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THE EFFECTS OF NATURAL VARIATION IN POLLINATOR VISITATION ON RATES OF POLLEN REMOVAL IN WILD RADISH, *RAPHANUS RAPHANISTRUM* (BRASSICACEAE)[1]

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Pollen removal from flowers is an important component of male fitness, but the effects of natural variation in visitation rates on pollen removal are poorly understood. We measured pollen removal over 2 yr in experimental field populations of wild radish, *Raphanus raphanistrum.* Pollen removal and pollinator visitation over 1-hr periods were measured on previously unvisited flowers. The effects of pollen production and visitation by different insect taxa on pollen removal were determined using multiple regression. Pollen removal rates were extremely high; a median of 84% of pollen produced was removed in 1 hr. Pollen production was far more important than visitation in determining the number of pollen grains removed. Pollen removal increased with increasing numbers of visits by honey bees and small native bees, but increased numbers of syrphid fly visits had no effect. Average visit duration had no effect on pollen removal in 1991, and a marginally negative effect in 1992.

Evolutionary studies of plants have long been hampered by an inability to measure male fitness. Plant biologists began to study pollen removal as one component of male fitness ≈20 yr ago. The early studies were on plants that package pollen in pollinia, making removal easier to quantify (Willson and Rathcke, 1974; Willson and Price, 1977; Schemske, 1980), but more recent studies of pollen removal have examined species with the far more common condition of loose granular pollen (e.g., Galen and Stanton, 1989; Wolfe and Barrett, 1989; Murcia, 1990; Young and Stanton, 1990; Wilson and Thomson, 1991; Stanton, Ashman, and Galloway, 1992; Harder and Barrett, 1993). In the past several years, biologists have begun to use molecular markers to measure paternity and thus male fitness directly (e.g., Broyles and Wyatt, 1990; Meagher, 1991; Devlin, Clegg, and Ellstrand, 1992), and the use of pollen removal as a surrogate for male fitness has been questioned (Stanton, Ashman, and Galloway, 1992; but see Broyles and Wyatt, 1990).

Regardless, pollen removal is the crucial first component of male fitness. Pollen must be removed from anthers before it has any chance of arriving at a stigma and fertilizing ovules, and therefore a full understanding of male fitness must include knowledge of the factors that affect pollen removal. There are two stages of pollen removal (cf. Waser, 1983): first, the flower must attract pollinators (visitation; Galen and Stanton, 1989; Young and Stanton, 1990) and then, when pollinators arrive, the flower must be effective at placing pollen on the pollinators' bodies. The latter process may depend on pollinator behavior such as time spent at the flower (Galen and Stanton, 1989; Young and Stanton, 1990). Most studies that have directly measured the effects of visitation on pollen removal, however, have used experimentally determined numbers of visits, so that the effects of natural variation in visitation is not well known (but see Young and Stanton, 1990).

In this paper we examine the effects of natural variation in visitation rate and time spent per visit on pollen removal in a 2-yr field study of *Raphanus raphanistrum.* We observed individual flowers for 1-hr periods, recording the number of visitors in each of three taxonomic groups and the time spent at the flower by each visitor. We estimated the amount of pollen removed over the 1-hr period. The separate influences of number of visitors and duration of visits of each taxon on pollen removal were determined using multiple regression.

MATERIALS AND METHODS

Field methods—Wild radish (*Raphanus raphanistrum,* Brassicaceae) is an annual weed that grows in disturbed habitats on six continents (Stanton, Bereczyky, and Hasbrouck, 1987). *R. raphanistrum* is self-incompatible (Sampson, 1964), and therefore requires insect pollination for successful reproduction. Pollen removal experiments were conducted in 1991 and 1992. Plants for both years were grown from seeds collected in a field near Binghamton, NY (Conner and Via, 1993). Experimental arrays of 64 plants with 1-m spacing were planted into a garden at the Phillips Tract natural areas northeast of Urbana, IL. This site is a mixture of old fields, mature forest, and restored prairie surrounded by agricultural land.

Pollen removal measurements were conducted from 6 September to 23 October 1991 and 16 July to 10 August 1992, using 54 and 48 plants in the 2 yr, respectively. The 1991 study dates were past the normal peak flowering period for *R. raphanistrum* in this area, but within the normal flowering time. In 1991 the main pollinators were honey bees and small native bees (55 and 32% of all visits, respectively) while in 1992 the main pollinators were small bees and syrphid flies (40% each; Conner, Rush, and Jennetten, 1996). The pollinators were all species that visit wild radish in upstate New York, and all effect pollination of wild radish (Conner, Davis, and Rush, 1995; G. Eickwort, personal communication). The honey bees foraged for both pollen and nectar,

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TABLE 1. Medians and quartiles for the variables measured. Pollen counts are for four of the six anthers; time per visit is in seconds.

Variable	1991 Median	1991 Quartiles	1992 Median	1992 Quartiles
Pollen available	—	—	72,028	57,406–79,080
Pollen left at 1 hr	10,014	5,621–35,918	11,210	6,345–29,070
Pollen left at wilting	4,932	3,355–7,856	—	—
No. of honey bee visits	1	0–2	—	—
Honey bee time/visit	7	5–15	—	—
No. of small bee visits	2	0–7	4	1–7
Small bee time/visit	39	18–57	37.0	27–57
No. of syrphid fly visits	—	—	2	0–4
Syrphid fly time/visit	—	—	33	13–138

while the small native bees and syrphid flies foraged almost exclusively for pollen.

Groups of buds were bagged with bridal-veil netting to exclude pollinators. When two flowers opened within the bag, these flowers were used to measure pollen removal. In both years the bag was removed and one of the two flowers was observed for 1 hr; the type of pollinator (honey bee, small bee, or syrphid fly) and the length of each visit were recorded. At the end of the hour pollen was collected from the flower. The other flower was treated differently in the two years of the study. In 1991 the second flower was left until it wilted and then its pollen was collected, allowing us to determine how much pollen remained at the end of a flower's life. In 1992 pollen was collected from the second flower as soon as the bag was removed, before any visitation occurred. The difference between this count of pollen before visitation and the amount of pollen left on the first flower after 1 hr of insect visitation was used as an estimate of the number of pollen grains removed over the hour (Harder, 1990; Young and Stanton, 1990). This relies on the assumption that the two adjacent flowers had similar pollen production. To test this, we collected pollen from two adjacent unvisited flowers using exactly the same methods as in the pollen removal experiments. Adjacent flowers were found to have similar pollen counts: 75% of the variance in pollen counts was between plants (N = 15 plants).

Pollen counting—Anthers were dried in a 37 C incubator with the vial lids removed for 1 wk to insure complete dehiscence, and then stored capped at room temperature until counting. Approximately 20 ml of 2% NaCl solution was then added to the vial (precise volume of NaCl solution was determined by weighing). Two cycles of vortex mixing (30 sec each, Vortex Genie) and sonication (2 min each) were used to strip the pollen from the anthers. Four 0.5 ml samples of the solution were then counted using a Coulter counter (model Z_{BI}, Coulter Electronics, Hialeah, FL). Immediately before the first and third counts the vials were vortexed for an additional 10 sec. The four values were averaged, and then this number was multiplied by two times the precise volume of NaCl solution (because the samples were 0.5 ml) to give the estimate for total pollen in the vial.

Data analyses—to determine the factors that affected either the number of pollen grains remaining on the anthers after 1 hr (1991) or the number removed in 1 hr (1992), multiple regression was performed. Independent variables were pollen available (1992 only) and the number of visits and visit duration for each pollinator taxon. Quadratic terms were included in initial models to test for diminishing returns, but none was significant so they were dropped from final models. Dropping these terms did not qualitatively alter the results for other variables, but did improve model fit in the 1991 analysis. All independent variables were standardized by z-transformation (mean = 0, variance = 1). Variance inflation factors (Neter, Wasserman, and Kutner, 1985) were always less than 1.5, indicating no multicollinearity problems. For the 1991 analysis the dependent variable, pollen remaining after 1 hr, was natural-log transformed. Residual plots of final models indicated little heteroscedasticity. JMP® version 3.0.2 (SAS, 1994) was used for all analyses.

RESULTS

In 1992 unvisited flowers produced a median of 72,028 pollen grains on four anthers, and 11,210 pollen grains remained after 1 hr of visitation (Table 1); thus, an average of 84% of pollen produced was removed in 1 hr. The 1991 plants had similar amounts of pollen remaining at the end of 1 hr as plants in 1992, with little remaining at wilting (Table 1).

The multiple regression models for 1991 and 1992 explained 43% and 74% of the variance, respectively ($P <$ 0.0001 in both models). The improved fit of the 1992 model was due to the addition of pollen available (when the 1992 data was analyzed based on pollen remaining the R^2 dropped to 34%). The number of pollen grains produced by the flower (pollen available) was far more important than the visitation variables in determining removal; the standardized regression slope for pollen available (18,358 ± 1865, $P < 0.0001$) was over three times as large as any of the standardized visitation slopes (Figs. 1, 2).

For small bees in both 1991 and 1992, and for honey bees in 1991, increased visitation increased pollen removal (i.e., a negative effect on pollen remaining in 1991; Fig. 1). The visitation rates of syrphid flies, however, did not affect pollen removal in 1992. In 1991 pollen remaining was not affected by average duration of visits by either pollinator type, while in 1992 pollen removal actually decreased with increasing duration (Fig. 2). This trend was significant for syrphid flies and marginally significant for small bees.

DISCUSSION

We found extremely rapid rates of pollen removal and very little pollen left at wilting, in agreement with previous studies of rates of pollen removal under natural visitation (Wolfe and Barrett, 1989; Young and Stanton, 1990; Wilson and Thomson, 1991; Stanton, Ashman, and Galloway 1992). Rapid removal rates imply that male fitness may depend more on rapid delivery of pollen to stigmas rather than the total number of pollen grains delivered, especially if flowers open synchronously (Stanton, Ashman, and Galloway 1992; Stanton, 1994), because there may be few unfertilized ovules available for pollen that is delivered later. Additional studies that measure rates of removal under natural conditions are needed to determine how often such rapid removal rates occur. In particular, studies of species that are not visited as heavily as *Raphanus* may show lower rates of removal.

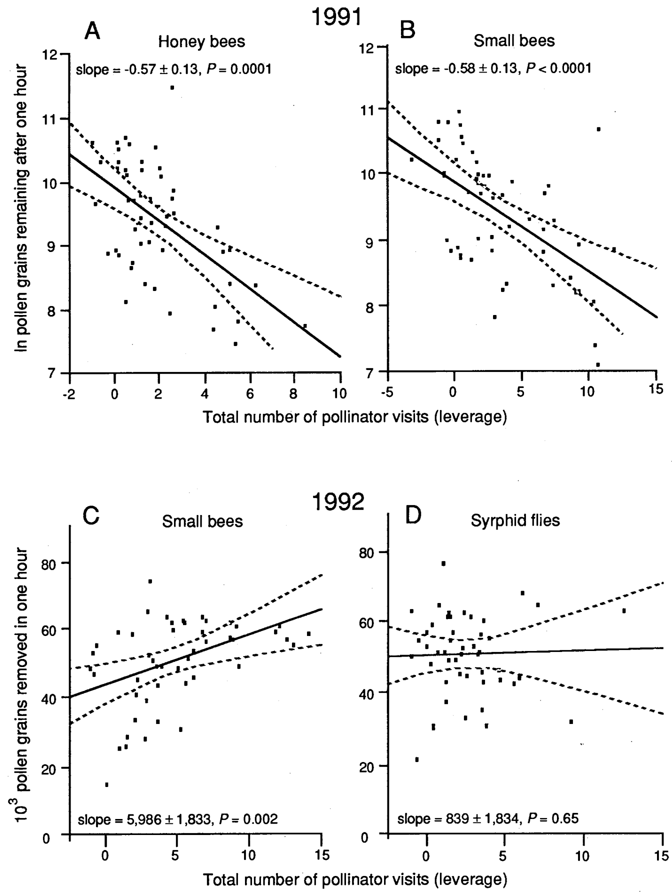

Fig. 1. Partial regression leverage plots (Sall, 1990) depicting the relationships between pollen removal and the number of pollinator visits after correcting for the other independent variables (see Materials and Methods). The solid lines are fitted partial regression lines, and the dashed lines are 95% confidence intervals of the lines. The dependent variable for 1991 was pollen remaining (ln-transformed), while the dependent variable in 1992 was pollen removed; therefore slopes with opposite signs in the different years indicate similar relationships. Standardized partial regression coefficients with their standard errors and significance levels (P) are shown. The negative values for numbers of visits were caused by the correction for other independent variables. Removing outliers or influential points did not alter the qualitative results in any case. N = 54 in 1991 and 48 in 1992.

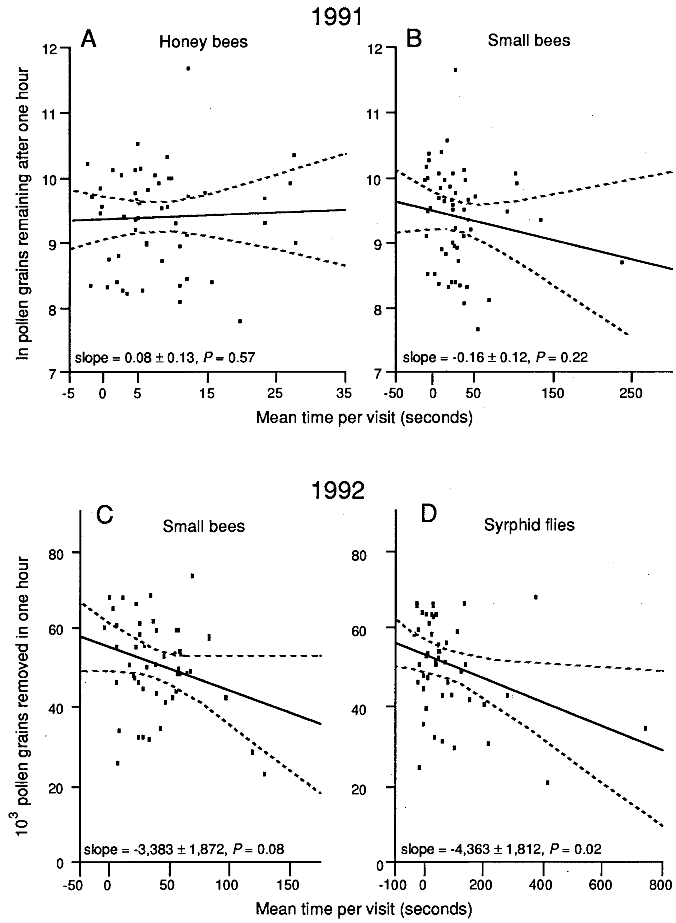

Fig. 2. Partial regression leverage plots depicting the relationships between pollen removal and the mean duration of pollinator visits after correcting for the other independent variables. See Fig. 1 for details.

Pollen production was far more important than visitation in determining pollen removal in 1992, in spite of the fact that visitation was more variable among plants than was pollen production (Table 1). If pollen removal is related to male fitness (as it is in *Asclepias*; Broyles and Wyatt, 1990), then there may be stronger selection to increase pollen production than to increase attractive floral traits, at least under the conditions in our study. This could result in the evolution of greater allocation to pollen at the expense of attractive traits.

While pollen removal was more strongly related to pollen production than visitation, visitation was also very important. Increased numbers of visits by honey bees in 1991 and small bees in both years led to strong increases in pollen removal (Fig. 1). There was no evidence for diminishing returns in this relationship (see Methods and Fig. 1), in contrast to previous studies (Galen and Stanton, 1989; Young and Stanton, 1990). This difference was not due to our plants receiving fewer visits than plants in the other studies (in most cases ours received more), but it may be due to differences in the pollinators. The pollinators in the studies cited above were primarily large bees foraging for nectar. Our syrphid fly and small bee pollinators were much smaller and were foraging almost exclusively for pollen, and our honey bees foraged for pollen as well as nectar. It is possible that diminishing returns are most likely when pollen removal is incidental, as with nectar feeding, since amounts of pollen picked up accidentally would decline with partial depletion of anthers. Conversely, when the pollinator is actively foraging for pollen, it may be more likely to remove similar amounts of pollen even after some depletion has occurred.

In contrast to the results for bees, the number of syrphid fly visits had no effect on pollen removal (Fig. 1). This result is surprising, since the syrphids were feeding on pollen and we have found that syrphids do remove substantial amounts of pollen in single visits (Conner, Davis, and Rush, 1995). It is possible that it is due to the low numbers of syrphid visits (Table 1), but the low slope and very high P value for the relationship between syrphid visitation and pollen removal (P = 0.65) suggests that if a positive relationship does exist, it is a very weak one. Harder and Barrett (1993) reported that increasing numbers of bee visits had no effect on total pollen removed from short and long anthers, but did affect removal from mid-level anthers, in the tristylous *Pontederia.* To our knowledge, no one has measured pollen removal by syrphid flies previously; further studies of pollen removal with multiple syrphid visits will be necessary to determine if the lack of relationship found in this study is generally true. If it is, then studies of pollen removal with controlled numbers of syrphid fly visits could be done (cf. Galen and Stanton, 1989), to determine the relationship between visitation and removal without other pollinators present.

Another result of our study that is difficult to explain was that longer average visit durations for all three pollinator taxa had neutral (1991) or marginally negative (1992) effects on pollen removal after correcting for the number of visits (Fig. 2). Studies of pollen removal in single pollinator visits have shown that increasing visit duration leads to increased pollen removal in a variety of species (Galen and Stanton, 1989; Harder and Thomson, 1989; Young and Stanton, 1990; Conner, Davis, and Rush, 1995). The discrepancy between our results and those of the single-visit studies suggests that the relationship between pollen removal and visit duration may be altered when multiple visits occur. To our knowledge, other multiple-visit studies have not examined the relationship between pollen removal and average visit duration after correcting for the number of visits.

Two possible hypotheses to explain the lack of a positive relationship between visit duration and pollen removal are not consistent with the facts. The first hypothesis is that pollinator's bodies became rapidly saturated with pollen so that additional visit time did not result in increased removal. For single visits, however, increased visit time of both small bees and syrphid flies increases pollen removal (Conner, Davis, and Rush, 1995). Also, since all the pollinators in our study were actively foraging for pollen, saturation with pollen long before the insect leaves the flower seems unlikely.

A second possible hypothesis is that the presence of a pollinator on the flower for longer periods may have inhibited other pollinators from visiting. Two facts argue against this. First, it implies a negative correlation between number of visits and time spent per visit; in fact, those correlations ranged between −0.01 and 0.33 for the 2 yr. Second, over the course of the 1-hr observations in 1992 visitation to the focal flower by all insects combined lasted for an average of slightly less than 7 min, leaving the flower unoccupied for an average of 53 min.

Therefore, it seems that there are real differences in our study involving multiple visits and the results of single-visit studies. Since many species like wild radish receive multiple visits in nature, more studies of pollen removal with multiple visits are needed to understand the importance of visit duration under natural conditions. First, additional studies are needed to determine if our negative results for mean time per visit are general. If they are, then experimental approaches to understanding the effects of visit duration with multiple visits will be needed. One possibility is to measure the number of pollen grains removed during each of a series of visits in which the visit durations are experimentally manipulated. It may be necessary to use trained pollinators, hand-held pollinators, or pollinator models (cf. Levin and Berube, 1972; Harder, 1990; Campbell, Waser, and Price, 1994) for these experiments.

In summary, our results showed that the number of pollen grains produced by a flower was far more important than visitation in determining the number of pollen grains removed. We found that pollen removal increased strongly and linearly with increased numbers of honey bee and small bee visits, but not with increased numbers of syrphid fly visits. Pollen removal did not increase with increased average time spent per visit by any pollinator taxon. Future experimental work could investigate why pollen removal was not related to the number of visits by syrphids or the time spent per visit by any taxon. Even more important are studies that relate pollen removal by different pollinator taxa to total male fitness. In particular, increasing pollen removal by pollen-feeding animals might decrease fitness, while pollen removal by nectar-feeding animals might increase fitness (e.g., Broyles and Wyatt, 1990).

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