

## MULTIPLE POLLINATOR VISITS TO *MIMULUS RINGENS* (PHRYMACEAE) FLOWERS INCREASE MATE NUMBER AND SEED SET WITHIN FRUITS<sup>1</sup>

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The timing and effectiveness of pollinator visitation to flowers is an important factor influencing mating patterns and reproductive success. Multiple pollinator probes to a flower may increase both the quantity and genetic diversity of progeny, especially if single probes deposit insufficient pollen for maximal seed set or if the interval between probes is brief. When pollen carryover is limited, sequential pollen loads may also differ markedly in sire representation. We hypothesized that these conditions help explain high levels of multiple paternity in *Mimulus ringens* fruits. We documented all bee visits to individual flowers, quantified resulting seed set, and determined paternity for 20 seeds per fruit. Most (76%) flowers received multiple probes, and the interval between probes was usually <30 min. Flowers probed multiple times produced 44% more seeds than flowers probed once. All fruits were multiply sired. Flowers receiving a single probe averaged 3.12 outcross sires per fruit, indicating that single probes deposit pollen from several donors. Multiple paternity was even greater after three or more probes (4.92 outcross sires), demonstrating that sequential visits bring pollen from donors not represented in the initial probe.

**Key words:** bumble bee; monkeyflower; multiple paternity; paternity analysis; Phrymaceae; pollination effectiveness; pollination intensity; seed set.

In most angiosperms, male gametes are dispersed by animal pollinators harvesting floral rewards. The foraging patterns of these pollinators often influence plant reproductive success, plant mating systems, and the spatial organization of genetic variation (Waser, 1983; Campbell and Waser, 1987; Karron et al., 1995a, 1997, 2004; Harder and Barrett, 1996; Barrett, 2003; Mitchell et al., 2004, 2005; Bell et al., 2005). For example, the number of pollinator probes received by flowers may affect the amount of pollen delivered and the number of ovules fertilized (Young and Stanton, 1990; Mitchell and Waser, 1992; Jones and Reithel, 2001). The efficiency of each probe in depositing pollen is also important (Snow, 1986; Mitchell and Waser, 1992) and may depend upon interspecific differences in pollinator morphology and behavior (Bertin, 1982; Herrera, 1987; Thomson and Thomson, 1992; Thomson and Chittka, 2001; Ivey et al., 2003), the duration of the probe (Young and Stanton, 1990), the proportion of conspecific and heterospecific pollen in the pollen load (Waser, 1978, 1983; Galen and Newport, 1988; Brown and Mitchell, 2001), and other factors. For many plant species, the amount of pollen deposited during a single probe may not be sufficient for maximal seed set (Bertin, 1982; Snow, 1986; Jahns and Jolliff, 1990). Pollen limitation of seed set following a single probe may be especially likely in flowers with many ovules (Bertin, 1982; Dudash and Ritland, 1991; Knight et al., 2005).

In self-compatible species, the number of probes to

individual flowers may also influence two aspects of the mating system: the proportion of outcross progeny within fruits and the patterns of paternity within fruits. As the number of probes to a flower in female phase increases, outcross-pollen deposition may rise more rapidly than self-pollen deposition, leading to a gradual increase in the outcrossing rate (Karoly, 1992). This is especially likely if much of the self-pollen deposition is due to prior selfing, competing selfing, and/or delayed selfing (Lloyd and Schoen, 1992; LeClerc-Potvin and Ritland, 1994); modes of selfing that occur without involvement of a pollinator and therefore are likely to show little response to the number of pollinator probes. If self progeny exhibit inbreeding depression, differences in outcrossing rate between single-probe and multiple-probe flowers may lead to differences in offspring performance.

The number of probes may also influence levels of multiple paternity within fruits. When pollen carryover is limited, pollen loads deposited sequentially on a stigma may differ markedly in sire representation (Mitchell et al., 2005). This is especially likely if successive pollinators approach a flower from distinct compass directions and bring in pollen loads from different donors (Harder and Barrett, 1996). Although pollen from the first probe may have a distinct advantage because of priority (Epperson and Clegg, 1987), if the interval between sequential probes is brief, pollen grains deposited during later probes may compete successfully for access to ovules (Spira et al., 1996; Snow et al., 2000). Successful fertilization by pollen from sires not represented in the initial pollen load would increase mate number for each fruit and would decrease genetic relatedness among siblings (Ritland, 1989). This increased level of multiple paternity may influence competitive interactions among developing seeds within fruits and among seedlings in the field (Ellstrand and Marshall, 1986; Karron and Marshall, 1990, 1993; Marshall, 1991; Paschke et al., 2002).

In an earlier study of the self-compatible wetland perennial *Mimulus ringens* L. (Phrymaceae), we demonstrated that nearly all fruits are multiply sired, with as many as nine donors ( $\bar{X}$  =

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4.63 ± 0.1 donors; Mitchell et al., 2005) siring the seeds in a single fruit. Because pollen carryover in *M. ringens* is limited and 95% of the pollen dispersed from a donor flower is deposited on one of the next four recipient flowers (Holmquist, 2005), we hypothesized that this high level of multiple paternity does not result solely from the simultaneous deposition of mixed pollen loads during a single floral probe. Instead, we predicted that the observed level of multiple paternity results from multiple probes to flowers, each depositing pollen from 1–4 sires. Properly characterizing the mechanism of multiple paternity was not possible in our earlier study, which recorded paternity, but did not collect data on the complete visitation history of individual flowers (Mitchell et al., 2005). Such data are necessary to determine whether flowers receiving a single visit differ in mate number from flowers receiving multiple visits.

Surprisingly few studies have quantified both the visitation history of individual flowers and the resulting reproductive success. A handful of studies (e.g., Young and Stanton, 1990) have examined the relationship between number of floral probes and pollen deposition or seed set. By contrast, the effects of visitation history on mating patterns are less well understood (Harder and Barrett, 1996; Karron et al., 2004; Mitchell et al., 2005). Indeed, we know of no published studies of the relationship between number of floral visits and number of sires per fruit. In this paper, we quantify the effects of single and multiple pollinator probes on seed set, outcrossing rates, and mate number in *Mimulus ringens*. Our study addresses the following questions: (1) Do fruits of multiple-probe flowers have more seeds than fruits of single-probe flowers? (2) Is the number of outcross seeds higher in fruits of multiple-probe flowers than in fruits of single-probe flowers? (3) Does the number of floral probes influence the outcrossing rate of individual fruits? (4) Is the number of sires higher for fruits of multiple-probe flowers than for fruits of single-probe flowers?

## MATERIALS AND METHODS

**Reproductive biology**—*Mimulus ringens* plants produce showy displays of 1–15 large (2 cm), blue, zygomorphic flowers, which last for half a day (Karron et al., 2004). Naturally occurring plants near our SE Wisconsin study site have a mean daily floral display (±1 SE) of 2.84 ± 1.42 flowers ( $N = 132$ ). Flowers in a single display are usually scattered across several separate stems. Although flower buds are frequently paired at leaf nodes, paired flowers generally open on different days.

Anthesis occurs at dawn, and individual flowers typically receive 1–4 probes by bumble bee workers between the hours of 0530 and 1130 Central Daylight Time (Mitchell et al., 2004, 2005). When probing flowers, bumble bees first contact the stigma and then the anthers with their faces (Fig. 1a). When pollen is deposited on the bilobed stigma, closure occurs gradually within 30–90 min (Mitchell et al., 2005). Note that *M. ringens* lacks the rapid (3–12 s) stigma closure (“sensitive stigma”) mechanism found in many other species of *Mimulus* (Ritland and Ritland, 1989; Fetscher and Kohn, 1999; Beardsley and Barker, 2005). By 1100 hours, most stigmas are closed, and corollas are shed by late afternoon.

*Mimulus ringens* pollen grains are very small (12 µm), and often accumulate in 2–3 layers on the stigma. Each layer may include several thousand pollen grains (Bell et al., 2005). As shown in Fig. 1b, the papillae increase stigmatic surface area for pollen germination. Grains germinate successfully in each layer and grow pollen tubes down to the stigmatic surface. Styles usually contain thousands of pollen tubes (Fig. 1c), which fertilize 700–6000 ovules. Nearly all flowers produce seed capsules (Fig. 1d).

In our main study population, controlled self and outcross hand-pollinations do not differ in number of seeds per fruit, seed mass, germination rate, or seedling survival (Karron et al., unpublished data). However, inbred progeny

have lower fitness at later stages of the life cycle, especially flower and fruit production. The overall fitness of self progeny is 20–30% lower than the overall fitness of outcross progeny (J. Karron et al., unpublished data).

**Experimental array of plants with unique marker genotypes**—To quantify the effects of floral visitation history on patterns of paternity within fruits, we established an experimental array consisting of 16 genets with unique multilocus combinations of homozygous allozyme genotypes. This design enabled us to unambiguously assign paternity to all sampled offspring, allowing us to quantify both the outcrossing rate and the number of sires of each fruit with a high degree of precision (Karron et al., 1995a, b, 2004; Mitchell et al., 2005).

We produced each of the homozygous genotypes used in our experiment by outcrossing 22 multilocus heterozygotes derived from a single naturally occurring population. This breeding program ensured that none of the marker genotypes resulted from a history of inbreeding. Further details concerning the breeding of these lines may be found in Karron et al. (1995a, 2004).

During the first week of June 2002, we planted an array of 36 ramets in a tilled garden at the University of Wisconsin-Milwaukee Field Station (Saukville, Wisconsin, USA). We planted the 36 ramets in a square grid with 0.8 m between plants. We chose this population size and spacing interval because it is typical of what we observe in natural populations. In the center of the array, we planted single ramets of each of 15 genets (Fig. 2). We surrounded these “central genets” with a buffer row of 21 ramets of genet “D” to minimize edge or boundary effects on patterns of pollinator visitation.

**Pollinator observation**—*Mimulus ringens* plants grow slightly larger in our experimental arrays than in field conditions. To ensure that floral display sizes were more representative of natural populations, we trimmed displays so that each plant had four open flowers in compass positions N, S, E, and W. To readily identify the flower in each compass position, we attached a small, colored wire to the base of each pedicel, using a different color for each compass position. We trimmed excess flowers with scissors at 0500 hours, before pollinators became active. We performed these floral display manipulations daily throughout the peak period of flowering, 7–12 August 2002, so that pollinators could acclimate to display sizes in the array.

On 11 and 12 August our team of four observers recorded the time and location of every floral probe in the array from anthesis (approximately 0550 hours) until closure of all stigmas (approximately 1130 hours). We observed a total of 762 floral probes by bees (almost exclusively *Bombus fervidus* workers) to 288 flowers over 2 days.

Because flowers typically receive multiple pollinator probes prior to stigma closure (Mitchell et al., 2005), we devised a strategy to ensure that one flower on each “central genet” only received a single pollinator probe. We randomly designated a single flower on each display to receive a fine mesh pollinator exclusion bag within 30 s following the first pollinator probe to that flower. These pollinator exclusion bags were removed at 1600 hours, after corollas were shed.

**Determining seed set, outcrossing rates, and mate number within fruits**—Three hours after all stigmas had closed, we tied labeled plastic tags to pedicels of all four flowers on each of the 15 central genets (60 flowers on each of 2 days). Flowers on the border rows were not tagged because we could not quantify the mating system precisely on those ramets. On 12 September 2002, we harvested each of the 120 labeled fruits. Slightly more than half of the fruits had already opened and therefore could not be used for analyses of seed set. Seeds in the remaining 45 unopened fruits were counted using a dissecting microscope.

Progeny arrays from 51 fruits (the 45 unopened fruits plus an additional six fruits that had only lost a small fraction of their seeds) were suitable for genotyping. The distribution in number of probes received by these fruits was not distinguishable from the distribution for all available fruits  $\chi^2 = 1.76$ ,  $df = 2$ ,  $P > 0.4$ , indicating that fruit loss was unbiased with regard to the number of probes received. We germinated progeny arrays in separate pots, then transplanted 2-wk-old seedlings into individual cells in plastic flats. Seed germination rates were high (>90%), and there was very little seedling mortality. After three additional weeks of growth, up to 20 ( $\bar{X} = 19.7$ ) randomly selected seedlings from each progeny array were genotyped at four allozyme loci using the methods of Karron et al. (2004). Because each central genet had a unique multilocus genotype, we were able to assign paternity unambiguously to all 1004 sampled seedlings. Therefore, we could determine the sire of each seed and whether seeds were self or outcross. This method necessarily



Fig. 1. Sexual reproduction in monkeyflower, *Mimulus ringens*. (a) *Bombus fervidus* worker exiting a *M. ringens* flower (photo: R. J. Mitchell). The white stripe of pollen on the face and proboscis contacts the stigma of each flower probed during a foraging sequence. (b) SEM showing pollen grains germinating on the surface of *M. ringens* stigma (photo: J. M. Bell). (c) *Mimulus ringens* pollen germination and tube growth, photographed under fluorescence microscopy (photo and microscopy: S. Miller and J. Thompson). The stigma surface is in the upper left, with pollen tubes growing down the style toward the ovary, which is out of sight toward the lower right. (d) Seeds in a capsule of *M. ringens* (photo: K. G. Holmquist). Fruits often contain several thousand tiny (0.008 mg) seeds.

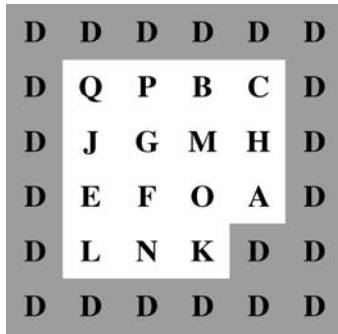


Fig. 2. Arrangement of *Mimulus ringens* plants with unique multilocus genotypes in the experimental array. Single ramets of 15 genets are shown on a white background. Multiple ramets of border genet “D” are shown on a gray background.

underestimates the number and diversity of mates because seeds sired by the 21 ramets in the border row cannot be distinguished from one another (Mitchell et al., 2005).

**Data analysis**—For all analyses we classified fruits in one of three approximately equally sized categories, depending upon the number of floral probes: (a) flowers receiving a single probe, (b) flowers receiving two probes, or (c) flowers receiving three or more probes. We then used ANOVA (SAS Institute, 2000) to test for differences among floral probe classes in (1) total number of seeds per fruit, (2) outcrossing rate, and (3) number of outcross seeds per fruit (calculated as outcrossing rate  $\times$  the total number of seeds). To test for differences in (4) number of outcross sires per fruit, we used the generalized linear model (GLM) (PROC GENMOD; SAS Institute, 2000) for a categorical analysis, appropriate for small integer data such as these.

The number of floral probes could potentially influence the evenness of outcross siring success as well as the number of outcross sires. If there is an exponential decline in pollen carryover to each successive flower in a visitation sequence, representation of pollen donors in a pollen load may be highly skewed (Holmquist, 2005; Mitchell et al., 2005). Additional probes depositing pollen of different donors in rapid succession may tend to even out donor representation. We calculated the Simpson index of evenness (Brower et al., 1998) for outcross sire representation in each fruit and used ANOVA to test for differences in evenness.

RESULTS

Approximately 93% of the 258 unbagged flowers received at least one pollinator probe, and 76% of the unbagged flowers received multiple probes. The mean number of probes per flower  $\pm$  1 SE was  $2.61 \pm 0.14$  on 11 August and  $2.92 \pm 0.14$  on 12 August. The interval between first and second probes was often quite brief (Fig. 3), with 80% of second probes within 30 min of the initial probe.

Total seeds per fruit varied significantly with the number of floral probes (ANOVA,  $df = 2, 42; F = 5.16, P < 0.01$ ), increasing from  $1633 \pm 178$  (mean  $\pm$  1 SE) seeds in fruits of single probe flowers to  $2345 \pm 152$  seeds in fruits of flowers probed three or more times (Fig. 4).

The number of outcross seeds per fruit also varied significantly with number of floral probes (ANOVA,  $df = 2, 42; F = 5.50, P < 0.008$ ), increasing from  $597 \pm 181$  (mean  $\pm$  1 SE) outcross seeds in fruits of single probe flowers to  $1367 \pm 154$  outcross seeds in fruits of flowers probed three or more times (Fig. 4). By contrast, the number of self seeds per fruit was nearly constant across the three floral probe classes ( $1036 \pm 142$  self seeds in fruits of single probe flowers;  $908 \pm$

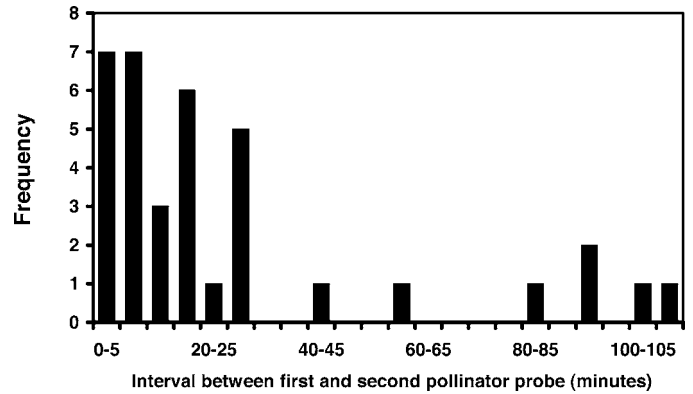


Fig. 3. Distribution of the length of time between first and second pollinator probes for each of 36 flowers of *Mimulus ringens* receiving two or more probes. Frequencies are reported in 5-min intervals. More than 80% of second probes occurred within 30 min of the first probe.

214 self seeds in fruits of flowers probed two times; and  $978 \pm 121$  self seeds in fruits of flowers probed three or more times (ANOVA,  $df = 2, 42; F = 0.13, P = 0.88$ ). Outcrossing rates (“*t*”) increased slightly with number of floral probes (single probe  $t = 0.40$ ; two probes  $t = 0.44$ ; three or more probes  $t = 0.54$ ), but the differences were not statistically significant (ANOVA,  $df = 2, 48; F = 2.29, P = 0.11$ ).

The number of outcross sires per fruit varied markedly with number of floral probes (GLM,  $\chi^2 = 8.71, df = 2, 48, p < 0.01$ ), increasing from  $3.12 \pm 0.44$  (mean  $\pm$  1 SE) sires of seeds in single probe fruits to  $4.92 \pm 0.37$  sires of seeds in fruits of flowers probed three or more times (Fig. 5). The

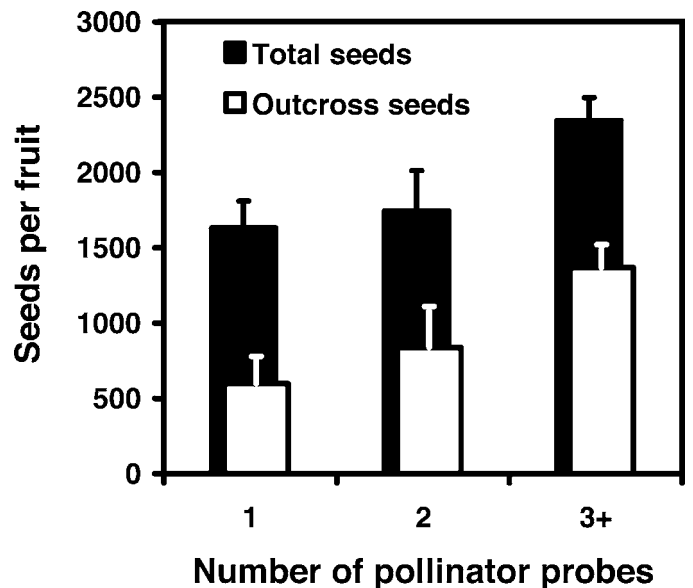


Fig. 4. Effect of number of pollinator probes on number of outcross seeds and total seeds per *Mimulus ringens* fruit ( $\pm$ 1 SE). Outcrossing rates for each fruit were determined by analysis of paternity for 20 progeny per fruit. The number of outcross seeds per fruit was then calculated by multiplying the outcrossing rate of each fruit times the number of seeds in that fruit. Number of fruits sampled from the three visitation classes were 17 fruits from single-probe flowers, nine fruits from two-probe flowers, and 25 fruits from flowers receiving three or more probes.

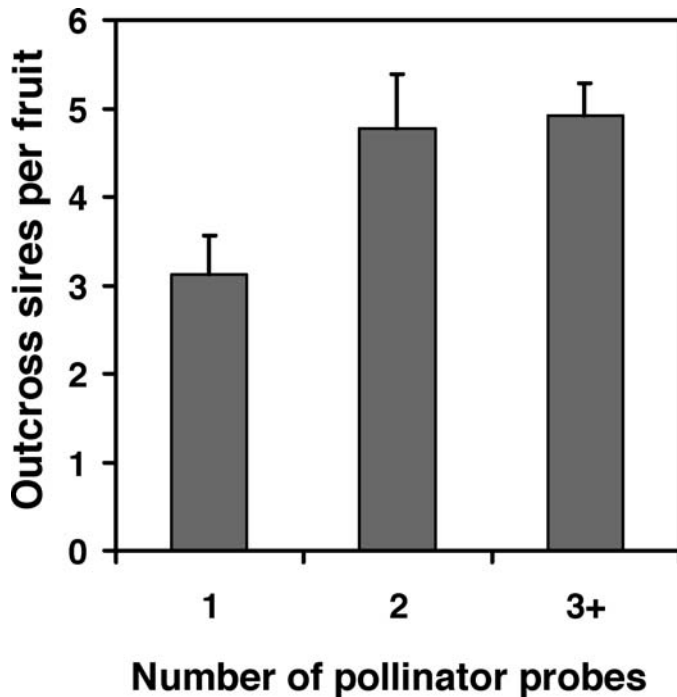


Fig. 5. Effect of number of pollinator probes on the mean number of outcross sires per *Mimulus ringens* fruit ( $\pm 1$  SE). Paternity based on samples of 20 seeds per fruit. Number of fruits sampled from the three visitation classes were 17 fruits from single-probe flowers, nine fruits from two-probe flowers, and 25 fruits from flowers receiving three or more probes.

evenness of outcross sire representation in each fruit did not vary significantly among floral probe classes (ANOVA,  $df = 2,45$   $F = 1.09$ ,  $P > 0.3$ ).

## DISCUSSION

The number of pollinator probes to *Mimulus ringens* flowers strongly and significantly affected seed set and mate number within fruits. However, we did not detect a significant effect of number of probes on the rate of outcrossing. We explore the implications of these findings below.

**Additional pollinator probes increase seed set**—*Mimulus ringens* flowers probed three or more times produced fruits with 44% more seeds than flowers probed a single time. This suggests that pollen loads deposited during initial probes were often insufficient to achieve maximal seed set in fruits with thousands of ovules. Dudash and Ritland (1991) reached a similar conclusion in a study of a congener, *M. guttatus*. They compared seed set in unmanipulated *M. guttatus* flowers, which typically remain open for 4 days, with seed set in flowers with corollas removed after a single day. Seed set was significantly higher in the longer-lived flowers, which presumably received more pollinator probes.

Seed set in *M. ringens* was not linearly proportional to the number of probes, with single-probe flowers producing 70% as many seeds as multiple-probe flowers. This pattern has been found by other researchers (e.g., McDade and Davidar, 1984; Galen and Stanton, 1989; Young and Stanton, 1990; Mitchell

and Waser, 1992) and may result from several factors, including (1) a reduction in the amount of pollen deposited during successive pollinator probes, (2) decreased effectiveness of successive pollen loads at fertilizing ovules, and (3) reduced availability of resources for provisioning seeds sired during later probes (McDade and Davidar, 1984; Young and Stanton, 1990).

**Multiple pollinator probes increase the number of sires per fruit**—When *Mimulus* flowers received single probes, the resulting fruits were typically sired by 2–4 outcross pollen donors. These multiply-sired fruits resulted from “simultaneous deposition” of pollen from several donors. This occurs when pollen from multiple donors accumulates on the head of the bee during a foraging sequence and some of this mixed pollen load is deposited on the next flower probed (Holmquist, 2005). Simultaneous deposition has been inferred for other plant species (Marshall and Ellstrand, 1985; Campbell, 1998), but to our knowledge this is the first direct confirmation in the field.

Our data also provide the first direct demonstration that “sequential deposition” of pollen during distinct pollinator probes also contributes to multiple paternity in *M. ringens*. Fruits of flowers probed three or more times had significantly more outcross sires (4.92) than fruits of single probe flowers (3.12). This finding is consistent with Dudash and Ritland’s (1991) observation that effective mate number in *M. guttatus* was higher for longer-lived flowers, which presumably received more pollinator probes than short-lived flowers.

Campbell (1998) noted that the relative importance of simultaneous and sequential deposition depends on two factors: (1) the extent of pollen carryover and (2) the interval between successive pollinator probes. For example, in *Ipomopsis aggregata* pollen carryover by hummingbirds is extensive (Price and Waser, 1982; Waser and Price, 1984), and the interval between successive pollinator probes is very long (around 24 h; Campbell, 1998). Campbell concluded that the high level of multiple paternity (4.4 sires per fruit) that she observed in *Ipomopsis* could be attributed entirely to simultaneous deposition of pollen from multiple donors during a single probe. In contrast to *Ipomopsis*, pollen carryover in *M. ringens* is limited (Karron et al., 1997; Holmquist, 2005), and multiple probes occur in rapid succession, often <15 min apart (Fig. 3). A short interval enhances the likelihood that fast-growing pollen grains from second and later probes can sometimes catch up to and outcompete pollen tubes from the first pollen load (Snow, 1986; Epperson and Clegg, 1987; Spira et al., 1996). This is especially likely if there is a large variance in pollen tube growth rate.

The extent of pollen carryover may vary with bee movement patterns. In our study system, floral display influences the proportion of within-plant and among-plant pollinator movements, which may affect the number of donors represented in the pollen load (Mitchell et al., 2004, 2005). We utilized a display size that closely resembles displays in natural populations in SE Wisconsin. Therefore, the balance between simultaneous and sequential deposition in our study is probably similar to that of natural populations. Note that interplant movements would be more common in populations with a smaller mean display size, potentially increasing the proportion of multiple paternity resulting from simultaneous deposition. By contrast, we would expect that sequential deposition would be more important in populations with larger display size.

Another factor likely to influence the extent of pollen

carryover is pollinator species composition. The predominant pollinator in the present study was *Bombus fervidus*. However, five other congeners (*B. bimaculatus*, *B. griseocollis*, *B. impatiens*, *B. nevadensis*, and *B. vagans*) co-occur sympatrically at this site (Mitchell et al., 2004), and in other SE Wisconsin populations. These bee species differ markedly in foraging patterns and the frequency of grooming (Mitchell et al., 2004); two factors likely to influence the extent of pollen carryover (Holmquist, 2005). Therefore, the relative importance of the two mechanisms of multiple paternity may vary among natural populations due to differences in the composition of pollinators.

**Effect of number of probes on self- and cross-pollination**—Outcrossing rate did not vary significantly with number of floral probes ( $P = 0.11$ ). This may reflect the limited sample size in each floral probe class or indicate that there is little difference in the proportion of self and cross pollen deposited during the first and subsequent probes. Interestingly, the total number of outcross seeds per fruit increased significantly with number of probes. This measure, which represents the product of outcrossing rate  $\times$  number of seeds, might be expected to increase if outcross pollen tubes tend to grow more rapidly than self pollen tubes (Bateman, 1956; Cruzan and Barrett, 1993; Rigney et al., 1993; Bernasconi, 2003). As pollen load size increases, there may be greater opportunity for outcross pollen to outcompete self pollen (Cruzan and Barrett, 1996).

**Conclusion**—The number of bumble bee probes to *M. ringens* flowers significantly influenced both mate number and seed set within fruits. Multiple paternity results from two mechanisms: “simultaneous deposition” of pollen from several donors during a single probe and “sequential deposition” of pollen from additional donors during subsequent pollinator probes. The relative importance of these two mechanisms is likely to vary considerably among plant species and even among populations within species.

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