

STIGMA BEHAVIOR IN *MIMULUS AURANTIACUS* (SCROPHULARIACEAE)¹

A. ELIZABETH FETSCHER^{2,3} AND JOSHUA R. KOHN²

²Department of Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0116

The bilobed stigma of many species in the order Scrophulariales closes in response to touch by an animal pollinator. In hummingbird-pollinated bush monkey flower, *Mimulus aurantiacus* (Scrophulariaceae), closure is rapid, occurring within seconds of tactile stimulus. We investigated the proximate causes of stigma closure and subsequent reopening in *M. aurantiacus*, as well as potential costs and benefits of stigma closure for female fitness. Stigma closure is elicited by both touch and pollen, but closure in response to pollen is much slower, requiring 0.5–1.5 h. Stigmata reopen within 2.5–4.5 h if touch, but no pollen, is applied. Upon receipt of pollen, most stigmata remain closed for the remaining lifetime of the flower, even if less pollen is received than is needed for full seed set. Those stigmata that do reopen after pollination generally require between 20 and 28 h to do so, much longer than for unpollinated stigmata. Reopening after pollination appears to be a response to low seed set rather than to low pollen load. Natural pollination of stigmata manipulated to prevent closure shows that closure does not increase capture of pollen or seed set. In fact, closure reduces the average pollen load received by flowers. Despite this, there is no evidence that stigma closure has any negative effect on female fitness in terms of seed set or germinability. Hypotheses for the adaptive significance of stigma closure are discussed. Understanding proximate causes of stigma closure and reopening is essential in the evaluation of these hypotheses.

Key words: behavior; hummingbirds; *Mimulus*; pollination; Scrophulariaceae; stigma sensitivity.

The bilobed stigma of *Mimulus aurantiacus* (Scrophulariaceae) closes, usually within 2 s, after being touched by a pollinator. As a result, the entire receptive surface of the stigma is enfolded between the outer surfaces of the two lobes, preventing further pollen receipt unless the stigma reopens (Fig. 1). Closure is somewhat slower in older flowers, or when ambient temperatures are low, but even under these circumstances, closure is complete in <10 s. Stigma closure is not limited to *M. aurantiacus*, but is common in at least four families in the order Scrophulariales: Scrophulariaceae, Bignoniaceae, Martyniaceae, and Lentibulariaceae (Newcombe, 1922). All species with touch-sensitive stigmata have hermaphroditic flowers with tubular corollas, a single pistil, and a stigma composed of two lobes. Time to closure of the stigma following tactile stimulation is quite variable among species, taking anywhere from 1 or 2 s to over an hour (Bertin, 1982; A. E. Fetscher, unpublished data). There appear to be no species with touch-sensitive stigmata outside the Scrophulariales. *Ipomopsis aggregata* (Polemoniaceae) has a trilobed stigma, which also closes, but receipt of pollen, rather than touch, is necessary to elicit this response (Waser and Fugate, 1986).

Literature dating at least as far back as the beginning of the century discusses physiological aspects of stigma closure (Burck, 1902; Lloyd, 1911; Newcombe, 1922, 1924; Guttenberg and Reif, 1958; Linskens, 1976), mostly focusing on the role of turgor as a proximate explanation of the mechanics of closure. Various hypotheses addressing the adaptive significance of stigma closure

have been proposed: stigma closure aids pollen capture and receipt (Linskens, 1976; Thieret, 1976), prevents intrafloral selfing (Kerner von Marilaun, 1895; Newcombe, 1922, 1924; Lloyd and Yates, 1982; Ritland and Ritland, 1989), and reduces interference between pollen receipt and export functions within the flower (Webb and Lloyd, 1986). However, no empirical tests of these hypotheses have been published.

This study asks three main questions. First, what triggers stigma closure in *Mimulus aurantiacus* and what factors determine whether the stigma reopens? We test the separate effects of touch and pollen on stigma closure. In addition, we use experiments to determine the effects of pollen load and fertilized ovules on the frequency and timing of stigma reopening. Second, does stigma closure enhance female fitness? One hypothesis for the adaptive significance of stigma closure is that it aids capture, adherence, or germination of pollen and thus enhances seed set (Linskens, 1976; Thieret, 1976). We compare pollen receipt and seed set of flowers with stigmata manipulated to remain open with that of flowers with stigmata that close. Finally, is stigma closure costly to female fitness? Stigmata that close and do not reopen prevent the flower from receiving additional pollen and, conceivably, pollen from additional donors. Stigma closure after receipt of insufficient pollen for full seed set would appear costly to female function. Even if full seed set were achieved, failure to receive additional pollen might be costly to female fitness if higher quality offspring result from larger pollen loads or pollen from more donors (Willson, 1979). We use experimental manipulations to examine whether additional pollen is received if stigmata do not close and how receipt of additional pollen affects seed number and quality.

MATERIALS AND METHODS

Except where noted, all field work was conducted at the University of California's Dawson-Los Monos Canyon reserve in Vista, California,

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³ Author for correspondence (fetscher@biomail.ucsd.edu).

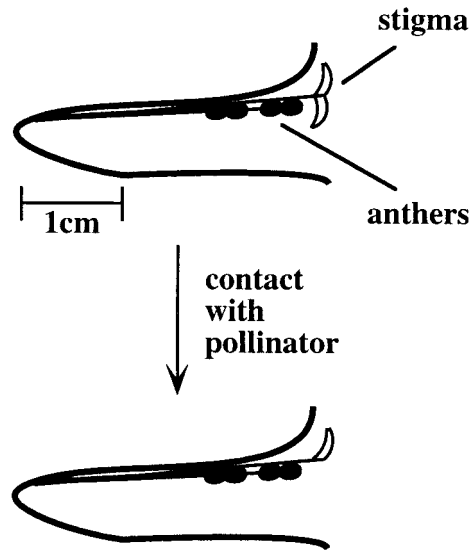


Fig. 1. Diagram of a *Mimulus aurantiacus* flower with its stigma in the open and closed positions. Though both stigmatic lobes respond to touch, pollinators usually only make contact with the lower lobe, such that it is the one that moves, resulting in the closed stigma being reflexed upward.

in April through August of 1996 through 1998, using *Mimulus aurantiacus* Curtis (bush monkeyflower), a self-compatible, hummingbird-pollinated, chaparral shrub. The stigma, which is exerted beyond the anthers (approach herkogamy), is spread open and receptive when the bud opens. The anthers, which are borne slightly below the style, dehisce 1 d later. The style and filaments are appressed to the top of the corolla (Fig. 1). Flowers are borne in pairs at nodes. Paired flowers tend to develop synchronously and last up to 8 d if unpollinated. At this study site, Anna's hummingbird (*Calypte anna*) is the primary pollinator of *M. aurantiacus*, but Allen's (*Selasphorus sasin*), Costa's (*Calypte costae*), Black-chinned (*Archilochus alexandri*), and Rufous (*Selasphorus rufus*) hummingbirds are also occasional visitors. In experiments involving "natural pollination," in which flowers in the field are tagged and sometimes manipulated and then left for observations or harvesting of stigmata or fruits later in the season, it is assumed that any pollination that may have occurred in the interim has been carried out largely by the above species.

Factors determining stigma closure and reopening—Reopening time of stigmata not receiving pollen was determined on 22 first-day flowers (across six bushes) by lightly touching stigmata with a finger free of pollen. Corollas and anthers had been removed the day before in order to discourage pollinator visitation. Preliminary studies show that removal of these organs has no effect on stigma behavior. All stigmata closed when touched, after which flowers were observed at half-hour intervals to determine whether their stigmata had reopened.

To examine the effect of pollen alone on stigma closure, pollen was gently sprinkled onto the stigmata of 28 first-day flowers across seven bushes. This technique does not induce the immediate closure characteristic of response to touch, presumably because stigmata are insensitive to touch below a certain amount of pressure. The stigmata were observed at half-hour intervals following addition of pollen and scored for whether they had closed.

The time lag to reopening of pollinated stigmata was estimated in 1998 at the UC San Diego field station in La Jolla, California, by hand pollinating (using uniformly light pollen loads) the stigmata of 77 first-day flowers, across 11 bushes, from which corollas and stamens had been removed. Unless otherwise noted, all hand pollinations used a roughly equal mixture of pollen from five randomly chosen donors

mixed on a microscope slide. The pollen mixture was then applied to stigmata by pressing them gently against the slide. All stigmata closed during hand pollinations. The stigmata were then observed at 2-h intervals from 20 to 32 h following pollination and scored for whether they had reopened.

To determine the effects of pollen receipt and seed production on stigma reopening and reclosing over a span of several days, stigmata of all first-day flowers across 20 bushes were hand pollinated in May through July 1997 with variable pollen loads. Corollas and stamens had been removed prior to pollination. All stigmata closed in response to contact during the application of pollen. Stigmata were then observed at either 1 and 3 d, 1 and 6 d, 2 and 4 d, 2 and 5 d, or 3 and 5 d following pollination and scored for whether they were open or closed. Upon senescence, stigmata were collected and stored in FAA (Kearns and Inouye, 1993). To count the number of pollen grains that had been applied during hand pollinations, stigmata were stained in 0.1% aniline blue in 0.15 mol/L K_2HPO_4 (pH 9.0) and mounted on microscope slides for viewing under ultraviolet illumination. Each pollen grain on the slide was counted by eye. Mature fruits were harvested for seed counts.

The time it takes pollen to access ovules was determined in 1998 by hand pollinating 155 flowers across six bushes, then removing each style at its junction with the ovary using a sharp forceps at some hourly timepoint between 14 and 31 h. Ovaries were observed 1 mo after pollination in order to deduce whether pollen tubes had reached the ovary as evidenced by development of fruits.

Costs and benefits of stigma closure for fitness through female function

In 1996, manipulations preventing stigma closure were performed in order to examine the effect of stigma closure on female fecundity and offspring quality. Stamens were removed, before anther dehiscence, from all flowers used in the experiment in order to avoid any possible confounding effects of intrafloral self pollen on seed number and quality. The possible effect of stigma closure on intrafloral pollen transfer was not being examined in the present study. Within each bush, stigmata from half of all first-day flowers (one from each nodal pair), across 16 bushes, were forced to remain permanently open, in a natural position, by anchoring the nonreceptive surface of each stigmatic lobe to the style with Elmer's® School Glue. This manipulation leaves the receptive surface of the stigma intact and in the same orientation as an unmanipulated, open stigma as depicted in Fig. 1. The remainder of the flowers were controls with unmanipulated stigmata. Natural pollination was allowed to ensue, and mature fruits from all flowers in the experiment were collected for seed counts. To estimate an early component of offspring quality, groups of 50 seeds/fruit were placed in petri dishes containing 5 mL sterile water, set on a laboratory shelf under ambient light, and every 2 d for a period of 14 d, positions of petri dishes were randomly rearranged. After 14 d, seeds were scored for germination.

The effect of stigma closure on the amount of pollen received was determined in 1997 by repeating the manipulation to prevent stigma closure as described above on one first-day flower at each node across 13 bushes, leaving the remaining flowers to serve as controls. As before, stamens were removed prior to anther dehiscence. Following natural pollination, stigmata were collected from manipulated and control flowers upon senescence, and pollen counts were made as described above.

Whether or not pollen arriving at a stigma subsequent to a previous pollen load is capable of contributing to seed set has important implications for whether stigma closure is costly to female fitness. Late-arriving pollen might be incapable of siring seed if earlier fertilized ovules outcompete later ones for maternal resources. To determine whether pollen loads received subsequent to the first would be able to sire seed, all first-day flowers (across ten bushes), from which stamens and corollas had been removed, were hand pollinated with small, relatively uniform, pollen loads. A second load of pollen was added to a subset of these stigmata randomly with respect to node on each bush either 2, 4, 8, 10, 22, 28, or 48 h after the first addition of pollen. For

TABLE 1. State of the stigma (open or closed) at timepoints following hand pollination with variable pollen-load sizes. Data are expressed as numbers of flowers falling into observation categories further stratified by state of the stigma during first and second observations.

Observation timepoints (d)	State of the stigma at each observation (1 st , 2 nd)				Totals
	Closed, closed	Closed, open	Open, open	Open, closed	
1, 3	9	2	9	1	21
1, 6	29	1	1	4	35
2, 4	99	0	1	5	105
2, 5	16	0	6	2	24
3, 5	13	0	1	0	14
Totals	166	3	18	12	199

all stigmata that had not reopened prior to the second addition of pollen, it was necessary to spread open the closed stigmatic lobes with a forceps and insert pollen with another forceps. Upon senescence of the flowers, stigmata were collected for pollen counts and fruits were harvested for seed counts.

If stigmata were to remain open following pollen deposition they could receive multiple loads of pollen and, potentially, pollen from additional sires. To determine whether multiple sires contributing to the total pollen received by a flower result in fitter offspring, all first-day flowers on 35 bushes were hand pollinated using roughly equal, saturating amounts of pollen from either one, or an equal mixture of five, randomly chosen donors. Corollas and stamens were removed from these flowers prior to hand pollination. Stamens only were removed from other first-day flowers that were otherwise unmanipulated to serve as naturally pollinated controls. Upon maturation, fruits were harvested, seeds were counted, and germination rates of seeds from each fruit were determined as described above.

RESULTS

Factors determining stigma closure and reopening—*Mimulus aurantiacus* stigmata on first-day flowers always closed within 2 s in response to touch. Pollen addition in the absence of pressure sufficient for immediate stigmatic response (when pollen was gently sprinkled onto stigmata) elicited closure between 0.5 and 1.5 h after pollination, similar to the pattern in *I. aggregata* in response to the addition of pollen (Waser and Fugate, 1986). All stigmata touched in the absence of pollen reopened between 2.5 and 4.5 h ($\bar{X} = 3.14$ h, $N = 22$) after closure. Time to reopening was generally much longer, and the frequency of reopening much lower, when stigmata had been pollinated than when they had been touched but no pollen was transferred. Of the 77 stigmata that were hand pollinated and observed at 2-h intervals from 20 to 32 h after pollination, one reopened sometime before 20 h after pollination, five reopened between 20 and 22 h, two reopened between 22 and 24 h, two reopened between 24 and 26 h, and two reopened between 26 and 28 h. The remaining 55 stigmata stayed closed for the duration of the experiment. For the long-term stigma behavior experiment in which stigmata were hand pollinated with variable loads and observed at two timepoints over a span of several days, 33 of 199 stigmata were found to be open at least at one observation timepoint. Twelve of these stigmata underwent a secondary closure, which occurred between 1 and 3 d after pollination in one flower and sometime between 3 and 6 d in all others (Table 1). Of

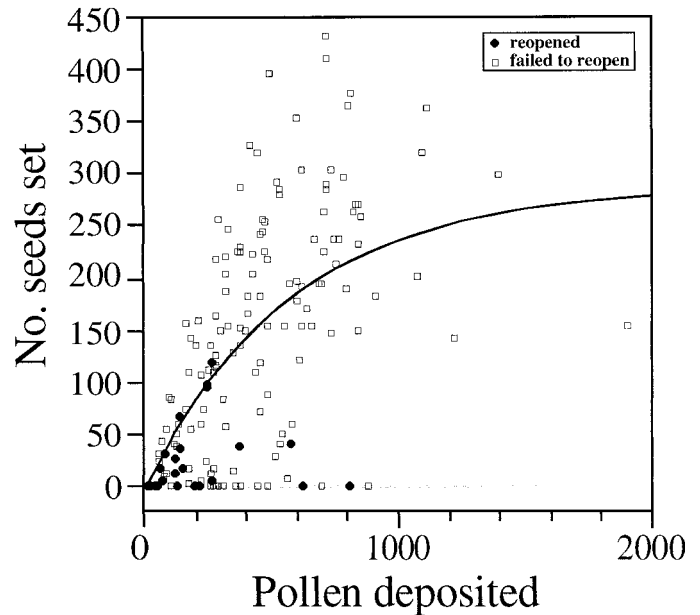


Fig. 2. Stigma reopening in *M. aurantiacus* as a function of pollen load and seed set. The relationship between number of pollen grains delivered during hand pollinations and the number of seeds set is indicated by a nonlinear regression curve (seeds = $289(1 - \exp^{-0.0017\text{pollen}})$; $R^2 = 0.50$; Mitchell, 1997). Solid diamonds represent flowers whose stigmata reopened at some point following pollination. Open squares indicate flowers whose stigmata remained closed for the life of the flower.

the flowers that were hand pollinated and then had their styles removed at some timepoint between 14 and 31 h after pollination, the ovaries of 14.2% showed signs of development into fruits a month later. Some of the ovaries whose styles had been removed as early as 15 h after pollination showed signs of development into fruits, indicating that pollen can reach ovules as soon as 15 h following deposition onto the stigma.

Seed set of hand-pollinated flowers was an increasing, asymptotic function of pollen load (Fig. 2). For flowers receiving ≥ 650 pollen grains, the slope of the regression relating pollen to seeds was indistinguishable from zero, indicating that receipt of pollen beyond 650 grains would not be expected to result in an increase in seed set. The mean seed set for flowers receiving ≥ 650 pollen grains was 247 (SE = 13.9; $N = 44$). Even below these numbers of pollen grains and seeds, stigmata usually failed to reopen. Pollen and seed numbers separately were each highly significant determinants of stigma reopening in simple logistic regressions ($P < 0.0001$ for both variables). However, multiple logistic regression showed that seed count remained a highly significant determinant of stigma reopening ($P = 0.0005$) when the effect of pollen was removed, whereas pollen count was not significant ($P = 0.0784$) when seed number was present in the model.

Costs and benefits of stigma closure for fitness through female function—For all experiments using paired treatments, only data from nodes at which pollen and seed (where applicable) counts could be determined for both flowers were analyzed. The mean number of pollen grains deposited by pollinators on control stigmata

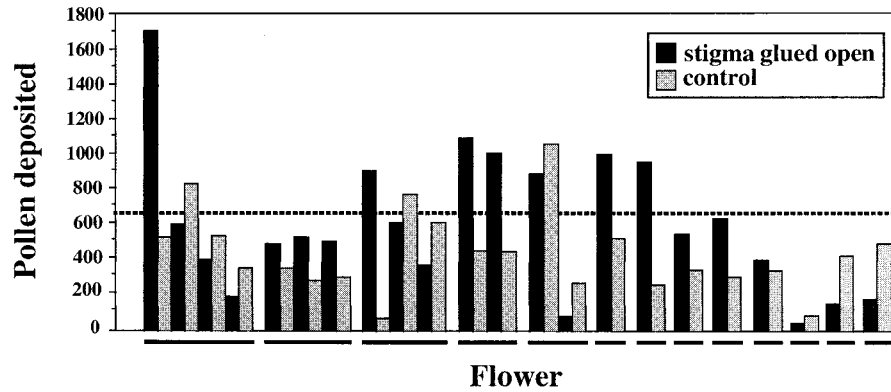


Fig. 3. Pollen deposition on manipulated (glued open) and control stigmas of *M. aurantiacus*. Each histogram represents the natural pollen deposition onto the stigma of a flower over its lifetime. Each pair of adjacent bars are nodally paired flowers receiving different stigma treatments, either glued open or unmanipulated, before exposure to natural pollination. Bars underneath the graph indicate data from the same bush. The line across the graph is plotted at 650 pollen grains, the approximate number of grains above which additional pollen has no measurable effect on seed number.

was 435 grains (SE = 49; $N = 22$) and the mean for stigmas which were glued open was 604 grains (SE = 86; $N = 22$; one-tailed paired t test, $P = 0.0351$; Fig. 3). Neither the number of seeds produced, nor the germination rates of these seeds, differed significantly between control flowers and those whose stigmatic lobes had been glued in place to prevent closure (Table 2).

Flowers that received a second load of pollen by hand produced significantly more seeds (127.5, SE = 70.4; $N = 47$) than control flowers (93.9, SE = 58.0; $N = 69$; unpaired two-tailed t test, $P = 0.0059$). Time of second addition had no effect on seed set (ANCOVA, $P = 0.9231$; Fig. 4). The number of pollen donors used in hand pollinating flowers had no significant effect on the germination rate of seeds. The mean germination rate of seeds sired by a mixture of five donors was 0.514 (SE = 0.014) and that for one donor was 0.516 (SE = 0.049), compared to a mean of 0.500 (SE = 0.018) for naturally pollinated fruits.

DISCUSSION

Two different stimuli, touch and pollen, are able to elicit stigma closure in *M. aurantiacus*, though possibly through different mechanisms. Response to touch is usually immediate, whereas response to the presence of pollen alone takes substantially longer. Stigmata always reopened when pollen had not been deposited. If stigmas were to reopen at all after pollen receipt, they took much longer to do so than when no pollen was received. Pollen is therefore not only able to elicit stigma closure, but also serves to maintain closure for an extended period, usually

permanently. Stigma closure has been attributed to the loss of turgor in cells comprising the stigmatic tissue. Pollen germination and subsequent tube growth, a process in which water from surrounding tissue in the pistil is taken up by the growing pollen tubes, probably maintain the stigma in a collapsed (closed) state (Linskens, 1976). What triggers secondary closure in some (nearly half) of the stigmas that reopen after hand pollination, a phenomenon that has been observed in several species in the Scrophulariales (Newcombe, 1922; Bertin, 1982), remains unclear. There was no obvious relationship between secondary closure and pollen or seed counts.

So long as some pollen was received, stigma reopening was not a direct function of the size of the pollen load, but was strongly dependent on the number of seeds produced (Figs. 2, 5). The relationship between seed count and stigma reopening suggests that the stigma may remain closed permanently in response to the onset of development of a sufficient number of fertilized ovules and reopen only when the number of ovules fertilized is low.

TABLE 2. Mean (SE) values for seed set, seed germination rate, and multiplicative fitness (product of seed set and germination rate per fruit) for fruits from control flowers and flowers on which stigmas had been glued open prior to natural pollination. All paired t tests were nonsignificant.

Stigma treatment	N (fruits)	Seed set	Germination rate	Multiplicative fitness
Glued open	26	143 (13)	0.399 (0.042)	59.9 (8.1)
Control	26	166 (15)	0.381 (0.046)	66.5 (10.8)

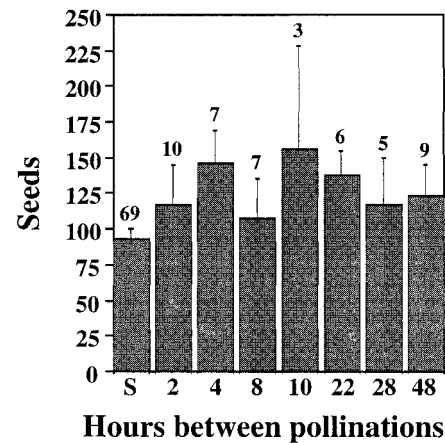


Fig. 4. Mean (1 SE) numbers of seeds set by flowers that received either a single (S), uniformly low dose of pollen delivered by hand, or this as well as a second dose a certain number of hours later (indicated below each bar). Numbers above error bars indicate sample size in each treatment group.

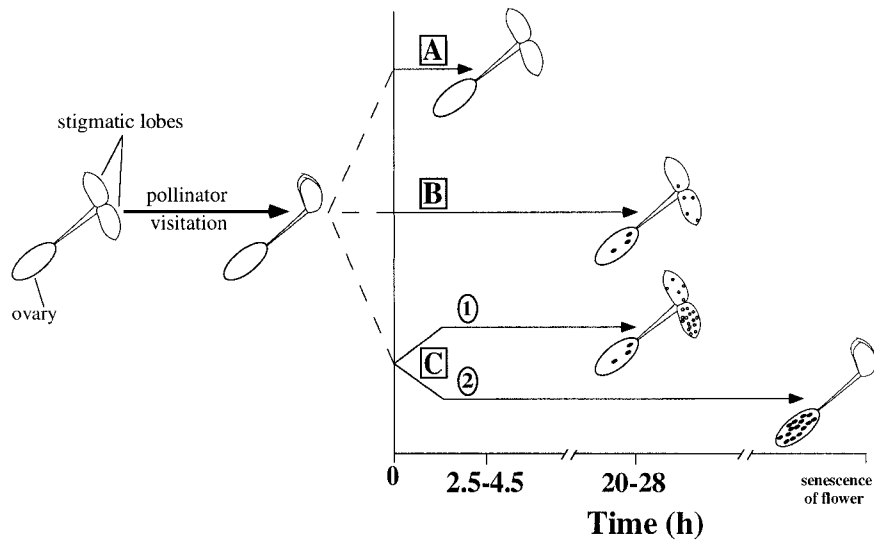


Fig. 5. Stigma behavior in *M. aurantiacus* in response to touch, pollen, and fertilized ovules. For simplicity, it is assumed that the pollinator contacted the stigma during visitation, causing the lobes to close together. (A) The stigma reopens quickly if no pollen has been deposited; (B) any reopening is delayed when a small quantity of pollen, resulting in few fertilized ovules, is deposited; (C) deposition of many grains results in (1) delayed reopening when there are few fertilized ovules and (2) permanent closure when there are many fertilized ovules. Note that, even in cases B and C1, stigmata often fail to reopen.

When a pollinated stigma does reopen, it takes longer to do so than the amount of time it takes pollen to reach ovules (within 15 h), providing additional support for the hypothesis that reopening of a pollinated stigma is a response to the number of ovules fertilized.

Apparently, above ~ 130 seeds set, stigmata never reopen. Even below this number, 72% of stigmata failed to reopen. The tendency of the stigma to reopen when touched in the absence of pollen, yet to remain closed after receiving pollen, has also been noted in *Proboscidea louisianica* (Martyniaceae) (Thieret, 1976), *Catalpa speciosa* (Bignoniaceae) (Stephenson and Thomas, 1977), and *Mimulus guttatus* (Dudash and Ritland, 1991).

One hypothesis for the adaptive significance of stigma closure is that it improves capture and germination of, and fertilization by, pollen (Linskens, 1976; Thieret, 1976). We found no support for this hypothesis. Stigma closure did not increase seed set nor germination rate. However, we have not assessed potential benefits of stigma closure to offspring quality post germination.

Stigma closure would seem likely to result in pollen limitation if closure were permanent even after receiving insufficient pollen for full seed set, which is often the case in *M. aurantiacus*. Despite this, we found no reduction in seed set for flowers whose stigmata closed, relative to flowers with stigmata manipulated to remain permanently open. Our failure to detect a female fecundity cost of stigma closure could not have been attributable to the inability of secondary pollen loads to sire seed. Pollen added up to 48 h after the first application of pollen can sire additional seed (Fig. 4). Stigma reopening following pollen deposition could therefore allow a second opportunity for the receipt of pollen that could fertilize additional ovules.

Several factors could account for our failure to detect a cost of stigma closure in terms of reduced seed set. First, limited visitation, such that flowers cannot reliably

expect a second pollen-depositing visit could partially explain the lack of cost. Stigmata tacked permanently open with glue and exposed to pollinators in a natural setting did, on average, receive more pollen than unmanipulated control stigmata. However, pollen receipt was highly variable across nodal pairs of treatment and control flowers. Only six of 22 of the glued open stigmata received more than twice the number of pollen grains found on control flowers, while at four nodes, the control flowers received a twofold or greater excess of pollen (Fig. 2). Second, much of the difference in mean pollen loads received by the two treatments occurred in flowers that received >650 grains. We can detect no increase in seed set from pollen loads above this value. Third, based on pollen loads found on naturally pollinated glued open and control stigmata, the regression line in Fig. 2 predicts mean seed sets of 164 for manipulated flowers and 142 for controls. This difference, though it potentially represents a significant cost of stigma closure to female function on an evolutionary scale, might not be detectable given the variation in the data and our sample sizes.

We found no measurable cost nor benefit of stigma closure in *M. aurantiacus* to seed production or quality. Two other hypotheses for the adaptive significance of this behavior are being examined. First, intrafloral selfing might be prevented if stigmata close before the pollinator has an opportunity to reach the anthers and move pollen back onto the stigma. Second, Webb and Lloyd (1986) suggest that stigma closure could represent "movement herkogamy" in which female organs, after pollen receipt, move to reduce interference with pollen dissemination. The architecture of *M. aurantiacus* flowers with tubular form, approach herkogamy, broad stigma lobes and anthers closely appressed to the style appears to make interference between female and male functions likely. The fact that pollinated stigmata are capable of reopening, but tend not to, also makes this a reasonable hypothesis. Such

behavior suggests that, if the open stigma interferes with pollen removal or export, it may benefit the plant to keep stigmata closed (and switch to a functionally male state) after receiving pollen, even though maximal seed set may not always be achieved.

Since pollen alone causes the stigma to close, as it does in *I. aggregata* (Waser and Fugate, 1986), rapid response to touch suggests that there is something to be gained by immediate response to pollinator visitation. We have shown that stigma closure is not necessary for pollen capture nor adherence, making such a function an unlikely explanation for rapid stigma closure. The other hypotheses for the adaptive significance of stigma sensitivity to touch, that it may prevent intrafloral selfing or reduce pollen–pistil interference, are both good candidates to explain the need for rapid closure of the stigma, because both hypotheses provide a context in which the advantage of stigma closure can be realized within a single visit by a pollinator to a flower. In fact, it appears that rapid stigma closure would be necessary for preventing intrafloral selfing. Stigma closure in *M. aurantiacus* usually occurs rapidly enough that closure could often be complete before the forager leaves the flower. It must also be taken into account, however, that many Scrophulariales with touch-sensitive stigmata do not respond as rapidly to touch. In many Bignoniaceae, for instance, stigma closure takes between 10 and 60 s, longer than a pollinator visit (Bertin, 1982; A. E. Fetscher, unpublished data), so prevention of intrafloral selfing is unlikely to be the universal explanation of stigma closure. Experiments are underway to test both the male–female interference and the intrafloral selfing hypotheses for the adaptive significance of stigma closure.

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