

## RESEARCH ARTICLE

# The timing of visits by large and small bees differentially affects pollination success in *Mimulus ringens*

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## Abstract

**Premise:** Cross-fertilization in most flowering plants is facilitated by mobile animals that transport pollen while foraging for floral rewards. The contributions of different visitors can vary widely, depending on the amount of pollen transferred during a single visit and on the frequency and timing of the visits of each pollinator taxon.

**Methods:** We used three approaches to measure the pollination value of bees that visit *Mimulus ringens*: pollinator interviews, field population observations, and caging studies.

**Results:** The single-visit effectiveness of small bees (primarily Halictidae) was only half that of larger bees (primarily *Bombus*) for pollen delivery and removal. In five field populations, we found substantial temporal and spatial variation in visitation and pollination. In most sites big bees were active before 08:00 hours, and by 10:00–11:00 hours, stigmas were usually fully pollinated and closed, and little pollen remained in anthers. Small bees seldom visited before 10:00 hours. Excluding big bees from plants confirmed that pollination is reduced and delayed in this ecological context.

**Conclusions:** Big bees are the primary pollinators of *M. ringens*, accounting for at least 75% of seed production. Not only are they more effective per visit, in most situations they also visit before small bees become active. Although small bees are not usually important pollinators of *M. ringens*, they have the potential to partially replace them as a “fail-safe” pollinator in contexts where big bees are not abundant. In a world where pollinator abundance is declining, such backup pollinators may be important for maintaining plant reproduction.

## KEYWORDS

bumble bee; effectiveness; Phrymaceae, *Mimulus*; pollination; pollinator visitation; solitary bee; timing of floral visits

Most flowering plants rely on mobile animals to disperse their pollen, and these visitors often vary in their contributions to pollination success (Herrera, 1987, 1989; Waser and Price, 1990; Sahli and Conner, 2007; Steenhuisen et al., 2012; King et al., 2013; Page et al., 2019, 2021; Johnson and Harder, 2023; Pearson et al., 2023). The “quality” of pollination services varies when pollinator taxa differ in the amount of pollen removed or deposited during a single visit (Herrera, 1987). Quality also varies with the proportion of self and outcross pollen deposited, and with the mate composition of the pollen load (Valverde et al., 2019). The “quantity” of pollination services varies with differences in visitation rate (Herrera, 1989). Together, the

quality and quantity of pollination services determines the relative importance of different pollinator taxa to a plant's pollination success by affecting the number and identity of pollen grains transported (Herrera, 1987, 1989; Ne'eman et al., 2010; Schupp et al., 2017; Valverde et al., 2019; Page et al., 2021). It is generally thought that pollinator importance primarily depends on the number of visits by each pollinator (quantity), with per visit quality playing a smaller role (Vázquez et al., 2005).

A less commonly evaluated factor in pollinator importance is the timing of pollinator visitation (Herrera, 1990). Pollen deposited during the first visit after a stigma becomes receptive

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will have a head start in pollen tube growth, and therefore may disproportionately sire ovules (Epperson and Clegg, 1987; Snow et al., 2000; Lankinen and Madjidian, 2011). By contrast, once a stigma is fully pollinated or closed, subsequent pollinator visits will have minimal contributions to seed set. Similarly, if the anthers of a focal flower have little or no remaining pollen, subsequent pollinator visits will not increase the siring contribution of the focal flower.

In past work with *Mimulus ringens* L. (Phrymaceae), we found that visitation by potential pollinators varies widely among sites (Christopher et al., 2021). Although *Bombus* species are usually the most common visitors to *M. ringens*, their visitation rates can substantially vary among sites, and other visitors may be locally more common. For example, visitation by small bees such as sweat bees (Halictidae: e.g., *Lasioglossum*, *Augochlora*), and small carpenter bees (*Ceratina*) sometimes can be substantial. Although the effectiveness of big bees (e.g., *Bombus* and *Anthophora*) in pollinating *M. ringens* is well established (Flanagan et al., 2009), it is not known whether smaller bees transfer pollen among *Mimulus* plants. Visually, small bees appear to gather a full load of pollen from one or a few flowers and do not seem to contact stigmas in ways that would transfer much outcross pollen. This behavior suggests that they may be ineffective pollinators and perhaps even pollen parasites, harvesting pollen without moving pollen among plants (Thomson and Thomson, 1992; Lau and Galloway, 2004; Parker et al., 2016; Koski et al., 2018; Danforth et al., 2019). Furthermore, *Bombus* forage earlier in the day than do smaller bees (Christopher et al., 2021), probably because of their large size and enhanced ability to thermoregulate (Bishop and Armbruster, 1999). Thus, at sites where *Bombus* are abundant, small bees may largely be visiting flowers whose stigmas have already been pollinated and whose anthers have been depleted of pollen. Therefore, these visitors may have little effect on plant reproduction. On the other hand, in ecological contexts where *Bombus* is not present or is in low abundance, these less-effective secondary visitors might provide important pollination services. Empirical data are needed to evaluate these possibilities.

To quantify the relative contributions of these two functional groups of visitors (small bees and big bees) to pollination success in *Mimulus ringens*, we addressed the following questions: (1) Do visitors to *M. ringens* differ in the quality of pollination services they provide? We addressed this question by assessing the amount of pollen transferred during single floral visits. (2) Do *M. ringens* visitors differ in the quantity of pollination services they provide? We investigated this by quantifying visitation rates and pollination rates in several field populations. (3) Does excluding large visitors affect pollination of *M. ringens*? Excluding larger bees such as *Bombus* allowed us to answer this question.

## MATERIALS AND METHODS

*Mimulus ringens* is a self-compatible, wetland perennial herb broadly distributed across eastern North America (Grant, 1924; Karron et al., 1997). It flowers at our Ohio (USA) field sites

during July–August. The 2–3-cm long, blue-purple zygomorphic flowers last a single day, opening before dawn and dropping corollas in the late afternoon. Daily floral displays range from one to six flowers per plant (Christopher et al., 2021). Entry to flowers appears to be easy for larger visitors such as several generalist species of *Bombus* and *Anthophora*, who are heavy and strong enough to open the tightly closed mouth of the corolla tube, allowing them to access both nectar and pollen. Smaller bees (in this study, primarily generalists in the genera *Lasioglossum*, *Augochlora*, *Halictus*, and *Ceratina*) are not large or strong enough to open the flower, and therefore squeeze through the small gap between the upper and lower petals. The four anthers almost always dehisce simultaneously in the early morning, so the amount of pollen available cannot increase over the day. Nectar is produced inside the corolla at the base of the ovary, primarily before anthesis, with a sugar concentration of 21.8% (mass/total mass,  $N = 26$ ). The two-lobed stigmas are open and likely receptive upon anthesis at dawn. *Mimulus ringens* stigmas show a variable but slow closing behavior upon pollination, transitioning from fully open to tightly closed within 3–300 min after pollen deposition. One to several *Bombus* visits are required for flowers to produce a full complement of 2000–4000 seeds/fruit (Karron et al., 2006). Seed production by *M. ringens* in wild populations (including several of the populations studied in the current work) is pollen limited, and the level of pollen limitation increases as visitation rate by *Bombus* decreased (Christopher et al., 2021). Inbreeding depression in field populations averages 45% (Christopher et al., 2021).

## Pollinator interviews

We used pollinator “interviews” (Thomson, 1988; Parker et al., 2016) to evaluate pollen deposition and depletion by different visitors. In June 2019, before flowering, we potted plants from a natural population at the Bath Nature Preserve (Summit County, Ohio, USA) and raised them in a nearby experimental garden. Flowering began in mid-July, and we maintained ~160 potted plants outdoors as a feeder arena to attract and maintain pollinator visitation. The evening before each day of interviews, we placed some plants in a pollinator-free screen tent and removed all open flowers, so that all newly opened flowers were unvisited. The following morning (between 06:00 and 12:00 hours, EDT) we used these flowers for our interviews, using wire pipe cleaners to affix one or two *M. ringens* flowers in natural postures on the end of a 30–90-cm bamboo stick. When we held such flowers adjacent to pollinators foraging in the arena, the bees visited them in a natural manner. We used a stopwatch to measure the amount of time each bee spent in a flower. Over 8 days, we sampled 139 visited flowers (97 *Bombus*, 16 *Anthophora*, 26 small bees) and 114 unvisited flowers.

Once the bee departed one of these flowers, we used fine forceps to dissect the flower and remove the anthers and stigma without disturbing the pollen present. We placed the

four anthers from the flower into one microcentrifuge tube and placed the stigma into a separate tube. We added 1 mL of 70% v/v ethanol to all stigma and anther samples to preserve the contents for analysis. We conducted these interviews between 06:00 and 12:00 hours on 8 days from 23 July to 8 August 2019. We collected two types of control flowers to compare with these visited flowers. Unhandled controls were simply collected in the screen cage and immediately processed as above. Handled controls were affixed to an interview stick and treated just as were visited flowers, but when no bees visited them within 5–15 min, we processed them as above. We found no significant difference among these controls in pollen deposition ( $\chi^2_1 = 2.11$ ,  $P > 0.14$ ) or pollen remaining ( $\chi^2_1 = 0.14$ ,  $P > 0.7$ ) and therefore combined them in analysis. We also measured the amount of nectar remaining in flowers using 5- $\mu$ L microcapillary tubes.

*Mimulus ringens* stigmas receive thousands of pollen grains in multiple layers that cannot be accurately counted using simple stigma squashes. Therefore, we used acetolysis (Kearns and Inouye, 1993), with modifications suggested by Jason Vizelka (University of Wisconsin-Milwaukee, personal communication) to dissolve stigma tissue and generate a liquid suspension of stigmatic pollen in a known volume. Just before counting with a compound microscope, we vortexed the sample for 20 s and then placed 100  $\mu$ L of the sample in a Palmer cell for counting. We repeated this for three separate counts of each tube and used that to estimate total pollen in the tube (accounting for dilution). Replicate counts from individual stigmas were highly repeatable ( $r = 0.84$ ,  $N = 246$ ). We rarely saw pollen from any other species in these samples.

To measure pollen remaining in anthers, we dislodged pollen from anthers using a sonication wand. We then dried the sample in a heated vacuum centrifuge and replaced the evaporated fluid with 1 mL of basic fuchsin stain in ethanol to facilitate counting. We used a compound microscope and three replicate hemacytometer slides from each tube to count pollen and used those counts to calculate mean number of pollen grains/flower. Replicate counts from a flower were highly repeatable ( $r = 0.93$ ,  $N = 192$ ). We adjusted mean counts based on dilution to assess total pollen remaining in that flower.

To analyze these data on single-visit pollen deposition and pollen remaining, we used generalized linear models implemented in PROC GENMOD in SAS v9.4 software (SAS Institute, Cary, NC, USA) to test for effects of visitor (*Bombus*, *Anthophora*, small bees, and control). We used day of the experiment as a fixed categorical blocking variable because each day of study differed in weather and other conditions. We chose error distributions for these analyses based on the Akaike information criterion (AIC) and patterns of residuals. For stigma pollen, we used a gamma distribution with log link, and for pollen remaining, we used a normal distribution with log link. Our primary interest was in evaluating whether small bees (*Lasioglossum*, *Augochlora*, and *Ceratina*) differ from big bees (*Bombus*, *Anthophora*) in pollen deposition and pollen remaining, and we therefore specified an appropriate a priori contrast statement.

## Wild population studies

To compare rates of pollination in different populations, we documented the time course of pollinator visitation, pollen removal, and pollen deposition in five natural *Mimulus ringens* populations in northeastern Ohio, United States during summer 2019. We chose these sites with the aim of providing a wide range of pollinator abundance and species composition (Appendix S1: Table S1, Figure S1), based in part on prior experience at some locations (Christopher et al., 2021). Each site had several hundred or more flowering *M. ringens* plants in an area of at least 200 m<sup>2</sup>. All sites are graminoid-dominated marshes or wet meadows within the Lake Erie Drift Plain ecoregion and have similar climate and edaphic characteristics. All are within 80 km of each other. At each site separately, we conducted observations on a single day, beginning at 06:00–7:00 hours, before most (or all) pollinator activity commenced. We made each of the measurements described below in rotation (45–60 min per rotation) until nearly all stigmas were closed (indicating that effective pollination for the day had finished). Stigmas typically close before noon, following 6–8 sampling rotations (Appendix S1: Table S1).

A round of observation started with collecting stigmatic pollen loads and anther contents from a sample of open flowers. To collect these pollen samples, we walked a haphazard transect through each site, stopping every 2 steps (>1 m) to collect stigma and anthers from a single flower, for a total of 10 flowers per transect. We later processed those samples using the same methods as for the pollinator interview study (above).

We then assessed pollinator visitation rates. Each of two or three observers chose a patch of 30–90 readily visible flowers within an area of approximately 1.5 m<sup>2</sup> and counted the number of flowers observed. We then recorded all flower visits, the number of visitors, and their identity during 15 min. We identified visitors on the wing, typically to genus or species. After completing observations, we occasionally took visitor voucher specimens to confirm the identification. These observations allowed us to calculate number of visits/flower/hour for each visitor taxon or group.

To analyze the field observation data, we used SAS V9.4 PROC GENMOD to test for fixed effects of site, time of day (1-h categories), and their interaction. We used a gamma distribution with a log link for these analyses, based on AIC and other diagnostics. For stigma pollen, we added 1 to all values to avoid zeros.

To evaluate the overall contribution of different-sized pollinators to pollen loads at these sites, we used a method based on the suggestion of Ne'eman et al. (2010) that the amount of pollen deposited by different visitors in a day can be calculated given knowledge of per visit deposition and visitation rate over time. Because *Mimulus* flowers cannot receive pollen after stigmas close, we modified the calculation to reflect this. Thus, for each hour, we calculated expected amount of pollen deposited on stigmas by each pollinator size category by multiplying the per visit deposition by the

visitation rate by the proportion of stigmas that were open during that time period. Summing these values for each pollinator category over time periods allows calculation of the portion of the pollen load on the stigma that was deposited by the pollinators in each size category.

## Caging study

To assess the contributions of small bees to pollination success in the absence of big bees, we conducted a caging study with triplets of potted plants matched by plant size, number of buds, and number of open flowers. In each of 15 triplets, we then randomly assigned each plant to one of three treatments: “open pollination” to allow all visitors, “bagged” to exclude all visitors, and “caged” to exclude big bees. Later analysis indicated no consistent differences in responses among triplets, so we ignored this blocking factor in the analysis. The open-pollinated treatment served as a control; all pollinators had access to these plants. The bagged-treatment plants were enclosed in mosquito netting with a mesh size <1 mm. These bags successfully prevented access for all potential pollinators. The caged treatment served to prevent access by big bees (*Apis*-sized and larger, including all *Bombus* and *Anthophora*) but allowed access by small bees (*Augochlora*-sized and smaller), thus acting as semipermeable membrane. To prevent big bee access, we enclosed each caged-treatment plant with an envelope of black plastic mesh (4-mm mesh) and supported it with wooden stakes. Internal struts kept the sides of the cage away from open flowers. This caging study was adjacent to the experimental gardens used for the pollinator interviews (above).

We placed these triplets in an array of blocks, with the three plants in a block 0.7 m apart and 1.5 m between blocks (Appendix S1: Figure S2). We allowed flower visitors to become accustomed to the array for a day and confirmed that they were foraging normally. We maintained these treatments from 3 to 8 August 2019. To prepare for a day of observation, each evening we removed and discarded open flowers so that all flowers were fresh the next morning. At ~6:00 hours, we counted open flowers on all plants, then sampled one flower from each plant, preserving stigmas and anthers in separate tubes as described earlier. We processed these pollen samples as for the other studies. We repeated this stigma and pollen sampling throughout the day after each round of pollinator observations.

To quantify pollinator visitation, we recorded the species of each floral visitor, the number of flowers visited in sequence, and the number of bees that visited each of the plants in a block in 15 min. There were 2–4 observers, each observing a different block. We recorded pollinator observations three times after each pollen sampling, rotating among blocks, which generated a total of 77 15-min observations across 3 days. We repeated pollen sampling and pollinator observations once every 2–3 h until most stigmas had closed. We observed pollinators on 4, 6, and 8 August 2019 and sampled pollen on 4 and 6 August.

We used generalized linear models (Proc GENMOD) to test for fixed effects of treatment and time of day on visitation, stigma pollen, and anther pollen contents. For visitation, we used a normal distribution with an identity link, for stigma pollen a gamma distribution with the canonical log inverse link, and for anther pollen, we used a gamma distribution with a log link. We used 1.5 h periods to categorize times in this analysis, since this generated suitable sample sizes for each period ( $N = 11$ – $20$ ), pooling across days.

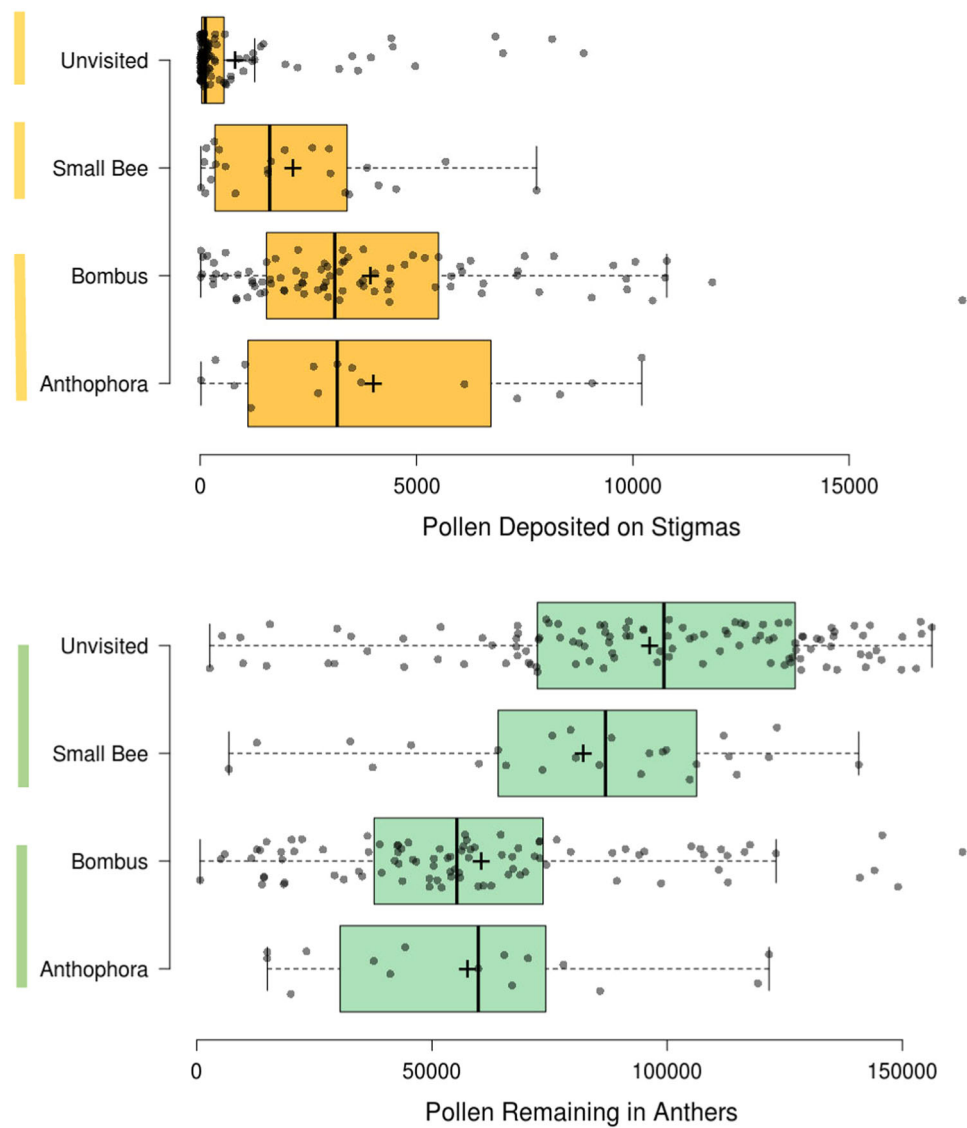
## RESULTS

### Pollinator interviews

The amount of pollen deposited on stigmas during a single visit varied significantly among visitor classes (Figure 1, Table 1; GENMOD  $\chi^2_3 = 69.8$ ,  $P < 0.0001$ , with day as a significant blocking factor). Big bees deposited significantly more pollen than did small bees (planned contrast  $\chi^2_1 = 6.2$ ,  $P < 0.02$ ). Big bees typically deposited about 4000 pollen grains on stigmas, nearly twice as much as did small bees. There was no significant relationship between pollen deposition and visit duration for any group of pollinators ( $P > 0.07$ ). Small bees spent substantially longer in flowers ( $90.8 \pm 8.5$  s) than did *Anthophora* ( $3.1 \pm 11.1$  s) or *Bombus* ( $4.7 \pm 4.5$  s;  $\chi^2_2 = 64.9$ ,  $P < 0.0001$ ).

The amount of pollen remaining in flowers after a single visit also varied significantly among visitor classes (Figure 1, Table 1,  $\chi^2_3 = 65.0$ ,  $P < 0.0001$ , with day as a significant blocking factor). Big bees differed significantly from small bees in pollen remaining (planned contrast  $\chi^2_1 = 12.2$ ,  $P < 0.0005$ ). Big bees left behind over half of the available pollen after one visit (60.6% for *Bombus*, 55.4% for *Anthophora*; Table 1). In contrast, small bees left behind much more of the pollen (87.6%). In fact, there was no significant difference in pollen remaining between flowers that were unvisited and those visited by small bees (Figure 1). Longer visit duration tended to reduce pollen remaining in flowers for all visitors. The increase in pollen remaining with visit duration was significant for *Bombus* (1962 grains/s;  $R^2 = 0.061$ ,  $F_{1,89} = 7.1$ ,  $P < 0.01$ ) and small bees (224 grains/s;  $R^2 = 0.266$ ,  $F_{1,26} = 9.02$ ,  $P = 0.006$ ), but not for *Anthophora*, for which we had a much smaller sample size (6222 grains/s;  $R^2 = 0.062$ ,  $F_{1,11} = 1.32$ ,  $P > 0.2$ ). Pollen transfer efficiency (percentage of pollen grains removed that were deposited) was remarkably similar for large and small bees (Table 1).

Nearly all bees visiting flowers during this study had visible pollen baskets (92.2% overall: 18/18 *Anthophora*, 97/105 *Bombus*, 28/32 small bees), confirming that these bees were foraging for pollen. At least some bees were gathering nectar as well as pollen; nectar remaining in flowers differed significantly among the four categories ( $\chi^2_3 = 14.6$ ,  $P < 0.002$ ). However, the amount of nectar in control flowers (LS mean  $\pm$  SE =  $1.73 \pm 0.13$   $\mu$ L) was only slightly greater than that found in bee-visited flowers (*Anthophora*:  $1.40 \pm 0.23$ , *Bombus*:  $1.07 \pm 0.12$ , small bees:  $1.67 \pm 0.24$ ), suggesting that nectar consumption was minimal.



**FIGURE 1** Pollen deposition and remaining after single visits by different visitors to *Mimulus ringens*. Center lines show the median; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; crosses represent sample means. Vertical bars to the left of the visitor names indicate groups that are not significantly different from one another based on Tukey comparisons.

**TABLE 1** Single-visit removal and deposition and pollen transfer efficiency (percentage of pollen grains removed that were deposited) for common flower visitors of *Mimulus ringens*. Unvisited flowers had  $99,451 \pm 3650$  pollen grains in anthers ( $N = 119$ ), and  $756 \pm 158$  on stigmas. Based on data from Figure 1.

Visitor	No. pollen grains remaining in anthers	No. pollen grains removed	Percent pollen removed	No. pollen grains deposited on stigmas	Pollen transfer efficiency (%)
<i>Anthophora</i>	57,574 (15)	41,877	42.1	4009 (15)	9.6
<i>Bombus</i>	60,352 (96)	39,099	39.3	3941 (90)	10.1
Small bees	82,085 (26)	17,366	17.5	2134 (24)	12.3

### Wild population studies

In 18.25 h of timed observations of *Mimulus ringens* at five sites, we recorded 10 visitor taxa (Table 2; Appendix S1: Table S2). *Bombus impatiens* and *Lasioglossum* spp. were

present at all sites, while other taxa were absent from some sites. We categorized *Bombus*, *Anthophora*, *Apis*, and *Xylocopa* (except *Xylocopa* that were robbing nectar without pollinating) as big bees, and *Lasioglossum*, *Ceratina*, and “green bees” (in the subfamily Augochlorinae) as small bees. Big bees typically

**TABLE 2** Generalized linear model analysis of the time course of visitation to *Mimulus ringens* by different visitor types (big bees vs. small bees) at different wild sites.  $N = 145$  observation periods of 15 min each.

Source	df	$\chi^2$	$P$
Site	4	95.4	<0.0001
Visitor type	1	47.4	<0.0001
Site $\times$ Visitor type	4	12.3	0.015
Time	5	130.6	<0.0001
Site $\times$ Time	19	82.2	<0.0001
Visitor type $\times$ Time	5	19.6	0.005
3-Way interaction	19	51.4	<0.0001

arrived when temperatures were 15–20°C (mean  $\pm$  SE = 18.9  $\pm$  1.3°C), while small bees typically arrived when temperatures were above 20°C (mean  $\pm$  SE = 23.4  $\pm$  1.6°C).

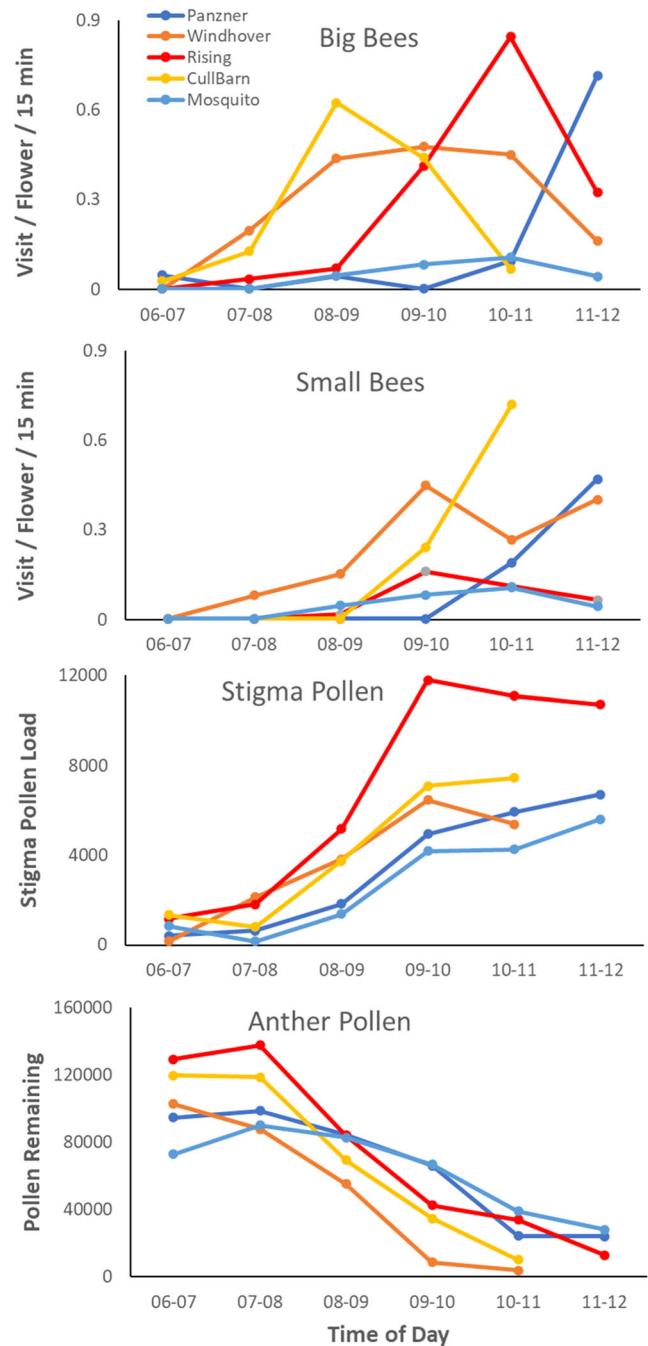
Overall pollinator visitation to *M. ringens* (across time periods) varied strongly among sites and visitor types (GENMOD; for site,  $\chi^2_4 = 44.2$ ,  $P < 0.0001$ , for visitor type  $\chi^2_1 = 9.9$ ,  $P < 0.001$ ). The significant interaction ( $\chi^2_4 = 9.5$ ,  $P < 0.05$ ) indicates that the relative visitation rate by big bees and small bees differed among sites.

The time course of visitation also varied strongly among sites and visitor types (Table 2, Figure 2). Visitation rates for big bees at three sites (Windhover, Rising Valley, Cull Barn) were high in the early morning, when small bees were rare or absent. At the other two sites (Panzner and Mosquito), there was comparatively little total visitation, and big bees were poorly represented at those sites (Figure 2). Small bee activity tended to occur later in the morning than did visitation by big bees.

The amount of pollen on stigmas grew steadily over the morning, and this pattern varied significantly among sites (for time,  $\chi^2_5 = 166.0$ ,  $P < 0.001$ ; for site,  $\chi^2_4 = 43.0$ ,  $P < 0.0001$ ; for interaction,  $\chi^2_{18} = 41.9$ ,  $P < 0.0012$ ; Figure 2). The three sites with higher visitation rates by big bees tended to achieve higher final pollen loads and did so earlier in the day. We found almost no stigmas with heterospecific pollen, although many other bee-visited plant species were flowering nearby at all sites.

Stigma pollen loads rose to near their maximum values by 09:00–10:00 hours at all sites, suggesting that most effective pollination was achieved by that time (well before small bees became active at all sites except Windhover; Figure 2). Maximum pollen loads on stigmas differed strongly among sites, and those with faster pollen accumulation tended to reach higher final stigma loads before small bee visitation increased later in the morning. Rates of stigma closure showed a similar pattern, with later stigma closure at the two sites with lower visitation (Panzner and Mosquito; see Appendix S1: Figure S3).

The amount of pollen remaining in anthers declined rapidly over the day, and this pattern also varied significantly among sites (for site,  $\chi^2_4 = 64.5$ ,  $P < 0.0001$ ; for



**FIGURE 2** Time course of visitation and pollination for *Mimulus ringens* at five field sites. Values shown are LS means. SE is not shown to avoid clutter. Warmer colors indicate higher overall visitation rates.

time,  $\chi^2_5 = 269.9$ ,  $P < 0.001$ ; for interaction,  $\chi^2_{18} = 97.94$ ,  $P < 0.0001$ ; Figure 2). Although less pronounced than for the increase in stigma pollen loads, there was a tendency for the sites with higher visitation rates to lose pollen more rapidly.

To evaluate the situation at the end of meaningful pollination activity, comparing the stigma and anther contents for the last sample of the day at each site is useful (Figure 2). These values differed significantly among sites

(stigmas,  $\chi_4^2 = 16.9$ ,  $P < 0.002$ ; anthers,  $\chi_4^2 = 15.7$ ,  $P < 0.03$ ). Stigma pollen loads were lowest at Mosquito and Windhover and highest at Rising Valley, differing nearly 2-fold among sites. Anther pollen remaining was much higher at Mosquito (29% of the values at the start of the day) and lowest at Windhover (2% of values at the start of the day).

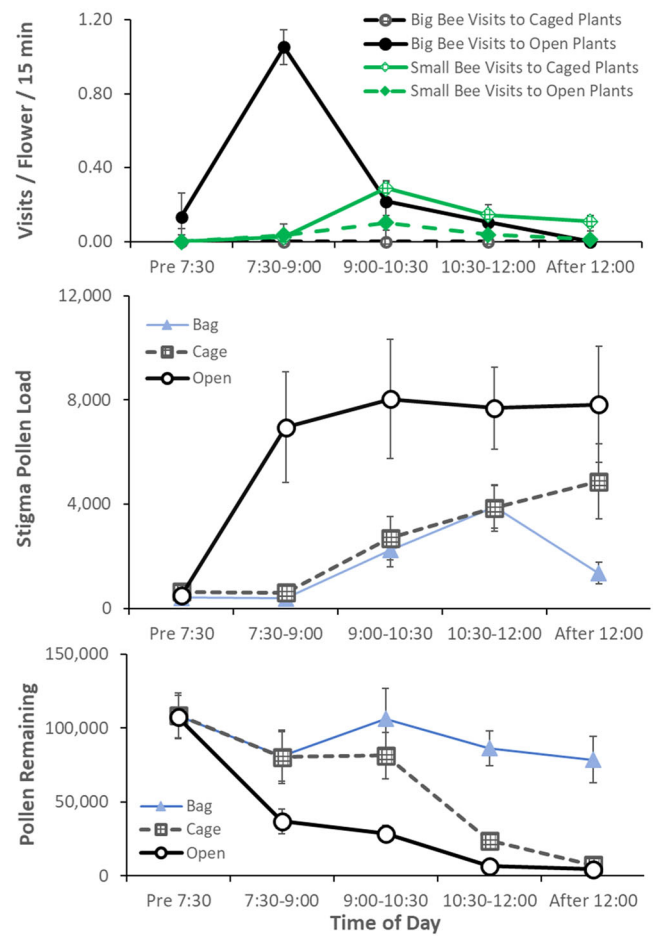
We evaluated the relative contributions of big bees and small bees to stigma pollen loads in each of our field populations using the conceptual framework of Ne'eman et al. (2010) as described in the methods (note that this accounts for temporal patterns of visitation, single visit pollen delivery, and stigma closure). These calculations indicate that big bees accounted for over 75% of pollen deposition in all field populations, even those where big bees were scarce (Panzner = 77.5%, Windhover = 80.8%, Mosquito = 92.8%, Rising Valley = 93.3%, Cull Barn 96.6%).

### Caging study

Caging strongly affected the time course of visitation to *Mimulus ringens* in our experimental garden (Figure 3). Visitation by big bees and by small bees varied strongly with treatment (for big bees,  $\chi_1^2 = 18.5$ ,  $P < 0.0001$ ; for small bees,  $\chi_1^2 = 14.5$ ,  $P < 0.0001$ ), with time of day (big bees,  $\chi_4^2 = 37.7$ ; small bees,  $X_4^2 = 38.0$ ;  $P < 0.0001$ ), and with the interaction of treatment and time of day (big,  $X_1^2 = 33.7$ ;  $P < 0.0001$ ; small,  $\chi_1^2 = 13.7$ ,  $P < 0.009$ ). Visitation by big bees to open-treatment plants peaked strongly in the early morning (07:30–09:00 hours) at over 1 visit/flower/15 min and was absent for caged plants. Small bee visitation peaked later (09:00–10:30 hours), and visitation to caged plants was 2.8-fold higher than that for open plants at that time. The increase in small bee visits to caged vs. open plants was even greater later in the morning (3.8- and 8.1-fold). No bees visited flowers on bagged plants.

Caging and bagging strongly affected the accumulation of stigma pollen loads over the morning (Figure 3; treatment,  $\chi_2^2 = 15.9$ ,  $P < 0.0004$ ; time,  $\chi_4^2 = 124.0$ ,  $P < 0.0001$ ; interaction,  $\chi_8^2 = 41.2$ ,  $P < 0.0001$ ). Stigmas on flowers in all treatments initially lacked pollen, but those in the open treatment quickly reached full pollen receipt (6924 pollen grains by the end of the period 07:30–09:00 hours). Indeed, pollen loads for open-treatment stigmas did not substantially increase after 09:00 hours, presumably because they had closed after the first few visits. In contrast, caged plants, only available to small bees, accumulated pollen later and slower. The amount of pollen on stigmas in the caging treatment at the end of the day (4872 pollen grains) was much less than that on stigmas open to big bees (7824). Stigmas on bagged plants accumulated small increases of pollen over the day, presumably from jostling within the bag.

Caging and bagging also strongly affected the time course of pollen remaining in anthers (Figure 3; treatment,  $\chi_2^2 = 133.1$ ,  $P < 0.0001$ ; time,  $\chi_4^2 = 195.5$ ,  $P < 0.0001$ ; interaction,  $\chi_8^2 = 110.7$ ,  $P < 0.0001$ ). All plants started with full



**FIGURE 3** Effects of treatments on visitation and pollination for the caging experiment. Values shown are LS means  $\pm$  SE. For visits by big bees to caged plants (dashed black lines in upper panel), all values are zero.

anthers, but flowers in the open treatment lost almost all their pollen between 07:30 and 09:00 (from an initial value of  $\sim 107,000$ , declining to  $\sim 37,000$ , or only 34.7% remaining for the 07:30–09:00 period). This period coincides with the time of maximal activity by big bees. Open treatment flowers continued to lose pollen through the rest of the morning, becoming nearly empty by the end of the morning (4.2% remaining). Flowers in the caged treatment lost some pollen in the early morning, but mostly lost pollen between 10:30 and 12:00 hours, after small bees became active. Again, the changes in pollen remaining in caged flowers, as for pollen deposition, was slower than that for flowers available to big bees. Flowers on bagged plants lost small amounts of pollen over time, presumably because of jostling within the bag.

### DISCUSSION

The identity of pollinators and the timing of floral visits strongly influence the quality and quantity of pollination services to *Mimulus ringens*. Pollinator species composition and rate of visitation vary substantially over the course of

the day and among sites. Thus, the relative importance of different floral visitors may be context dependent. Experimental exclusion of big bees strongly affected both pollen deposition and pollen removal. We consider these findings and their consequences in more detail below.

### Quality of pollination services from single visits

Single visits by big bees deposited and removed approximately twice as much pollen per visit as did single visits by small bees (Table 1). However, big bees and small bees had similar pollen transfer efficiencies (proportion of grains removed that were deposited; Table 1), delivering between 9.6 and 12.3% of the pollen they removed to stigmas (Table 1). Thus, although small bees deposit less pollen per visit than big bees, they may be effective pollinators and are not necessarily acting as pollen parasites. Given the similar pollen transfer efficiencies of these two functional groups, their relative importance to *Mimulus* pollination largely depends on the rate and timing of visitation.

Small bees may also deliver a higher proportion of self-pollen to stigmas than do big bees. Although we were not able to test this directly, we know that small bees (1) spend much longer than big bees in flowers (see above), (2) usually emerge from flowers with scopae nearly completely full of pollen, and (3) are much less likely to move between plants compared to big bees. All these behaviors increase the likelihood that small bees are delivering more self-pollen compared to big bees.

### Quantity and timing of pollination services in wild populations

Big bees and small bees also differed in the quantity and timing of pollination services they provided. Big bees had higher per flower visitation rates than small bees had and usually visited earlier in the morning. Many small bee visits occurred after stigmas had closed and anthers were largely depleted of pollen. For example, see the Mosquito and Panzner populations in Figure 2 for 10:00–11:00 hours. However, note that there was marked variation among sites in pollinator species composition, relative abundance, and timing of peak visitation.

At sites where big bees were abundant, anthers were largely depleted of pollen by 09:00–10:00 hours. From a pollen forager's point of view, the expected resource value gained from visiting a flower declined rapidly because of this depletion. From a plant's point of view, when big bees were common, nearly all pollination (arrival of pollen on stigmas and export to open stigmas) was achieved by 09:00–10:00 hours (Figure 2). Any visitation after that period was unlikely to deliver pollen to a receptive stigma. Indeed, our calculations (Ne'eman et al., 2010) indicate that over 75% of pollen arriving on *M. ringens* stigmas was the result of visitation by big bees. This result suggests that

although small bees often visit *M. ringens*, in contexts where big bees are frequent, they seldom have an opportunity to act as effective pollinators. By the time small bees begin visiting *Mimulus* (typically after 09:00 hours), most flowers have already been fully pollinated by big bees, and stigmas are closed.

Our study highlights the importance of temporal differences in visitation by different pollinator functional groups (Herrera, 1990; Ashman et al., 1993; Stone et al., 1998; Pisanty et al., 2016; Kendall et al., 2022; Štenc et al., 2023). As just noted, when big bees are abundant, frequent visitation by small bees to flowers with closed stigmas will have little influence on *M. ringens* pollination success. Indeed, in this context, small bees may gain pollen resources without pollinating, because few or no stigmas are available to receive the remaining pollen. In contrast, when big bees are scarce, small bees may be a valuable but inferior backup pollinator. Our findings underscore the importance of evaluating the timing of visitation by different pollinator classes when studying pollinator importance.

In addition to variation in the timing of visitation, there can also be substantial spatial variation in pollination services to *M. ringens* (Figure 2). Among-site variation in pollinator species composition and visitation rate is common in other systems and can strongly affect the levels of pollination experienced by plants (Thompson, 2001; Ollerton and Cranmer, 2002; Kalisz and Vogler, 2003; Parker et al., 2016; Waser and Price, 2016). In our study, we found that small bees were common at all sites later in the morning, while big bee abundance ranged widely. At sites where big bee visits are uncommon, small bees may act as fail-safe pollinators (see below). Furthermore, the species composition within functional groups also varied substantially (Appendix S1: Table S2, unpublished data). Such a spatial mosaic of interactions, if maintained over time, could provide an opportunity for local adaptation and coevolution (Thompson, 1994). Indeed, our studies in prior years at several of the sites reported here showed consistent site-specific patterns of pollinator visitation and species composition (Christopher et al., 2021).

### Excluding big bees: Is the role of small bees context dependent?

The caging experiment allowed us to explore pollination services when only small bees were present. We found that exclusion of big bees reduced pollen receipt, and delayed pollination because of differences in visitor identity, visitation rate, visitation effectiveness, and timing. As in many wild populations, in the caging study most big bee visits to open plants occurred between 07:30 and 09:00 hours, whereas visits by small bees peaked later in the morning. Furthermore, small bees appeared to avoid the depleted flowers on open plants and were more abundant on caged plants, suggesting that they were responding to preemptive (earlier) pollen consumption by big bees. The



pattern of pollen receipt for caged and uncaged plants matches the patterns of pollinator visitation and suggests that small bees were only partially able to replace the function of the absent big bees. This matches patterns in seed set (unpublished data). Thus, in contexts where big bees were not present, late-visiting small bees partially made up for pollination by big bees.

Excluding pollinators by size to determine their relative contributions is a common and useful approach for contrasting contributions of markedly different classes of pollinators such as birds vs. bees (Lange and Scott, 1999; Fenster and Dudash, 2001; Robertson et al., 2005; Symes et al., 2009; Steenhuisen et al., 2012). However, selective exclusion of bees by size is relatively infrequent (e.g., Heard, 1994; Campbell et al., 2018; Lassen et al., 2018). The strong differences we detected among bees of different sizes suggest that further studies of this sort on more plant species would be informative and useful. In our study, 4-mm mesh excluded big bees and allowed good access for small bees, achieving our intended manipulation. The large differences in the amount and temporal pattern of pollen deposition and removal between big and small bees were a valuable confirmation of expectations for context dependence generated by our other studies.

## CONCLUSIONS

This research provides insight into how the identity and abundance of floral visitors affects plant reproduction. Pollinator visitation rates and timing of visitation for *Mimulus ringens* varies widely among sites. Thus, the pollination environment is a spatial mosaic, opening the possibility for altered ecological interactions and evolutionary trajectories (Thompson, 1994).

Our results suggest that the effects of a general pollinator decline may be quite different from those predicted if a single pollinator functional group or species declines (Pauw, 2007; Brosi and Briggs, 2013; Hallett et al., 2017). Not all pollinators have equal effects on plant reproduction, and the effect can depend upon the ecological context. Floral visits by big bees are important for reproductive success in the *M. ringens* populations we studied. However, in contexts where *Mimulus* populations have very few big bees, small bees may act as a fail-safe pollinator (Figure 3), providing critical pollination services.

## AUTHOR CONTRIBUTIONS

R.J.M., D.S., V.F., and J.D.K. conceived the ideas and designed methodology; R.J.M., D.S., and V.F. collected the data; R.J.M. analyzed the data; R.J.M. and J.D.K. led the writing of the manuscript.

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## DATA AVAILABILITY STATEMENT

Data are available at Figshare: <https://figshare.com/s/3fd5bb2724d88d3cde92>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

### Appendix S1

**Figure S1.** Map of northeastern Ohio.

**Figure S2.** Cage experiment setup.

**Figure S3.** Time course of stigma closure for *Mimulus ringens* at five field sites.

**Table S1.** Field sites for study of *Mimulus ringens* pollination in Northeastern Ohio USA.

**Table S2.** Visitors to *Mimulus ringens* during timed observations at five field populations.

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