Fluorescent dye particles are good pollen analogs for hummingbird-pollinated *Silene virginica* (Caryophyllaceae)

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Abstract: We tested the utility of fluorescent dye particles as pollen analogs for hummingbird-pollinated *Silene virginica* (Caryophyllaceae) by comparing the movement of pollen and fluorescent dye across sequentially visited emasculated flowers. We found no differences in either the intercept or the slope of the regressions of the two particle types on floral visitation sequence. In addition, the presence of fluorescent powder on the stigmas of a flower was a good indicator of pollen transferred to that flower. Both pollen and dye particles were transported almost identical distances in the flower sequence. These data indicate that fluorescent powder is a good pollen analog for *S. virginica*. We compare our findings with previous studies examining the utility of fluorescent dye as a pollen analog.

Key words: pollen analog, fluorescent dye, pollen carry-over.

Résumé : Les auteurs ont évalué l'utilité de particules de colorant fluorescent comme analogues polliniques pour le *Silene virginica* (Caryophyllaceae) pollinisé par les oiseaux-mouches, en comparant le mouvement du pollen et du colorant le long d'une séquences de visites effectuées sur des fleurs émasculées. Les auteurs n'ont trouvé aucune différence soit dans le point d'interception ou soit dans les pentes des courbes de régressions des deux types de particules sur la séquence des visites florales. De plus, la présence de poudre fluorescente sur les stigmates d'une fleur constitue un bon indicateur du transfert de pollen à cette fleur. Les pollens aussi bien que les particules de colorant sont transportés sur des distances presqu'identiques dans les séquences florales. Ces données indiquent que la poudre fluorescente est un bon analogue du pollen pour le *S. virginica*. Les auteurs comparent leurs résultats à ceux d'études antécédentes, en examinant l'utilité d'un colorant fluorescent comme analogue du pollen.

Mots clés : analogue pollinique, colorant fluorescent, dépacement de pollen.
[Traduit par la rédaction]

Introduction

Knowledge of pollen dispersal patterns is fundamental to a complete understanding of plant reproductive ecology and evolution. A number of methods have been used to track pollen: pollen marking using neutron-activation analysis (Handel 1976) or radioactive markers (Colwell 1951; Schlising and Turpin 1971; Reinke and Bloom 1979; Stanton et al. 1992); the use of natural pollen polymorphisms, such as pin and thrum pollen (Richards and Ibrahim 1978) and color dimorphism (Thomson and Plowright 1980; Thomson and Thomson 1989); genetic markers (Shaal 1980; Ellstrand and

Marshall 1985; Meagher 1986; Broyles and Wyatt 1990; Barrett et al. 1992; Fenster 1995); labelling orchid pollinia with unique numeric codes (Nilsson et al. 1992); and fluorescent dye powders (Waser and Price 1982; Campbell 1985).

Each method has its limitations. Artificial labelling techniques are often expensive and technically difficult. Natural pollen polymorphisms are not widespread. While paternity analysis using genetic markers summarizes both pollination and fertilization events, it is often difficult to ascribe precise paternity in natural populations (e.g., Meagher 1986). Individually labelling pollen grains will be restricted to organisms that transfer their pollen in masses, e.g., Orchidaceae and Asclepiadaceae. Fluorescent dyes are the least expensive and most accessible technique available to ecologists interested in quantifying pollen movement.

Despite the ease of using fluorescent dye powder as a pollen analog and the insight gained on factors affecting pollen removal and transfer through its use (Price and Waser 1982; Campbell 1985, 1989; Svensson 1985, 1986; Murawski 1987; Campbell et al. 1994), there are relatively few published studies that have verified the accuracy of the pollen distributions inferred from this method (Waser and Price 1982; Campbell 1985; Thomson et al. 1986). The objective of this study was to determine whether fluorescent dye is a good analog of pollen in hummingbird-pollinated *Silene virginica* (Caryophyllaceae) by comparing the movement of dye particles with pollen grains across sequentially visited

flowers. We wished to extend the findings of other workers using *Silene virginica*, which differs in its reproductive morphology compared with plants used in previous investigations and is pollinated by the Ruby-throated Hummingbird, which has not previously been the focus of investigation. We use similar methodology as previous studies to facilitate cross-study comparisons.

Methods

Organism

Silene virginica is a short-lived perennial found in eastern North America. It flowers from late May to early July near Mountain Lake Biological Station, Giles Co., Virginia. The flowers are protandrous, with the male phase lasting 2 days. On each of these first 2 days a rank of five (sometimes four) anthers emerges from the corolla. At onset of the female phase, on day 3 or 4, three styles emerge from the throat of the corolla and the anthers are physically displaced from the styles. The papillose stigmatic surface, approximately 0.2 mm in diameter, is at the terminus of a long (20–30 mm) filiform style and extends 1–5 mm down the style. Most pollinations are the result of visitation by the Ruby-throated Hummingbird (*Archilochus colubris*), with a smaller proportion from visits by syrphid flies and small bees (M.R. Dudash and C.B. Fenster, unpublished data).

Experimental methods

We examined the similarity of pollen and dye movement in a series of experimental runs. The flowers used in this experiment were obtained from a site near the Biological Station or from potted plants collected the previous year from the same site and another nearby natural population. The potted plants were maintained at the University of Maryland greenhouse and were brought to Mountain Lake Biological Station just prior to flowering. Both field flowers and potted plants were kept in screened cages to prevent visitation of flowers. To determine the amount of pollen transferred to stigmas within the cages, or "background noise", several stigmas were collected from the screened flowers on each day experiments were conducted and examined under a compound microscope at 100×. *Silene virginica* does not autogamously self-fertilize under field or greenhouse conditions (C.B. Fenster and M.R. Dudash, unpublished data), and no pollen grains were observed on the collected stigmas.

The experimental runs were conducted in a 3 × 3.3 × 3 m tent. Hummingbirds were enticed to feed within the tent by placing a hummingbird feeder filled with a 20% sucrose solution just within the open door of the tent and rolling the flap up to just above the opening of the tent. Once accustomed to feeding in the tent, the hummingbirds were easily captured by simply waiting quietly beside the door and pulling the canopy down over the opening after a hummingbird had entered the tent.

Each hummingbird was cleaned of pollen by pressing the head and neck areas with scotch tape immediately following its capture. The hummingbird was then allowed to acclimate to the enclosure for approximately 1 h. All feeders in the tent were then removed and an experimental array of flowers was presented to the hummingbird in the tent. First the hummingbird was offered an array of three to four emasculated flowers in female phase as its only food source. These flowers were held at arm's length and were usually visited by the hummingbird after approximately 20–30 min. The number of flowers in the array was gradually decreased to a single hand-held flower. These initial flowers were subsequently examined and found to have no *Silene virginica* pollen on their stigmas. Then a single male-phase flower with dye applied to two of its five anthers was presented to the hummingbird. Green, orange, and

chartreuse dyes from Magruder Color Company (1029 Newark Ave., Elizabeth, N.J.) supplied by Radiant Color (2800 Radiant Ave., Richmond, Calif.) were applied to dehiscing anthers with the flat end of a wooden toothpick. Preliminary experiments conducted in nearby natural populations demonstrated that hummingbird visitation was unaffected by the presence or color of the fluorescent powders (e.g., Dudash 1991). Dye particle sizes are approximately 3–10 μm in diameter, considerably smaller than *Silene virginica* pollen grains (40–50 μm in diameter).
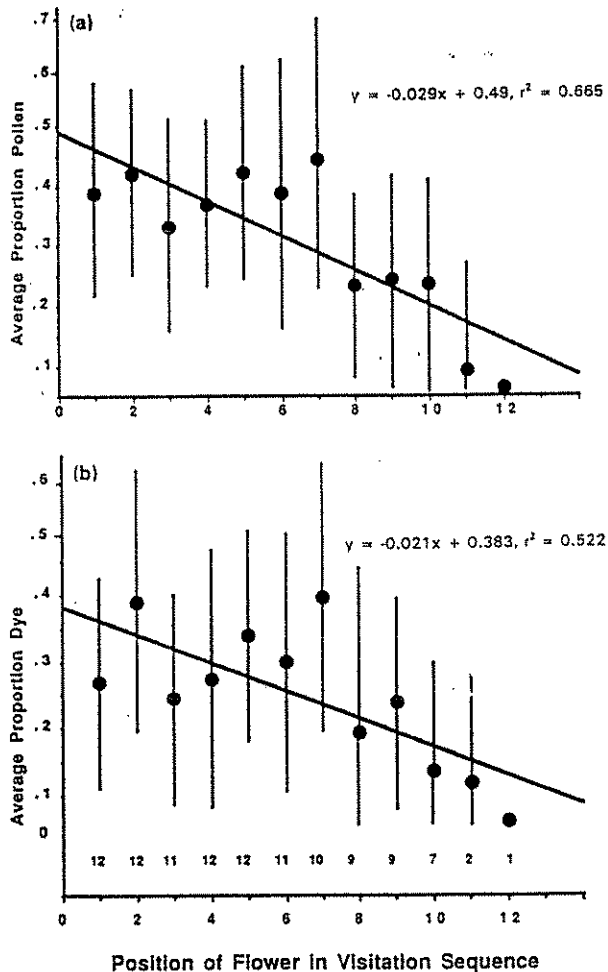
After visiting the dyed male-phase flower, the hummingbird was presented sequentially uncontaminated, emasculated female flowers from the caged plants. The flowers were placed upright in 1.5-mL microcentrifuge tubes immediately after visitation and the stigmas were transferred to slides following the completion of an experimental run. Following the run, the hummingbird was cleaned with scotch tape and allowed to visit a feeder before being released. A total of 12 runs were conducted of 5, 5, 7, 9, 9, 10, 10, 10, 10, 10, 11, and 12 flowers (totalling 108 flowers). Run lengths depended on the bird's "willingness" to visit the flowers. By clipping a specific tail feather after each run we determined that nine different hummingbirds were used in the 12 runs.

The stigmas were collected from each run and both particle types were counted using a compound fluorescent microscope at 100×. Blue light was used to count the fluorescent dye particles and white light was used to count the pollen. All microscope work was performed by one investigator to minimize error variation due to observer error.

Statistical methods

To determine whether fluorescent dye powder is a good analog of pollen we compared the movement of dye particles with pollen grains across the sequentially visited emasculated female flowers. To control for large differences in amount of pollen and dye picked up by each bird in each run, the total number of pollen grains or dye particles deposited on each flower (summed across the three stigmas) was divided by the maximum number of pollen grains or dye particles on the stigmas of a flower observed in that run (e.g., Waser and Price 1982). Two types of regression analyses with the adjusted values of dye and pollen deposition were used to determine if the two particle types moved similarly across the floral visitation sequence: (*i*) each flower was used as a replicate, and (*ii*) the adjusted values of dye and pollen deposition were averaged across runs to obtain mean adjusted values of dye and pollen deposition. We had little or no replication for sequences 11 and 12, only two and one runs, respectively, raising concerns for overweighting data from flowers in the 11th and 12th positions for the regression based on the mean adjusted values. Therefore the regression analysis based on the mean adjusted value was conducted with and without data from the 11th and 12th positions. To determine if the intercepts and slopes of the regressions of pollen grain and dye particles on flower visitation sequence differed, an ANOVA (PROC GLM, heterogeneity of slopes model, type III SS; SAS Institute Inc. 1985) was conducted to determine the presence of a main effect of type of particle (either pollen or dye) on the stigma and an interaction effect between type of particle and flower number in the sequence. In this analysis each flower represented a data point. Mean and median sequence or transport distance for pollen and dye were determined for each run and contrasted using paired *t*-tests. Finally, Spearman rank correlations were conducted between number of pollen grains and dye particles deposited on the stigma. Because nine birds were used in the 12 runs, the runs were not completely independent if different birds differentially affected pollen and dye behavior across the *Silene virginica* flowers. Thus we also analyzed the data by using bird means for those runs that overlapped with bird identity. Since no differences were observed in the analyses using 9 or 12 independent runs, we only present the analyses with the full 12 runs.

Fig. 1. Linear regression of (a) deposition of pollen and (b) fluorescent powder based on mean deposition for each position in the floral visitation sequence of emasculated female flowers of *Silene virginica*. Values shown represent mean adjusted values for number of dye particles and pollen grains reaching flowers at a given position in the sequence, ± 2 SE. Numbers under the error bars of the bottom figure give number of observations for that sequence position.

Fig. 2. Correlation between loads of pollen and fluorescent dye particles on individual emasculated *Silene virginica* flowers.
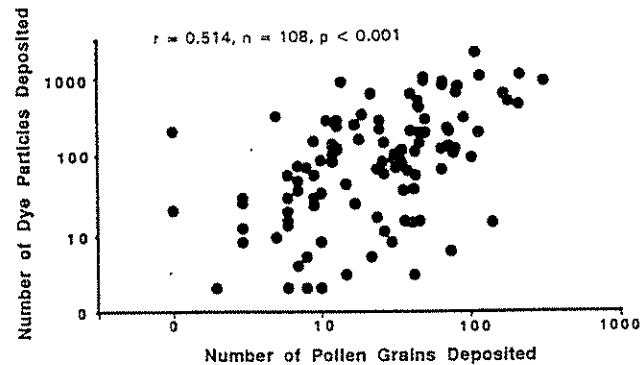

Table 1. Heterogeneity of slopes ANOVA (type III SS) of the effect of particle type and floral visitation sequence on adjusted amounts of pollen and dye deposited on *Silene virginica* flowers by Ruby-throated Hummingbirds.

Source	df	MS	F	P > F
Sequence	1	0.497	4.96	0.0270
Particle type	1	0.122	1.22	0.2702
Sequence × particle type	1	0.016	0.16	0.6911
Error	212	0.100		

Results

Adjusted amounts of dye and pollen deposited on stigmas decreased slightly during the floral visitation sequence by hummingbirds (Fig. 1). Using each visit as a replicate, the slopes of the adjusted amount of pollen and fluorescent dye on floral visitation are similar, with $b = -0.19$ ($F_{[1]} = 3.865$, $P = 0.052$) and $b = -0.14$ ($F_{[1]} = 1.513$, $P = 0.222$), respectively. There were also similar significant negative regressions for both fluorescent dye ($b = -0.021$, $F_{[1]} = 10.910$, $P = 0.008$) and pollen grain ($b = -0.029$, $F_{[1]} = 19.865$, $P = 0.001$) on floral visitation sequence based on the means across runs (Fig. 1). When positions 11 and 12 were removed from the analysis based on the means across runs, there was still a significant negative regression of number of pollen grains on floral visitation sequence ($b = -0.018$, $F = 5.83$, $P = 0.0422$), but the negative regression of number of fluorescent dye particles on floral visitation sequence was no longer significant ($b = 0.013$,

$F = 2.523$, $P = 0.1509$). There was no improvement of the r^2 when an exponential regression was performed using either each visit as the replicate or the means across runs.

There was no effect of particle type, nor was there an interaction between particle type and position in the floral visitation sequence on the adjusted amount of pollen or dye deposited on stigmas (Table 1). In this model there is a significant effect of sequence, probably reflecting the greater sample size by including both particle types in the analysis compared with the regressions based on the single particle types. These results indicate that the regressions of pollen and dye on sequence did not differ in either intercept (no significant effect of particle type) or slope (no significant interaction between particle type and sequence) and that deposition rates of both particles decreased across the floral visitation sequence.

Further evidence that dye and pollen moved similarly across the visitation sequence is demonstrated by the significant correlation between dye and pollen deposition on stigmas (Fig. 2). In 11 of the 12 runs the correlation between number of pollen and dye particles deposited on stigmas was either moderate or high (Spearman rank correlation $r \geq 0.57$ in 11 out of 12 correlations). In 106 of 108 flowers, both dye and pollen were found on stigmas. For the two discordant flowers, one of which was early in the floral visitation sequence and the other later, pollen was absent and dye was present. Looking at each of the three stigmas of a flower separately (instead of the sum of pollen and dye across the stigmas of a flower), the number of discordances increased to 63 of the 324 stigmas, with no bias towards either pollen present, dye absent (36 stigmas) or pollen absent, dye present (27 stigmas, $\chi^2_{[1]} = 1.285$, $P > 0.35$). Averaged

across all 12 runs, the mean transport distance for pollen and dye were 4.7 and 4.6 flowers, respectively (paired $t_{[1]} = 0.615$, $P = 0.55$). Median transport distances were identical for both particle types (4.7 flowers).

Discussion

Fluorescent dye is a good analog for pollen in *Silene virginica* based on all criteria in our study. The decline in deposition of dye and pollen across flowers in a floral visitation sequence was similar, and the mean and median flowers reached in the visitation sequence were nearly identical. These results indicate that fluorescent dye can be used to determine both relative amounts of pollen transferred among individuals and absolute distances of pollen traveled in *Silene virginica*.

Our results on *Silene virginica* do differ in some ways from previous studies that demonstrated dye to be a reasonable analog of pollen. In contrast with previous studies on *Brassica campestris* (Handel 1983), *Delphinium nelsonii* (Waser 1988), *Ipomopsis aggregata* (Waser and Price 1982), and *Erythronium grandiflorum* (Thomson et al. 1986), we observed that the presence of dye was a good indicator of the presence of pollen and that pollen and dye moved similar distances. Several explanations may account for this. First, hummingbird visits are often brief and birds do not probe the flower but rather stay in a single plane of orientation. Therefore, it is likely that if dye and pollen particles are segregated on even the smallest scale, then discordances between the presence and absence of dye and pollen will arise. However, unlike the previous experimental organisms (cited above) that have a single lobe and stigma, *Silene virginica* has three styles, allowing a larger area of the pollinator to be sampled than stigmas attached to a single style. Although there were comparable rates of discordances for single stigmas compared with the above-mentioned studies, the discordances were greatly reduced when averaged across the three stigmas. Dudash (1991) also observed that presence and absence scores of fluorescent dye particles were informative of pollen on stigmas of *Sabatia angularis* (Gentianaceae), which has large bilobed stigmas. Second, pollen and dye are deposited on the forehead of Ruby-throated hummingbirds visiting *Silene virginica*. In contrast, birds visiting *D. nelsonii* and *I. aggregata* (Waser 1988) have pollen and dye adhering to their slender bill, which often may not contact stigmas. Finally, all of our runs were considerably shorter than other studies. Thus, if the birds had cooperated by visiting longer sequences of flowers, we may have observed greater discordances. However, the runs of up to 12 flowers correspond to observations of the number of flowers the birds commonly visit in the field (C. Fenster and M. Dudash, personal observation). The overall lack of discordances and similar distance moved by dye and pollen indicate that we loaded the anthers with the correct amount of dye and the dye subsequently behaved similarly to pollen.

The slow decay of pollen and dye amount across the floral visitation sequence suggest that pollen carry-over may be extensive in *Silene virginica*. Similar findings were reported by Waser and Price (1982) in their study of pollen carry-over in *I. aggregata*. In contrast, a greater rate of decrease was observed in bumblebee-pollinated *E. grandiflorum* (Thomson et al. 1986), ant-pollinated *Scleranthus perennis* (Svensson 1985, 1986), and *Stellaria pubera* pollinated by bees, flies, and small bees (Campbell 1985). A comparison of pollen and dye transfer on bee- and hummingbird-pollinated *D. nelsonii* (Waser 1988) directly demonstrated a greater rate of decrease of dye across the floral visitation sequence for bees. These results indicate that pollen dispersal is likely to be greater with nongrooming vectors.

The close correspondence between dye and pollen movement observed here and in previous studies demonstrate the utility of using fluorescent powder as an analog for pollen. Further studies with other taxa, both plant and pollinator, should be conducted to further explore the adequacy of this technique.

Acknowledgements

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