all three models (Appendix S2d). The SE around the intercept was .41 and 0.01 around the slope (fixed effects) (Table 2) while the STD was 1.37 for the intercept and 0.15 for the slope (random effects) (Table 3).

Pattern of pollen deposition all flowers visited

Flower model – bumble bee—When all visited flowers were included (tripped and untripped flowers), there were 784 pollen deposition data points over the 15 bumble bee runs and a median of 44 flowers visited per run. The best model was the Poisson log model with an AIC= 6771.3 (Table 1), with decreasing pollen count in successively visited flowers (Fig. 2A). The equation for the model was $\log_e$ (mean count GUS pollen grains) = 2.02 – 0.56 $\log_e$ (Flower) with SE = 0.33 for the intercept and 0.14 for the slope (fixed effects) (Table 2). The STD was 1.25 for the intercept and 0.53 for the slope (random effects) (Table 3). The three top models were the models with RIRS (Appendix S3a).

Flower model – leafcutting bee—With 215 pollen deposition data points and 13 leafcutting runs, the best model was the Poisson sqrt model with RIRS and an AIC = 614.2 (Table 1). The equation was $\log_e$ (mean count GUS pollen grains) = 1.94 – 1.07 sqrt (Flower) with SE = 0.65 for the intercept and 0.25 for the slope (fixed effects) (Table 2). The STD was 2.06 for the intercept and 0.73 for the slope (random effects) (Table 3). The median number of flowers visited per run was 14. The models with RIRS were the three best models (Table 1, Appendix S3b).

Distance model – bumble bee—With the 784 observations and 15 runs, there were two best models for bumble bees, the Poisson log model with an AIC = 6802.8 and the Poisson sqrt model with AIC = 6803.7, both with RIRS (Table 4; Fig. 2C). The equation for the Poisson log model was $\log_e$ (mean count GUS pollen grains) = 1.60 – 0.22 log (Flower) with SE = 0.29 for the intercept and 0.05 for the slope (Table 2). The STD was 1.09 for the intercept and 0.19 for the slope (Table 3). The equation for the Poisson sqrt model was $\log_e$ (mean count GUS pollen grains) = 1.67 – 0.08 sqrt (Flower) with SE = 0.31 for the intercept and 0.02 for the slope (Table 2). The STD was 1.19 for the intercept and 0.09 for the slope (Table 3). The three top models all included RIRS (Appendix S3c).

Distance model – leafcutting bee—With the 13 runs and 215 observations for leafcutting bees, the best model was the Poisson log model with RIRS and an AIC = 649.7 (Table 4). The number of GUS pollen on stigmas decreased with increasing distance traveled by a bee (Fig. 2D). The equation for the fixed effects of the model was $\log_e$ (mean count GUS pollen grains) = 0.23 – 0.32 $\log_e$ (Flower) with SE = 0.33 for the intercept and 0.08 for the slope (fixed effects) (Table 2) and STD of 1.09 (intercept) and 0.23 (slope) (random effects). The best three models all included RIRS (Table 4; Appendix S3d).

Comparing models between bee species

The same Poisson model represented the best fit to the pollen deposition data for both bumble bee and leafcutting bee in three cases, the standard Poisson model for the Flower model for tripped flowers; the Poisson sqrt model for Distance for tripped flowers; and the Poisson log model for Distance for all visited flowers (Tables 1 and 4). In addition, we added a comparison for the Poisson log model for Flower

TABLE 4. The best Poisson model(s) to describe pollen deposition by bumble bees (Bbee) and leafcutting bees (Lcbee) over cumulative distances, based on AIC values. The six Poisson models are described in the text and compared here for tripped flowers only and for all flowers visited (tripped and untripped). The best model(s), the model with the lowest AIC value, is in bold. A difference of 2 in AIC value between models indicated different fits to the data. N is the sample size or the total number of racemes visited over all foraging bouts.

	Tripped flowers only		All visited flowers	
Model	Bbee (N = 330)	Lcbee (N = 138)	Bbee (N = 784)	Lcbee (N = 215)
Standard Poisson with random intercept	3878.5	531.5	7260.9	853.6
Standard Poisson with random intercept and random slope	3769.7	462.3	6894.5	776.5
Poisson log with random intercept	3909.4	455.6	7104.8	676.0
Poisson log with random intercept and random slope	3747.7	441.7	**6802.8**	**649.7**
Poisson sqrt with random intercept	3873.4	467.7	7191.8	697.8
Poisson sqrt with random intercept and random slope	**3734.3**	**436.3**	**6803.7**	756.2

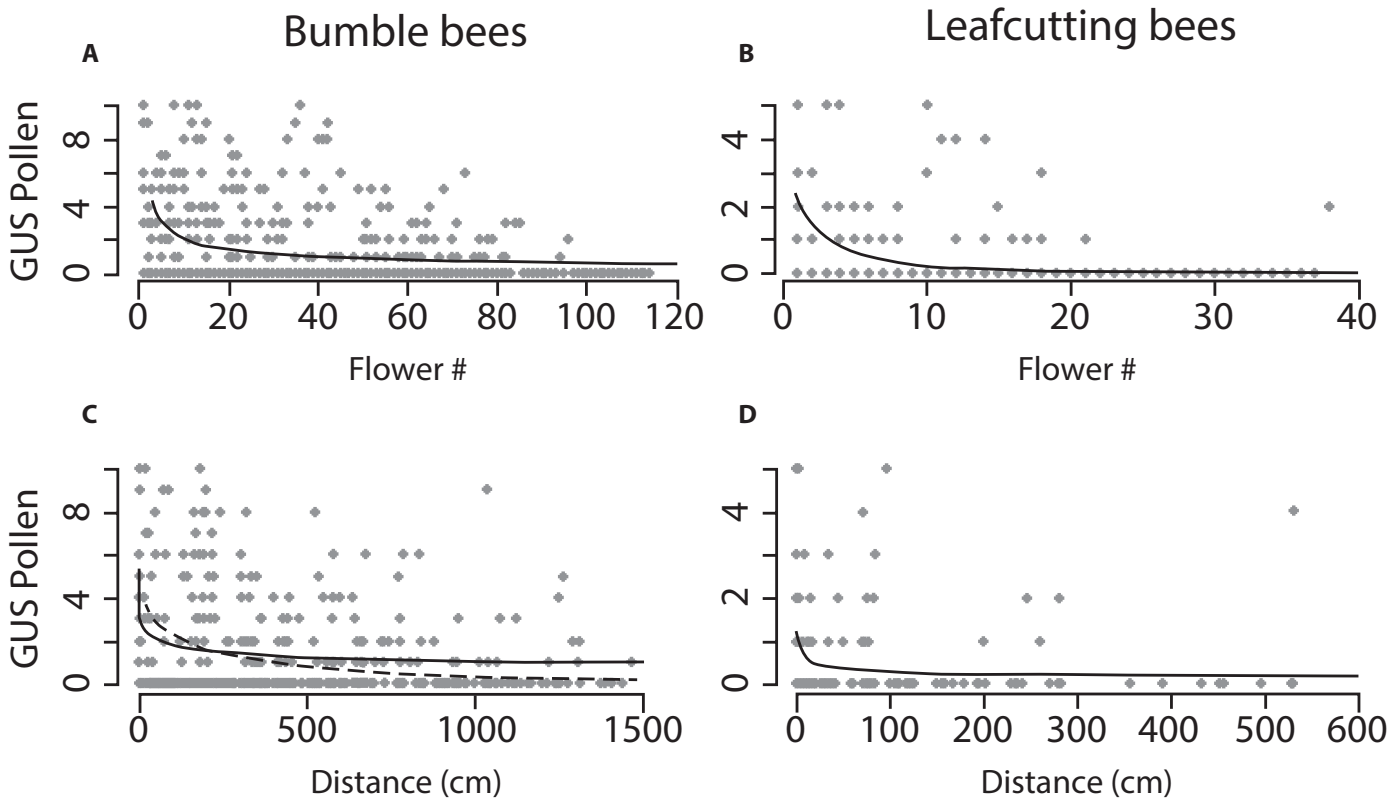

FIGURE 2. The best models for pollen deposition curves for all visited flowers (tripped and untripped) with individual pollen deposition data. The graph illustrates the untransformed number of GUS pollen grains on a stigma and the order in which a flower was visited in succession during a foraging bout (Flower model) or cumulative distance traveled by a bee between visited flowers (Distance model). For the number of flowers visited in succession (Flower model) for (A) bumble bee, Poisson log; (B) leafcutting bee, Poisson sqrt; for the cumulative distance traveled by a bee (Distance model) for (C) bumble bee, there are two best models, log Poisson (—) and sqrt Poisson (---); and (D) leafcutting bee, log Poisson.

for all visited flowers (Table 2) because that model for leafcutting bee was only a 2.1 AIC difference from the best Poisson sqrt model (Table 1). For both the Flower and Distance models with tripped flowers, the intercepts and the slopes were statistically different between bee species (Table 5). Fewer GUS pollen grains were deposited on the first visited flower (intercept) by leafcutting bees relative to bumble bees and GUS pollen decayed more rapidly when it was carried by leafcutting bees relative to being carried by bumble bees (Table 5; Fig. 3A, C). When all visited flowers were considered, for the Flower model, the slope but not the intercept differed between bee species (Appendix S3A, B) while the reverse was true for the Distance model, where the intercept but not the slope differed between bee species (Fig. 3D) (Table 5).

Differences in foraging metrics between bee species

Bumble bees visited significantly more flowers per foraging bout (no revisits) (mean ± SE) (44.7 ± 7.6) relative to leafcutting bees (12.3 ± 1.8) ($F_{1,26} = 16.35$; $P = 0.0002$). However, bumble bees tripped a smaller proportion of the visited flowers (52.1% ± 5.2 for BB vs. 86.9% ± 2.9 for LCB) ($F_{1,26} = 31.05$; $P < 0.0001$). In an average foraging bout, bumble bees tripped 22.0 flowers and deposited 154.5 ± 55.8 GUS pollen grains while leafcutting bees tripped 10.6 flowers and deposited 19.2 ± 8.1 GUS pollen grains ($F_{1,26} = 4.98$; $P = 0.03$ for GUS pollen grains). When examining only the first flower visited in the sequence, bumble bees deposited considerably more GUS pollen grains (25.7 ± 9.1) relative to leafcutting bees (6.7 ± 2.4) ($F_{1,28} = 5.78$, $P = 0.02$). Bumble bees and leafcutting bees had similar number of grooming episodes per foraging bout (5.7 ± 1.1 for bumble bee and 3.5 ± 1.1 for leafcutting bee) ($F_{1,26} = 3.59$; $P = 0.07$), and similar number of flower revisits (7.6 ± 1.5 flowers for bumble bees and 4.2 ± 1.6 for leafcutting bees) ($F_{1,26} = 3.69$; $P = 0.14$).

DISCUSSION

This is the first report of a difference in pollen deposition curves between two bee species that are both grooming pollinators. Previously, differences in pollen deposition curves have been predicted and observed between grooming and non-grooming pollinators but no differences have been predicted between grooming pollinators such as distinct bee species (Thomson, 1986; Castellanos et al., 2003; Richards et al., 2009). When examining pollen deposition curves, the slope illustrates the rate of pollen decay over consecutive flowers visited or over cumulative distance. In most cases, we observed steeper pollen deposition curves when pollen was carried by leafcutting bees relative to being carried by bumble bees. The only exception was for the Distance model for all flowers (tripped and untripped) where, although not statistically significant, the slope for leafcutting bees (–0.32) was still steeper than the slope for bumble bees (–0.22) (Table 2). Bee species with steeper pollen

TABLE 5. Contrasting the intercept and slope (fixed effects) between the best fitted Poisson model for bumble bees and the corresponding best model for leafcutting bees. For the Flower model, all visited flowers, the best bumble bee model was contrasted to the corresponding second best model for leafcutting bees (Table 1). A species effect in the model indicates a difference in intercept and an interaction Flower:Species effect suggests a difference in slope between the two Poisson models. The estimates for the species and the interaction terms compare leafcutting bees (LC) relative to bumble bees.

Model	Run Type	Best Model	Variable	Estimate	Std. Error	z value	Pr(>|z|)
Flower	Tripped flowers	Standard Poisson	Intercept	2.44	0.35	7.05	<0.0001
			Flower	−0.14	0.05	−2.77	0.006
			Species LC	−1.16	0.53	−2.18	0.029
			Flower:Species LC	−0.17	0.085	−1.97	0.049
	All visited flowers	Poisson log	Intercept	2.02	0.34	5.84	<0.0001
			Log Flower	−0.56	0.14	−3.84	0.0001
			Species LC	−0.90	0.52	−1.73	0.084
			Log Flower:Species LC	−0.49	0.24	−2.10	0.037
Distance	Tripped flowers	Poisson sqrt	Intercept	2.41	0.31	7.70	<0.0001
			Sqrt Distance	−0.07	0.02	−3.03	0.002
			Species LC	−1.52	0.48	−3.20	0.001
			Sqrt Distance:Species LC	−0.11	0.04	−2.70	0.007
	All visited flowers	Poisson log	Intercept	1.60	0.29	5.55	<0.0001
			Log Distance	−0.22	0.06	−3.97	<0.0001
			Species LC	−1.35	0.43	−3.12	0.0018
			Log Distance:Species LC	−0.12	0.09	−1.333	0.18243

deposition curves are expected to carry pollen shorter distances relative to bee species with less steep pollen deposition curves and this is what we observed (Castellanos et al., 2003; Richards et al., 2009). In all cases examined, GUS pollen grains carried by leafcutting bees were depleted faster (steeper slope) than GUS pollen grains carried by bumble bees. In addition, leafcutting bees deposited fewer GUS pollen grains on the first visited flower (asymptote) and on a stigma, on average, over a foraging bout. They deposited fewer total GUS pollen grains over a foraging bout. Leafcutting bees visited fewer flowers per foraging bout and, despite having a greater tripping rate, they tripped fewer flowers in a foraging bout relative to bumble bees. These behaviors would all lead to leafcutting bees moving fewer genes shorter distances relative to bumble bees when visiting *M. sativa* flowers.

Related to the difference in pollen deposition curves, we expect leafcutting bees to create less gene flow relative to bumble bees because they deplete pollen from a specific donor or patch faster, i.e., after visiting fewer flowers or traveling shorter distances. In agriculture, gene flow is usually considered a risk and, with genetically engineered (GE) crops, this could represent the probability of moving GE genes into non-GE fields (Smith and Spangenberg, 2016). For example, adventitious presence, i.e., the unwanted presence of GE genes, in an organic field would negatively impact the organic market. In addition, gene flow between cultivar varieties can threaten cultivar purities. The GE genes can also move from a crop to feral populations or to cross-compatible weedy or wild relatives with potentially negative consequences (Snow et al., 2003; Ellstrand et al., 2013; Greene et al., 2015). In natural systems, gene flow plays an important role as it homogenizes the genetic diversity of plant populations and can lead to introgression and the potential merging of species (Campbell, 2004; Ellstrand, 2014). Distinct pollinators have been previously shown to differentially influence female and male reproductive success and can differentially impact selection on plant traits (Brunet and Holmquist, 2009; Sahli and Conner, 2011; Kulbaba and Worley, 2012, 2013). When comparing distinct bee species, differences have been detected on seed set (Bauer et al., 2017) and on selection of floral traits (Sahli and Conner, 2011; Brunet et al., 2021). We show here that distinct bee species can also affect pollen deposition curves and thus pollen dispersal and subsequent gene flow (Brunet et al., 2019). The impact of distinct bee species on gene flow, population differentiation and introgression deserve further investigation.

The steeper pollen deposition curve of leafcutting bees was associated with a higher tripping rate. Leafcutting bees tripped a greater proportion of visited flowers (86.5%) relative to bumble bees (51.6%) on *M. sativa* flowers. Variation in tripping rate among bee species has been previously linked to gene flow risk, with lesser risk associated with higher tripping rate (Brunet et al. 2019). Because pollen tends to get deposited on tripped flowers, a bee species that trips more flowers is expected to get rid of the pollen from a specific donor faster; in other words, after visiting fewer flowers or traveling a shorter distance, as we observed here. To confirm that pollen remains on the bee's body following visits to untripped flowers, a future study should determine how much pollen remains on a bee following the tripping or lack of tripping of a flower and compare such proportion among bee species. It would also be of interest to examine the relationship between the number of revisits to a flower, prior and after tripping, and the number of pollen grains deposited on a stigma. In addition to tripping rate, grooming is known to influence pollen deposition curves (Harder and Wilson, 1998; Brunet and Holmquist, 2009; Holmquist et al., 2012). We did not observe any differences in the frequency of grooming between the two bee species, although we did not compare its intensity, and it is not clear whether distinct bee species have different grooming habits (Parker et al., 2015). Differences in hairiness among bee species can affect the number of pollen grains deposited in a single visit (Stavert et al., 2016) and could help explain differences in the intercepts between bee species and in the average number of pollen grains deposited on a stigma. However, hairiness affects the interaction between the bee and the stigma and this interaction should not change as a bee visits consecutive flowers. We therefore do not expect differences in hairiness among bees to influence the rate of pollen decay. Future studies may identify other differences in bee behavior or

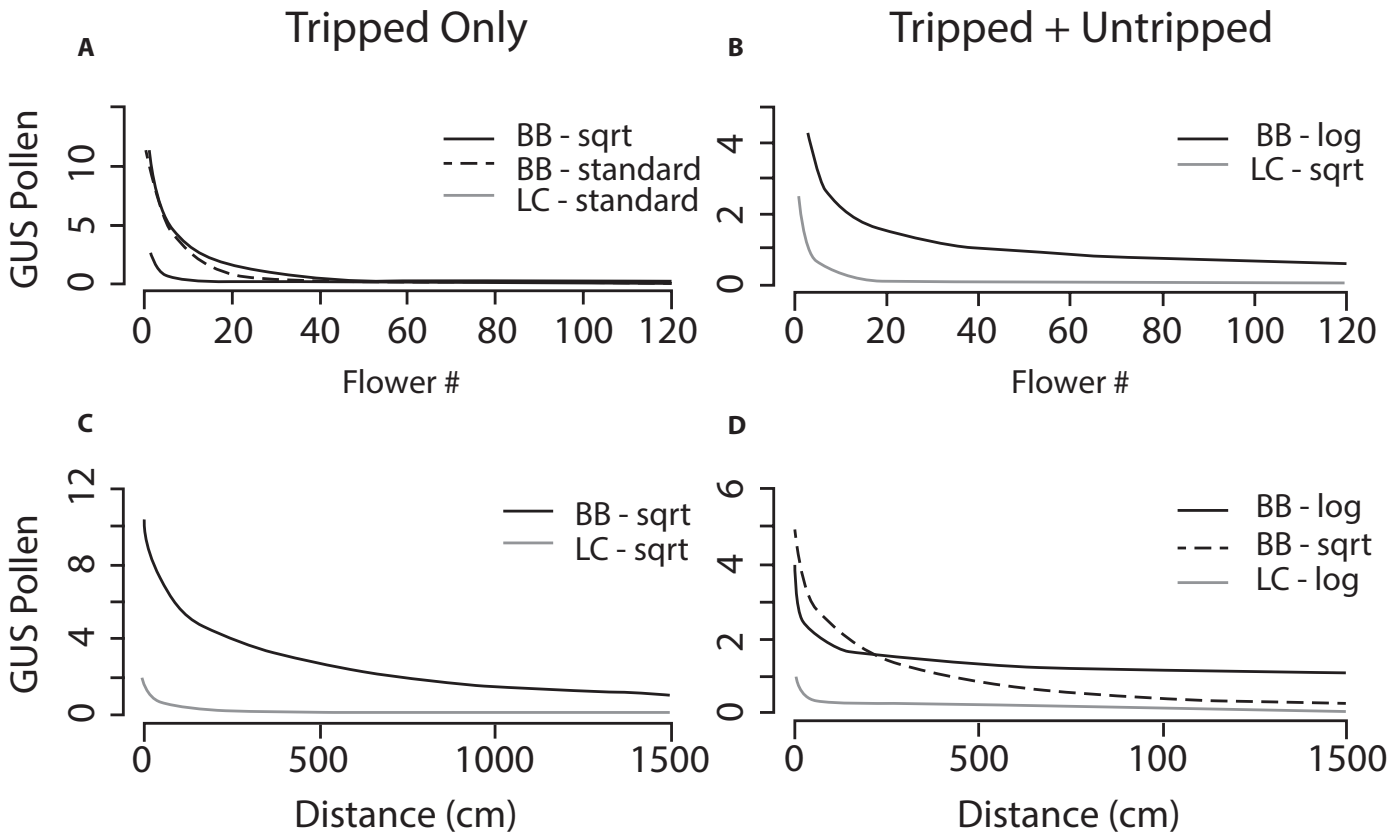

FIGURE 3. Best models for pollen deposition curves between bee species. Comparing bee species for flowers visited in succession (Flower model) for (A) tripped flowers and (B) tripped and untripped flowers (all flowers); for cumulative distance traveled by a bee (Distance model) for (C) tripped flowers; and (D) tripped and untripped flowers. Figures 1 and 2 are placed on a similar scale here to facilitate bee species comparisons.

bee morphology that could influence the rate of pollen decay and thus affect pollen dispersal and gene flow among bee species.

We originally expected the contrast of the pollen deposition curves between tripped only and all visited flowers to illustrate the impact of the tripping rate on the pollen deposition curve. In retrospect, we realize that this is not the case and that contrasting these two curves mainly demonstrates the impact of adding zeros to a pollen deposition curve. For example, a difference in tripping rate would modify the cumulative distance traveled between tripped flowers with lower tripping rate having greater distances between tripped flowers. However, combining tripped and untripped flowers does not change the cumulative distances between tripped flowers. It simply adds distances with zero pollen counts between the existing cumulative distances between tripped flowers. In addition, an increase in tripping rate would increase the number of tripped flowers for a given number of visited flowers. Combining tripped and untripped flowers to the Flower model does not increase the number of tripped flowers but mostly stretches the curve. For instance, a second tripped flower can become the fourth visited flower with two added untripped flowers in between, each with zero pollen count. Thus, comparing the curves of tripped only to all visited flowers does not reflect a difference in tripping rate. As expected, we observed larger differences between pollen deposition curves of the tripped only versus the all flowers visited for bumble bees relative to leafcutting bees. This is to be expected because the lower tripping rate of bumble bees implies a greater number of untripped flowers with zero pollen counts. In order to directly quantify the impact of the tripping rate on the pollen deposition curve, we suggest contrasting the pollen deposition curves of a single bee species with variable tripping rates. This is possible because the tripping rate of leafcutting bees and honey bees, but not bumble bees, visiting *M. sativa* flowers is affected by temperature (Cane, 2002; Pitts-Singer and Cane, 2011; Brunet et al., 2019). Thus, one could contrast the pollen deposition curves of leafcutting bees at two temperatures, hence with two tripping rates.

Environmental conditions, through their impact on the tripping rate, could affect the gene flow potential of some bee species. For example, with a lower tripping rate at cooler temperatures (Cane, 2002; Brunet et al., 2019), leafcutting bees would be associated with a higher gene flow risk at cooler than at higher temperatures. A lower tripping rate would also mean lower seed set or yield per bee. The use of specific managed pollinators in distinct climates could therefore have strong implications for the maintenance of genetic purity and for promoting coexistence of biotech and organic markets (Smith and Spangenberg, 2016; Brunet et al., 2019). One must, of course, consider the potential impact of wild pollinators in the crop fields on gene flow. Plant species in the family Fabaceae, including various crops such as alfalfa, clover, soybeans, beans, peas, chickpeas, lentils, and tamarind, have a tripping mechanism. Some of these crops, such as soybeans, beans, and peas, are mostly self-pollinated although pollinators can increase seed set (Milfont et al., 2013; Blettler et al., 2018). Thus, to understand the potential impact of distinct bee species on pollen dispersal and gene flow for plant species with a tripping mechanism, it is important to determine whether distinct

bee species typically have different tripping rates, whether tripping rate is affected by temperature or other environmental factors and how the tripping rate impacts the pattern of pollen deposition and subsequent gene flow.

In this study, we minimized the unintentional creation of longer dispersal tails known to occur in the study of pollen deposition curves by using plants transformed to express the GUS gene in pollen and by fitting different Poisson distributions with different rates of pollen decay to the pollen deposition data. Our results should encourage the use of similar approaches in future studies of pollen deposition curves. The statistical models were applied to all runs combined and took bee to bee variation into consideration by including a random intercept and a random slope as factors in the model. In all cases the model with RIRS proved superior to a model with just a random intercept (equivalently, with just a random bee effect). Statistical packages are widely available to run statistical models similar to the ones used in this study. As a caution, the plants used in this study represent non-deregulated transgenic material and thus require proper permits. The GUS gene, expressed to various degrees in seedlings or in pollen, has previously been used as a genetic marker to examine the distance traveled by pollen or seeds from a source (Paul et al., 1995; Messeguer et al, 2004; Harst et al., 2009). Only two studies to date, have used the GUS gene expressed in the pollen grains to study pollen deposition curves. Richards et al. (2009) utilized selfed progeny of a TI transgenic line of *Brassica napus* L. (Brassicaceae), where plants had multiple hemizygous insertions of the GUS gene and each plant was tested for GUS expression prior to the experiment. The current study is the first to use plants with a single insertion event and multiple copies of the allele, which ensures the stability of the GUS gene in the progeny and eliminates the need to test each plant for full GUS expression in pollen. The finding that distinct bee species can have different pollen deposition curves should stimulate further research on the impact of distinct pollinators, in particular bee species, on pollen dispersal and gene flow.

CONCLUSIONS

This study demonstrates differences in pollen deposition curves between two bee species that are grooming pollinators and differ in their tripping rates, with implications on pollen dispersal and gene flow. The bee species with the higher tripping rate, the leafcutting bee, had a steeper pollen deposition curve suggesting lower pollen dispersal and gene flow. Because many plant species, including crops, have a tripping mechanism and different bee species are likely to vary in their tripping rates when visiting a plant species, the variation in pollen deposition curves among bee species could be more common than is currently believed. Future research should examine the impact of temperature on tripping rate and gene flow as it bears implications for the impact of climate change on gene flow by distinct bee species. Finally, studies should determine whether other foraging behaviors, besides the tripping rate, could create differences in pollen deposition curves among grooming or among non-grooming pollinators.

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AUTHOR CONTRIBUTIONS

J.B. conceived the study, formulated the hypotheses and supervised the project; M.S. transformed the plants; H.R., M.S., and E.S.M. performed crosses and tests to obtain plants with three or more copies of the GUS genes; E.S.M, C.dC, and J.B. designed the pollen deposition experiment; E.S.M. and C.dC. performed the pollen deposition experiments and processed samples; E.S.M., M.K.C., J.B, and A.F. contributed to data analyses with a major input from A.F.; J.B. wrote the manuscript and E.S.M., C.dC. M.K.C., A.F, and M.S. provided comments.

DATA AVAILABILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.ncjsxkstj (Santa-Martinez et al., 2021).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Pollen deposition over successive flowers visited by a bumble bee during a foraging bout. The three panels (a–c) illustrate separate foraging bouts. Revisited flowers are highlighted and the number of GUS pollen grains deposited on stigmas of a flower visited in a given order in a foraging bout is similar between revisited flowers and flowers that received a single visit.

APPENDIX S2. The standard Poisson, the Poisson log and the Poisson sqrt models for tripped flowers for flowers visited in succession (Flower model) for (a) bumble bee and (b) leafcutting bee and for cumulative distance traveled by a bee (Distance model) for (c) bumble bee and (d) leafcutting bee. The three models are described in the text.

APPENDIX S3. The standard Poisson, the Poisson log, and the Poisson sqrt models for all visited flowers (tripped and untripped), for flowers visited in succession (Flower model) for (a) bumble bee and (b) leafcutting bee, and for cumulative distance traveled by a bee (Distance model) for (c) bumble bee and (d) leafcutting bee. The three models are described in the text.

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