

Effects of pollination and postpollination processes on selfing rate in *Mimulus ringens*¹

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PREMISE OF THE STUDY: Selfing rates vary widely within and among populations of self-compatible flowering plants. This variation is often attributed to differences in the amount and timing of self and outcross pollen deposition on stigmas, as well as to the influence of postpollination mechanisms that control fertilization success. This study explores the relative importance of pollination and postpollination processes in determining selfing rates in monkeyflower, *Mimulus ringens*.

METHODS: We hand-pollinated flowers on 17 unrelated mothers with pollen from one of three experimental treatments intended to replicate field conditions: (1) simultaneous deposition of 50% self pollen and 50% outcross pollen from 5 unrelated donors; (2) self pollen followed 15 min later by application of an equal amount of outcross pollen from five unrelated donors; and (3) outcross pollen from 5 unrelated donors followed 15 min later by application of an equal amount of self pollen. We genotyped 757 progeny at 8 polymorphic microsatellite loci and used paternity exclusion to determine whether each seedling was selfed or outcrossed.

KEY RESULTS: When self and outcross pollen arrived simultaneously, and when self pollen arrived 15 min prior to outcross pollen, the observed proportions of self and outcross progeny did not deviate from the expected 1:1 ratio. However, when outcross pollen was applied 15 min prior to self pollen, there was a significant excess of outcross progeny.

CONCLUSIONS: Selfing rate in *Mimulus ringens* is influenced by small differences in the timing of pollen arrival, but not by nonrandom postpollination sorting.

KEYWORDS: monkeyflower; pollen competition; pollination; postpollination; selfing rate; siring success

Selfing rates of animal-pollinated flowering plants often vary widely within and among populations (Cruzan and Barrett, 1996; Zalucki et al., 2013; Itagaki et al., 2016; Yin et al., 2016). This variation has been attributed to pollination events that affect the amount and timing of self and outcross pollen deposition on stigmas (Williams and Mazer, 2016) and to postpollination events that influence fertilization success (Cruzan and Barrett, 2016). Both pollination and postpollination events can influence offspring quality and population genetic structure (Karron et al., 2012), but they are rarely studied concurrently and therefore their relative contributions to variation in selfing rate remain largely unexplored.

Pollination events, mediated by interactions between plants and pollinators, influence the amount and proportion of self and outcross pollen deposited on stigmas (Mitchell et al., 2004, 2013; Devaux et al., 2014) and the order of self and outcross pollen arrival (Epperson and Clegg, 1987; Spira et al., 1996; Burkhardt et al., 2009). Earlier arriving pollen typically has a siring advantage over late-arriving pollen, and this difference is often positively associated with the interval between pollinator visits to a flower (Epperson and Clegg, 1987; Burkhardt et al., 2009).

Postpollination events can also affect the realized selfing rate (Snow and Spira, 1991; Kruszewski and Galloway, 2006; Ruane, 2009). Selfing rates are often lower than expected based on the relative amounts of self and outcross pollen deposited on stigmas (Johnston, 1993; Cruzan and Barrett, 2016). This discrepancy may be the result of competitive interactions among pollen grains and pollen tubes, or of female choice and interactions between male gametophytes and stylar tissue. These postpollination processes may influence both the relative fertilization success of self and outcross pollen, and also the relative success of pollen from different outcross donors

¹ Manuscript received 1 April 2016; revision accepted 20 June 2016.

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doi:10.3732/ajb.1600145

(Marshall and Ellstrand, 1986; Marshall and Folsom, 1991; Karron and Marshall, 1993; Rigney et al., 1993; Mitchell and Marshall, 1998). For instance, if outcross pollen tubes grow more rapidly than self-pollen tubes, they may sire a disproportionate number of ovules, even if deposited well after the arrival of self pollen on the stigma (Lloyd, 1992; Spira et al., 1996; Nemeth and Smith-Huerta, 2002; see also Broyles and Wyatt, 1993). Competition favoring outcross pollen may decrease the proportion of self-progeny, reducing the expression of inbreeding depression and altering population genetic structure (Baker and Shore, 1995; Karron et al., 2012; Lankinen et al., 2016).

Investigating the relative influence of pollination and postpollination events on mating patterns requires detailed knowledge of pollinator visitation patterns, pollen-load sizes, and pollen load composition (e.g., proportion of self pollen). Such integrated studies are not common (but see Barrett et al., 1994; Lau and Galloway, 2004), and require a wide array of field and laboratory techniques (Karron et al., 2012). The most informative investigations of the influence of pollination and postpollination events on selfing rate and mating patterns should use pollen mixes and pollination intervals similar to those that occur in nature.

This study explores the relative importance of pollination and postpollination processes in determining selfing rates in *Mimulus ringens*. Our experimental design closely matches patterns of pollen delivery and composition that occur in the wild (Karron et al., 2004, 2006; Holmquist et al., 2012). By altering the order of arrival of pollen deposition on stigmas, this study addresses two questions: (1) do self and outcross pollen differ in siring success? and (2) do female selfing rates depend on the order of arrival of self and outcross pollen on stigmas?

MATERIALS AND METHODS

Study species—*Mimulus ringens* L. (Phrymaceae) is a diploid perennial herb native to wet meadows of central and eastern North America. The showy purple hermaphroditic flowers open before dawn and last for half a day. Populations typically have female selfing rates of 25% to 40% (Karron and Mitchell, 2012; Karron et al., unpublished data). Controlled single-donor self and outcross hand-pollinations with abundant pollen do not differ in number of seeds per fruit, germination rate, or seedling survival (Karron et al., unpublished data), but selfed offspring exhibit substantial inbreeding depression later in the life cycle (Karron et al., unpublished data).

Prior work with *Mimulus ringens* provides the detailed knowledge of pollinator visitation patterns, pollen-load sizes, and pollen-load composition necessary to design relevant pollination experiments. Pollinators often probe more flowers sequentially on large floral displays (geitonogamy; Mitchell et al., 2004; Eckert et al., 2010), leading to a significant increase in the rate of self-fertilization (Karron et al., 2004, 2009). The proportion of self pollen deposited on a stigma during a single probe often varies widely (e.g., 20% self to 75% self; Karron et al., 2009), and single visits typically bring in pollen from 2 to 5 donors (Karron et al., 2006). A bumblebee visit to a *Mimulus* flower deposits 6000 to 10,000 pollen grains (Flanagan et al., 2009), and most flowers receive a second bee visit within 15 min of the initial probe (Karron et al., 2006). Flowers receiving a second probe produce more outcross seeds and have higher mate diversity than flowers receiving a single probe. This indicates that pollen deposited by a second probe even a short time after the initial probe may successfully fertilize ovules (Karron et al., 2006).

Experimental design—We grew pollen donor and recipient plants from seed in the University of Wisconsin-Milwaukee greenhouse from 20 April to 31 August 2014. All plants were generated by crosses between unrelated plants derived from a single large natural population in Akron, Ohio, USA. The five pollen donors and 17 pollen recipients in the experimental crosses shared no parents.

Before pollinations we emasculated three randomly chosen buds on each of 17 pollen recipient plants to prevent autonomy. The morning after emasculation we hand-pollinated each open flower with an equal mixture of self and outcross pollen applied in one of three ways (experimental treatments): (1) simultaneous deposition of 50% self pollen and 50% outcross pollen from 5 unrelated donors. This 1:1 mixture was applied twice, separated by 15 min; (2) self pollen followed 15 min later by application of an equal amount of outcross pollen from five unrelated donors; and (3) outcross pollen from the same five unrelated pollen donors followed 15 min later by application of an equal amount of self pollen. Each experimental treatment was performed once on each pollen recipient. Pollinations on any one plant were performed in random order, and flowers were randomly assigned to treatments. We performed these pollinations during 12–16 July 2014. This corresponded to the middle of the flowering phenology for these plants, ensuring synchrony of donor and recipient plants and preventing assortative mating by flowering time.

We prepared fresh mixtures of outcross pollen each morning, immediately prior to controlled pollinations. We used a microspatula to scoop pollen from an equal number of anthers from each of the five donors. The pollen was mixed thoroughly on a microscope slide. We then used a calibrated microspatula to apply a mean of $10,094 \pm 337.9$ pollen grains (mean \pm SD; $N = 15$) to each stigma. The left half of both lobes of the stigma always received the first pollen load, whereas the right half of both lobes received the second pollen load. For the simultaneous treatment, equal quantities of self and outcross pollen were mixed and applied simultaneously. Thus, in each pollination we deposited a mean of 10,094 self pollen grains, and 10,094 cross grains (a 50:50 ratio), so in all cases the proportion of self pollen across both applications was almost exactly 50%. This proportion is well within the wide range of self pollen receipt experienced in field populations (Karron et al., 2009). Stigmas of *Mimulus ringens* do not exhibit the rapid stigma closure found in some other members of the genus, and instead close gradually over a 90 min window (Karron et al., 2006).

We collected mature fruits 45 d after pollination, germinated seeds from all sampled fruits and harvested leaf tissue from 8-wk-old seedlings. Germination rate and seedling survival exceeded 95% for all treatments. A total of 757 progeny, 14 to 15 from each of three treatments on 17 maternal plants, were genotyped at eight microsatellite loci (Miri24, Miri27, Miri40, Miri46, Miri58, Miri78, Miri82, and Miri95), following the protocols of Nunziata et al. (2012). Each locus had 4 to 9 alleles. Maternal genotypes were determined from samples of young leaves processed and analyzed in the same way as for offspring. Progeny genotypes were compared to genotypes of the 17 maternal plants and five pollen donors. We used simple paternity exclusion to determine whether each seedling was the product of self- or cross-fertilization. Every seed could be unambiguously identified as either selfed or outcrossed. For 78% of the outcross progeny, we were also able to unambiguously determine the identity of the male parent.

To quantify pollen production for each of the five pollen donors, we sampled five flowers per plant on a single day during the five-day window of hand-pollination. We placed anthers from each

flower into separate microfuge tubes with 70% ethanol solution, mixed thoroughly, and counted subsamples on a depression slide. Pollen production averaged 158,996 grains per flower, with plant means ranging significantly among donors ($F_{4,29} = 6.2$, $P < 0.002$; $R^2 = 0.50$) from 121,332 to 193,010 grains.

Data analysis—To test for effects of pollination treatment on selfing rate we used a Generalized Linear Mixed Model with a binomial distribution and a logit link function. We treated Maternal Plant as a random factor and Pollination Treatment as fixed using Proc GLIMMIX in SAS version 9.4. This analysis also uses a t test to directly and separately test for a selfing rate of 0.5 (a mean logit value of 0) for each treatment. For the subset of outcross progeny for which we were able to unambiguously determine the identity of the male parent, we tested for equal relative sire success using a Generalized Linear Mixed Model, with Treatment and Male as crossed fixed factors, and Maternal Plant as a random effect. We then used a ‘contrast’ statement to test for equal relative sire success by choosing coefficients that differed linearly according to the absolute pollen production for each male.

RESULTS

Fertilization success of self and outcross pollen—Pollination treatment significantly affected selfing rate ($F_{2,32} = 6.57$, $P < 0.005$). When equal quantities of self and outcross pollen were applied simultaneously to the stigma, the least squares mean selfing rate (backtransformed from logit values) across mothers was 49.8% (Fig. 1; 95% CI = 43.2–56.3%). Thus, self and outcross pollen were equally successful at fertilizing ovules, matching the expected 1:1 ratio based upon proportions of self and outcross pollen on the stigma ($t_{32} = -0.08$, $P > 0.9$). When self pollen was applied to the stigma 15 min prior to application of outcross pollen, the observed selfing rate was 55.0% (CI = 48.4–61.4), which did not differ significantly from the expected 1:1 ratio ($t_{32} = 1.54$, $P > 0.13$). When outcross pollen was given a 15 min head start, the observed selfing rate was 38.7% (CI = 32.5–45.4) which differed significantly from the expected 1:1 ratio ($t_{32} = -3.43$, $P < 0.002$).

Siring success of outcross-pollen donors—Across all treatments, the proportion of outcross-seeds sired varied markedly among the five pollen donors (Fig. 2; $F_{2,225} = 14.52$, $P < 0.0001$). However, proportional success of donors in siring offspring did not vary among treatments ($F_{2,225} = 0.02$, $P > 0.9$), and there was no interactive effect of treatment and donor ($F_{8,225} = 0.9$, $P > 0.5$). Because some of the paternity share variation may reflect differences in pollen production, we also tested for proportionality using a linear contrast statement (coefficients adjusted to reflect the representation of each outcross donor in the pollen mix). Even with this adjustment, observed outcross-sire ratios differed significantly from the expected sire ratios ($F_{1,225} = 15.03$, $P < 0.001$). For example, pollen donor D sired 3 times more seeds than donor A, even though they had similar pollen production per flower.

DISCUSSION

Variation in selfing rates within and among flowering plant populations has been attributed to pollination events that influence the amount and timing of pollen deposition on stigmas, and

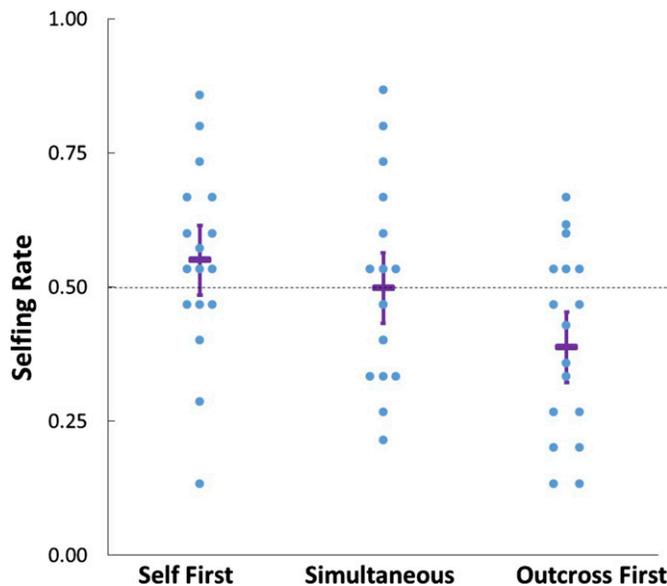


FIGURE 1 Selfing rates following three pollination treatments: (1) self pollen deposition followed 15 min later by an equal application of outcross pollen; (2) simultaneous deposition of 50% outcross and 50% self pollen; and (3) outcross pollen deposition followed 15 min later by an equal application of self pollen. For each treatment, blue data points represent the selfing rates for a single fruit from an individual maternal plant ($N = 17$). Least Squares Mean from Generalized Linear Mixed Model analysis is shown with purple horizontal bar, and 95% CI is shown with purple vertical bar. Dashed line indicates null expectation of 50%.

postpollination events that affect the relative fertilization success of self and outcross pollen (Medrano et al., 2012; Cruzan and Barrett, 2016; Lankinen et al., 2016; McCallum and Chang, 2016). Although pollination and postpollination events can markedly affect offspring quality and population genetic structure, these processes have rarely been studied concurrently to determine their relative

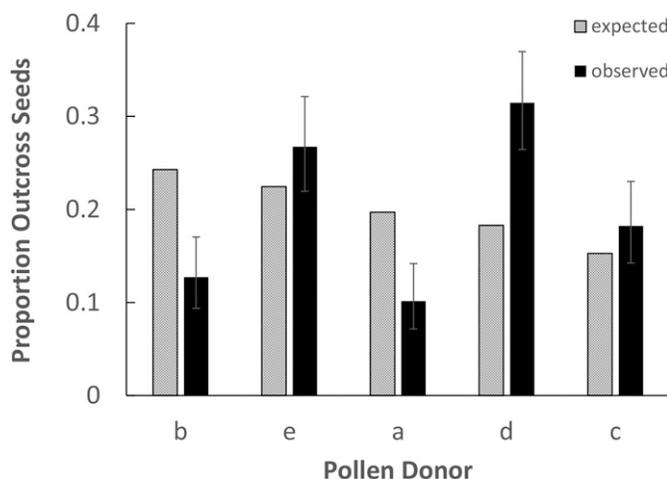


FIGURE 2 Expected and observed proportions of progeny sired by each of five unrelated outcross pollen donors (Sire A—E) pooled across pollination treatments. Expectations are based on the relative pollen production of each pollen donor. Pollen donors are presented in order of representation in the pollen pool. The observed paternity shares (with 95% CI) differed significantly from the expected paternity ($F_{1,225} = 15.03$, $P < 0.001$).

contribution to selfing-rate variation. The findings of the current study suggest that for *Mimulus ringens*, selfing rates are largely influenced by the order of arrival of self- and outcross-pollen grains, with minor contributions of postpollination sorting.

Importance of pollination events—Pollinator foraging patterns have previously been shown to influence the selfing rate in *Mimulus ringens* (Mitchell et al., 2004; Karron et al., 2004, 2006). Such pollination events can influence the order of arrival of self and outcross pollen on the stigma, and time lags may then determine relative siring success of different pollen donors (Epperson and Clegg, 1987; Spira et al., 1996). For example, in *Ipomoea purpurea* (L.) Roth, pollen arriving 30 min following first pollinations fertilized 14% fewer ovules (Epperson and Clegg, 1987). Late-arriving pollen also fertilized significantly fewer ovules in *Hibiscus moscheutos* L. (Spira et al., 1996). In the current study, we altered the order of arrival of self- and outcross-pollen loads with a time lag of 15 min that matches the interval between floral probes in nature. When applied first, outcross pollen had a statistically significant 11% siring advantage over self-pollen. When self pollen was applied first, the selfing rate did not differ significantly from the null expectation. Since *M. ringens* pollen tubes typically take 8 to 10 hours to fertilize ovules (Mitchell unpublished data), a 15 min head start corresponds to just 3% of the fertilization window. Thus, a small change in the timing of pollen arrival can have a large effect on paternity.

Our estimate of the influence of pollination events is probably an underestimate because we applied the first and second pollinations adjacent to one another, rather than layering them as would occur following bee visitation. Layering is likely to reduce the success of later arriving pollen (Mitchell and Marshall, 1995), so effects of the timing of pollen delivery might well be stronger in the field.

Importance of postpollination events—When *Mimulus ringens* received an equal mix of self and cross pollen, the resulting selfing rate was 49.9%, suggesting that postpollination sorting does not discriminate against self pollen. However, studies of several other species suggest that selfing rates may often be strongly affected by postpollination processes (Cruzan and Barrett, 2016). Reduced siring success of self pollen has been attributed to competition among pollen grains as well as interactions between pollen and the style (Aizen et al., 1990; Snow and Spira, 1991; Nemeth and Smith-Huerta, 2002). For example, when equal quantities of self and outcross pollen were applied to stigmas of *Campanulastrum americanum* (L.) Small, the percentage of self progeny ranged from 6 to 34% across maternal plants (Kruszewski and Galloway, 2006). Similarly, when self and outcross pollen were applied simultaneously to *Erythronium grandiflorum* Pursh stigmas, the selfing rate was 32% and significantly lower than expected (Rigney et al., 1993).

In *Mimulus ringens* self and outcross pollen arriving simultaneously on the stigma were equally successful in siring ovules. This suggests that in this species there should be a close correspondence between rates of self-pollination and self-fertilization. Indeed, this finding is consistent with our previous studies showing a close correlation between geitonogamous pollinator movements and geitonogamous self-fertilization (Karron et al., 2004; 2009). Populations of *M. ringens* are often pollinated by several sympatric species of *Bombus* that differ in the extent of geitonogamous pollinator movements (Mitchell et al., 2004). Changes in the relative abundance of these pollinators could shift proportions of self and outcross pollen delivery, leading to both spatial and temporal variation in selfing rates.

Siring success of outcross pollen donors—Postpollination events have also been shown to influence the relative fertilization success of different outcross pollen donors. For example, in a study of *Raphanus sativus* L. pollen donors differed in pollen-tube growth rate and number of seeds sired (Marshall and Diggie, 2001). In our study, pollen donors differed significantly in the proportion of progeny sired (Fig. 2). Paternity shares of the five donors differed significantly from expected proportions, even following adjustment for differences in pollen production of each donor (Fig. 2). This suggests that postpollination processes may discriminate among sires even though mean performance of outcross pollen did not differ from mean performance of self pollen.

CONCLUSIONS

Our experimental design closely matches patterns of pollen delivery and pollen-load composition that occur in the wild, allowing us to make meaningful comparisons of pollination and postpollination events. For *Mimulus ringens*, pollination events such as the timing of arrival of pollen on the stigma and the proportion of that pollen that is self are most important in influencing selfing rate under realistic conditions. Indeed, there is substantial variation among flowers in both of these factors, much of it related to floral display (Karron et al., 2004). Specifically, many-flowered plants experience much more between-flower selfing, and also produce a higher proportion of offspring that are selfed (Karron et al., 2004). Although such variation in pollinator behavior is common for many flowering plants, their relative importance in determining selfing rates will depend on the range of pollinator behaviors and services and how they balance against the magnitude of postpollination sorting. Our results underscore the importance of the dynamics of the pollination process for plant mating systems. Subtle variation in the timing of self and outcross pollen arrival may lead to considerable differences in selfing rates among flowers, even when subsequent pollinator probes deliver pollen loads differing markedly in composition.

ACKNOWLEDGEMENTS

The authors thank P. Engevoold and T. Schuck for assistance in propagating *Mimulus ringens* in the UWM Greenhouse. F. Alberto, C. Wimpee, the associate editor, and 2 reviewers provided helpful comments on earlier drafts of the manuscript. This study was supported by a UWM Research Growth Initiative award (JDK), and by the University of Akron Glenn Endowment (RJM).

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