

EFFECTS OF SEQUENTIAL POLLINATION ON THE SUCCESS OF “FAST” AND “SLOW” POLLEN DONORS IN *HIBISCUS MOSCHEUTOS* (MALVACEAE)¹

ALLISON A. SNOW,^{2,3} TIMOTHY P. SPIRA,⁴ AND HONG LIU^{4,5}

³Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, 1735 Neil Avenue, Columbus, Ohio 43210-1293 USA; and ⁴Department of Biological Sciences, Clemson University, Clemson, South Carolina 29634-1903 USA

Competition among pollen grains for the chance to fertilize ovules typically involves two stages: arrival times on stigmas and/or the growth of pollen tubes through styles. In a previous study of *Hibiscus moscheutos*, we found that individual pollen donors often differed in pollen tube competitive ability. Here we determined whether short delays in pollen arrival time altered the average success of “fast” and “slow” pollen donors when both types of pollen experienced the same delays. Hand-pollination experiments were carried out using four pairs of pollen donors that differed in competitive ability. We allowed delays of 15 or 30 min between the first and second pollen donor and then determined seed paternity using allozyme markers. The second donor typically sired fewer seeds than pollen that arrived earlier, but, contrary to expectation, “faster” pollen did not always sire significantly more seeds than “slower” pollen when each was applied after delays of the same duration. In two of the four pairs of donors, differences that were seen following simultaneous pollinations disappeared when each type of pollen was applied following identical delays of 15 or 30 min. This unexpected response suggests that the dynamics of pollen tube competition are more complex than anticipated.

Key words: allozyme marker; *Hibiscus*; male fitness; Malvaceae; pollen tube competition; pollination; timing of pollen deposition.

In many animal-pollinated species, excessive amounts of pollen are deposited on stigmas and pollen tubes compete for access to unfertilized ovules (e.g., Mulcahy, Curtis, and Snow, 1983; Snow, 1986; Snow and Roubik, 1987; Levin, 1990; Spira et al., 1992). When this occurs, plants with the greatest success as pollen donors may be those whose pollen arrives earliest on the stigmas of newly open flowers and/or those whose pollen is able to sire seeds most quickly after pollen mixtures are deposited. These two types of competition often take place during the first few hours after anthesis and are influenced by the complex dynamics of pollinator movements, availability of ovules, and variable rates of pollen tube growth. Despite a large number of studies on nonrandom fertilization in plants, we know little about whether this process influences male fitness in natural populations (Stephenson and Bertin, 1983; Lyons et al., 1988; Marshall and Folsom, 1991; Walsh and Charlesworth, 1992; Snow, 1994).

Hibiscus moscheutos, commonly known as wild rose mallow, is a well-studied species in which pollen competition may affect male fitness (Snow and Spira, 1996). At our study sites in Edgewater, Maryland, USA, bumble bees (*Bombus* spp.) and a specialist anthophorid bee (*Ptilothrix bombiformis*) make frequent visits to the large, 1-d flowers. More than half of the consecutive pollinator visits we observed occurred within 15 min of each other, and pollinators typically deposited surplus pollen (more than ~350 pollen grains) within 2–3 h of anthesis (Snow and Spira, 1991a; Spira, Snow, and Puterbaugh, 1996). Pollen carryover, frequent stigma contacts by pollinators, and the close proximity of neighboring plants suggest that

stigmas often receive pollen from multiple donor plants, either simultaneously or following brief delays between visits (Spira et al., 1992).

When excessive amounts of pollen from several donors are deposited on stigmas simultaneously, the speed of pollen tube growth down the 5–6 cm style of *H. moscheutos* can determine the siring success of competing pollen donors. Using two-donor hand-pollination experiments, we found that individual pollen donors often differed in the proportion of seeds sired, in some cases by ratios exceeding 2:1 (Snow and Spira, 1996). Furthermore, these differences were consistent across a range of recipient plants. Competitive hierarchies were also consistent among donors, allowing us to identify “fast” and “slow” pollen donors relative to the population as a whole. Therefore, we concluded that pollen competitive ability may be an important component of male reproductive success in these populations (Snow and Spira, 1991b, 1996). Differential siring success is likely due to corresponding differences in pollen tube growth rates (Snow and Spira, 1991a), although this was not examined in all of our studies.

A complicating factor in evaluating the fitness consequences of pollen tube competition in *H. moscheutos* is the fact that several pollinator visits were often required to foster strong competition for ovules, giving a head-start advantage to pollen that arrived early (Spira et al., 1992). For pollen donors with similar competitive abilities, we found that delays of only 15–30 min between visits ensured that ~60–75% of a flower's ovules were fertilized by the first of two donors, as compared to ~50% from each donor following simultaneous deposition (Spira, Snow, and Puterbaugh, 1996). However, these time-dependent inequalities would not necessarily offset differences in pollen competitive ability if the order of arrival is random with respect to pollen tube growth rates, as is likely to occur in nature. The penalty of arriving late should be greater for slow donors than for fast ones. Therefore, we undertook the present study to test the assumption that the average siring success of fast pollen donors is greater than that of slow do-

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² Author for reprint requests.

⁵ Current address: Department of Biology, Florida International University, Miami, Florida 33199 USA.

TABLE 1. Summary of pollen donors, pollen recipients, and genetic markers used in each set of sequential pollinations. GPI marker refers to allozyme markers used to assign seed paternity.

| Donor pair | | Fast donor | Slow donor | Recipients | Year |
|------------|------------|------------|------------|---------------|------|
| I | ID number | 1 | 29 | 103,110,120 | 1994 |
| | GPI marker | BB | AC | BB, BB, BB | |
| II | ID number | 129 | 57 | 90, 97, 108 | 1994 |
| | GPI marker | AA | BB | BB, BB, BB | |
| III | ID number | 17 | 57 | 5, 108, 124 | 1995 |
| | GPI marker | AA | BB | BB, BB, BB | |
| IV | ID number | 18 | 57 | 128, 144, 145 | 1996 |
| | GPI marker | AC | BB | AA, AA, AA | |

nors when each is subjected to a 15 or 30 min delay relative to the other. We hypothesized that when influenced by similar time delays, fast pollen donors would sire significantly more seeds than slow donors.

MATERIALS AND METHODS

Hibiscus moscheutos (Malvaceae) is a self-compatible, herbaceous perennial that occurs in fresh and brackish marshes of the eastern United States (Spira, 1989). Individual plants consist of multiple 1–2 m tall stems that emerge each spring from a woody rootstock. Genets can be propagated vegetatively, but they are not clonal under natural conditions. Flowering occurs in mid- to late summer. Automatic self-pollination is prevented by spatial separation of the anthers and stigmas, and outcrossing rates are estimated to be ~64% (Snow and Spira, 1991b; Snow et al., 1995). The showy white or pink flowers are visited frequently by bees and typically produce ~120 seeds per fruit. The round, hard-coated seeds are often destroyed by insect seed predators; undamaged seeds are buoyant and appear to be dispersed by water (Spira, 1989).

We collected plants from the Mill Swamp population in Edgewater, Maryland, as described in our previous studies, and planted them in 20-L pots kept outdoors at the South Carolina Botanical Garden at Clemson University, Clemson, South Carolina, USA. Although this site is distant from the source population in Maryland, the climate is similar and the plants became dormant and resprouted each year on a schedule similar to their counterparts in Maryland (but plants grown in South Carolina flowered ~1 mo earlier than those in Maryland). Each genotype was cultivated in the same manner from 1993 to 1996, and in many cases these genotypes were propagated as several potted ramets to increase the numbers of available flowers per genotype. These genotypes (hereafter referred to as pollen donors or pollen recipients) had three Mendelian alleles for GPI (glucose phosphate isomerase), based on starch gel electrophoresis with Gottlieb's (1981) extraction buffer and procedures described in Soltis et al. (1983). Plants used as competing pollen donors differed in allozyme markers, such that the siring success of each donor could be determined by scoring seeds from experimental crosses (Table 1).

Experimental methods for sequential pollinations were essentially the same as those described in Spira et al. (1996). Pollen donors were designated as "fast" or "slow" based on their competitive ability in previous experiments (Snow and Spira, 1996; unpublished data). We carried out mixed-donor hand-pollinations using each of four pairs of pollen donors in the following treatments: (1) no delay—fast and slow pollen applied simultaneously, (2) 15-min delay with either fast pollen first (F-15) or slow pollen first (S-15), or (3) 30-min delay with either fast pollen first (F-30) or slow pollen first (S-30).

Flowers on recipient plants were bagged just prior to anthesis and rebagged after hand-pollination to exclude pollinators. On newly opened flowers, two of the five stigmas received pollen from each donor in a pair, with the fifth stigma left unpollinated. Each stigma was coated with pollen by gently brushing it with pollen-laden anthers held in forceps. This resulted in more than twice the amount of pollen from each donor required to fertilize all available ovules (~800 grains/donor, ~1600 grains per flower; Snow and Spira, 1991a). We did not attempt to mix pollen from the two sources prior to application,

as advocated by Mitchell and Marshall (1995), because we wanted both early- and late-arriving pollen to be in contact with clean stigmatic surfaces. Thus, pollen from the two donors did not have the opportunity to interact on the surface of the stigma, although their pollen tubes converged ~1–2 cm below the stigma, at the base of the stylar branches (Snow and Spira, 1991a). In similar studies by other investigators, later pollen loads were placed directly over earlier pollen to test for stigma "clogging" (e.g., Galen, Gregory, and Galloway, 1989). We opted not to use this approach because the five large stigmas of each *Hibiscus* flower can accommodate a total of ~2000 pollen grains (Snow and Spira, 1991a), so stigma clogging may be rare, and in any case we wanted to focus on pollen tube competition as a possible mechanism for nonrandom fertilization.

For each pair of competing donors, we pollinated eight flowers per treatment on each of three recipient plants, taking care to alternate which of the five treatments was started first on different days (4 donor pairs \times 5 treatments \times 3 recipients \times 8 flowers = 480 flowers). Pollinations were carried out between 0830 and 1100 on days that were not rainy or unusually cool. About 4 wk later, mature but undehisced fruit capsules were collected and stored in labeled envelopes. To induce germination, seeds were nicked and placed on moist filter paper in petri dishes at room temperature. We then scored 150 seedlings for each treatment for electrophoretic markers to assign paternity (3 recipients \times 5 fruits \times 10 seedlings per fruit). This measure of paternal success likely reflects fertilization success rather than differences in seed abortion, germination, or seedling survivorship (Snow and Spira, 1991a, b). Aborted embryos were extremely rare, seed germination exceeded 90%, and very few seedlings died prior to sampling, so we assumed that survivorship at these life stages did not affect paternal reproductive success.

RESULTS

With simultaneous pollination, the proportions of seeds sired by fast vs. slow pollen donors were generally in the ratio of ~62:38 (Table 1, Fig. 1). In two of the four pairs of donors, fast donors sired significantly more seeds than slow donors when each was subjected to delays of 15 or 30 min, as expected (donor pairs I and III; Fig. 1). However, the other two donor pairs did not show differences in siring ability under these conditions (donor pairs II and IV; Fig. 1). Pooling data from the four delay treatments (S-15, F-15, S-30, F-30) showed that the advantage of fast donors seen with simultaneous pollinations diminished and was not significant at $P < 0.05$ in two of the four pairs of pollen donors (Table 2).

Delays of 15 min typically resulted in a decrease in the siring success of the second donor, but the penalty associated with this delay was not statistically significant for the slow donors in donor pairs II, III, or IV (Table 3). In most cases the longer delays of 30 min did not diminish the proportions of seeds sired beyond what occurred due to 15-min delays (15 vs. 30 min), and no penalty was seen for the slow donor in donor pair IV after 30 min (0 vs. 30 min; Table 3). Some of these inconsistencies may be due to small sample sizes (150 seeds per treatment), but they suggest that the dynamics of competition between sequential pollen loads may be more complex than we anticipated.

DISCUSSION

The most surprising result from this study was that fast pollen donors did not consistently outperform slow donors when both were subjected to similar delays of 15 or 30 min in sequential hand-pollinations. Instead, only two of the four pairs of pollen donors (I and III) exhibited predictable differences in siring success. These inconsistencies do not appear to be associated with particular growing seasons, since donor pairs I and II were both used in 1994 (Table 1), nor are they likely

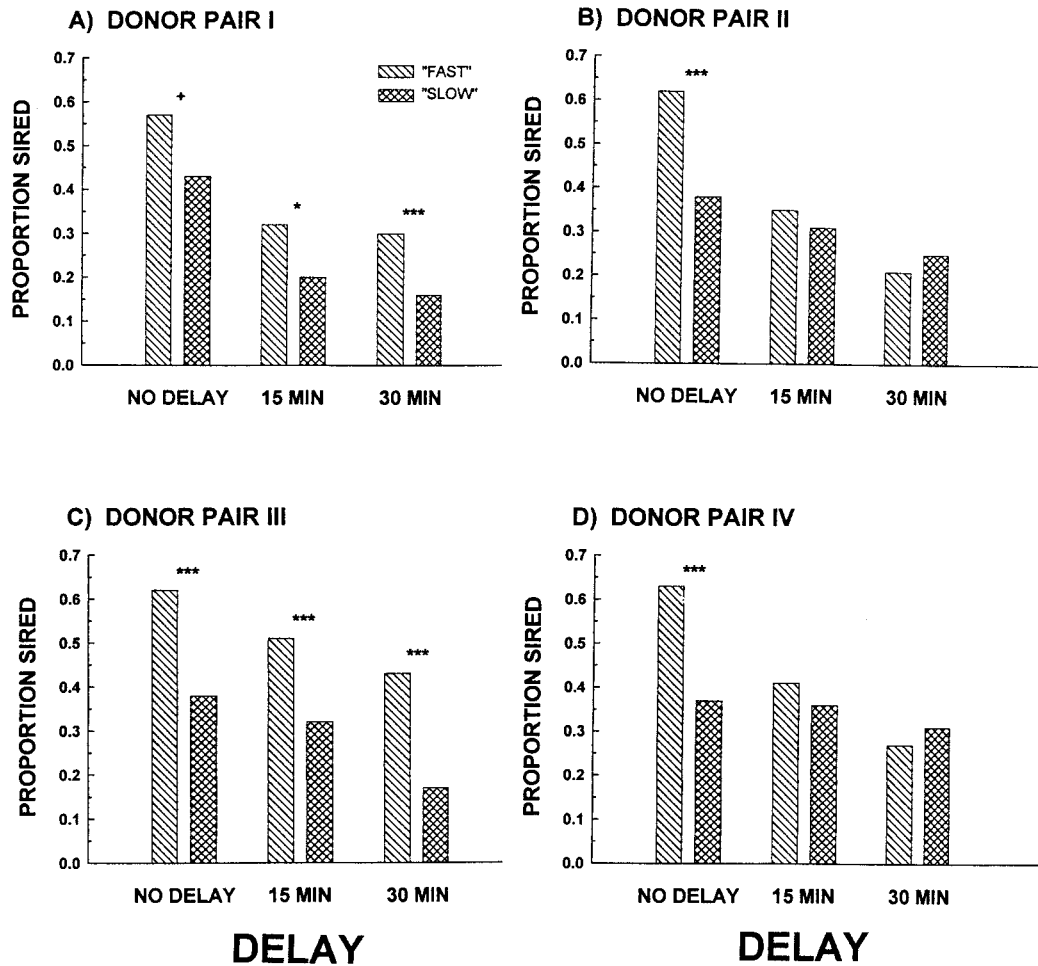


Fig. 1. Proportion of seeds sired by fast vs. slow donors following simultaneous or sequential pollinations (delays of 15 or 30 min). For sequential pollinations, success of the second donor is shown. Siring success from simultaneous pollinations was compared to an expected ratio of 1:1 using chi-square tests; siring frequencies from delayed pollinations were compared using *G* tests; + indicates $P < 0.10$, * $P < 0.05$, *** $P < 0.001$; 150 seeds per treatment category. See Table 3 for significance tests of delay effects for each donor in a pair.

to be due to environmental conditions such as temperature, which was relatively constant throughout the study. Nonetheless, it would be easier to identify possible causes of our results if all donor pairs had been compared in the same year.

Flower age could also contribute to the variation we ob-

served in the effects of delayed pollination. In a previous paper, we reported that the penalty of arriving late decreased when pollinations were started at 1130 as opposed to 0930 (Spira et al., 1996). In the present study, however, treatments were assigned at random throughout the morning and gener-

TABLE 2. Summary of siring success of fast pollen donors following simultaneous pollinations (no delay) and pooled data from the other four treatments (delays of 15 vs. 30 min, two in which fast pollen was applied first and two in which slow pollen was applied first). Chi-square tests were used to compare observed values with the expectation of 0.50 if siring success of fast and slow donors is equal; *N* = number of progeny scored.

| Donor pair | Type of pollination | Proportion sired by fast donor | <i>N</i> | <i>P</i> |
|------------|---------------------|--------------------------------|----------|----------|
| I | no delay | 0.57 | 150 | <0.10 |
| | delays | 0.57 | 600 | <0.001 |
| II | no delay | 0.62 | 150 | <0.001 |
| | delays | 0.50 | 600 | NS |
| III | no delay | 0.62 | 150 | <0.001 |
| | delays | 0.60 | 600 | <0.001 |
| IV | no delay | 0.63 | 150 | <0.001 |
| | delays | 0.54 | 600 | NS |

TABLE 3. Effects of delayed pollination on the siring success of fast and slow pollen donors. For each donor in a pair, *G* tests were used to test for significant effects of three time intervals (0 vs. 15 min, 15 vs. 30 min, and 0 vs. 30 min) on the proportion of seeds sired by the second donor. * $P < 0.05$, *** $P < 0.001$, NS = not significant. Same data set as shown in Fig. 1.

| Donor pair | Pollen donor | Time interval | | |
|------------|--------------|---------------|---------------|--------------|
| | | 0 vs. 15 min | 15 vs. 30 min | 0 vs. 30 min |
| I | fast | *** | NS | *** |
| | slow | *** | NS | *** |
| II | fast | *** | *** | *** |
| | slow | NS | NS | * |
| III | fast | * | NS | *** |
| | slow | NS | *** | *** |
| IV | fast | *** | * | *** |
| | slow | NS | NS | NS |

ally resulted in lower siring success after a time delay, suggesting that flower age was not responsible for the inconsistencies in our results.

Other factors that could affect pollen competitive ability are the genotypes of the three pollen recipients used with each pair of donors, or possible donor-by-recipient interactions (Table 1). Snow and Spira (1996) found that differences in siring ability were significant across samples of 11–15 recipients, but no attempt was made to test for variation in siring success due to possible interactions between pollen donors and recipients. Here, with different groups of recipients for each donor pair, it is possible that such interactions could have had unanticipated effects on the dynamics of competing pollen tubes, although *G* tests (not shown) revealed few differences among recipients within treatments. Also, donor-by-recipient interactions would have to account for differences in relative siring success between delayed pollinations and simultaneous applications.

Regardless of the underlying reasons for our unexpected findings, this research shows that competition between pollen grains from different pollinator visits is more complicated than we initially assumed. Staggered pollen arrival times are common in natural populations, so earlier arriving pollen often has a substantial head start over later arriving pollen in sending pollen tubes to the ovary (e.g., Mulcahy, Curtis, and Snow, 1983; Spira et al., 1992). In *H. moscheutos* this head start weakens the intensity of competition among pollen genotypes and sometimes changes the outcome of competition based on what is expected following simultaneous pollinations (Fig. 1; note, however that all significant differences between pairs of donors were in the predicted direction). Here, pollen from relatively fast pollen donors did not always sire more seeds than pollen from slow donors with the same delay in arrival. Delays of only 15 min sometimes masked differences in siring success that were seen following simultaneous pollinations. Perhaps a larger sample of competing pollen donors (and more seeds sampled per donor) would have identified plants that exhibit greater or more consistent differences in siring success. In conclusion, results from this study indicate that fitness consequences of pollen competition in natural populations of *H. moscheutos* may be negligible or difficult to detect.

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