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POLLEN VIABILITY, VIGOR, AND COMPETITIVE ABILITY IN *ERYTHRONIUM GRANDIFLORUM* (LILIACEAE)[1]

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To determine how the capability of pollen is affected by lengthy exposure to field conditions, we obtained pollen samples (from *Erythronium grandiflorum*) that were fresh (from newly dehisced anthers) or aged, from 6 to 24 hr. Without pretreating the collected grains, we compared their abilities 1) to retain viability, as indicated by the Heslop-Harrison fluorochromatic reaction (FCR) test; 2) to retain germinability, as indicated by pollen-tube growth in vitro on Brewbaker-Kwack medium; 3) to sire seeds when used as a pollen donor on recipient flowers in the field; and 4) to sire seeds competitively when mixed with another donor pollen in competitive pollinations. FCR scores declined drastically with pollen age. Germinability typically declined with age, but less drastically; the correlation between FCR and germinability ranged from strong to nil in different trials. The sharp decline in FCR was probably due to nonlethal, reversible desiccation. Seed-siring success, both in competitive and noncompetitive trials, bore no relation to pollen age or to germinability. Any loss in seed-siring ability in *E. grandiflorum* pollen over 24 hr is apparently overwhelmed by other sources of variation. The lack of correlation between seed-siring ability and FCR or germinability scores means that those tests should not be used to estimate overall pollen competence in this species. They should be so used in other species only if experiments have demonstrated their greater reliability.

Pollen grains are small, delicate organisms. Their ability to carry out their functions may be compromised by environmental stresses, with various implications for ecological and evolutionary questions. Assessing the general importance of pollen viability is complicated, however, by definitional and technical confusion. How do we measure pollen viability? Do different techniques measure the same thing? If not, which techniques are most appropriate for which current questions in reproductive biology?

Pollen viability, sensu lato, has been treated differently by different groups of botanists. Systematists, when concerned, for example, with assessing the constitution of a hybrid, have traditionally assessed "pollen viability" by stains that differentiate pollen with cytoplasm from empty, therefore inviable, grains (Alexander, 1980). Physiologists have tended instead to assess viability as the ability of filled grains to retain their metabolic functions in the face of environmental stresses such as desiccation. This tradition uses metabolic indications, such as the fluorochromatic reaction (FCR) test (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison, Heslop-Harrison, and Shivanna, 1984), which assays the ability of a cell's membranes to function normally, by concentrating fluorescein diacetate. The test simultaneously assays the presence of functional esterases, which cleave the compound to detectable fluorescein. Recently, Shivanna and colleagues, noting that there is sometimes a poor correspondence between FCR scores and measures of actual pollen performance (e.g., the speed of germination, the vigor of tube growth in artificial medium or cultured styles, and seed-siring ability) have proposed a distinction between viability and vigor. They restrict the former term to describe a grain's eventual ability to germinate, and argue that the FCR test is a reliable assay (Shivanna, Linskens, and Cresti, 1991a). They have shown in several taxa, however, that stresses of temperature and humidity have marked effects on germination time and tube growth, without any decline in FCR viability (Kumar, Chaudhury, and Shivanna, 1988; Shivanna and Cresti, 1989; Shivanna, Linskens, and Cresti, 1991a). They use the term vigor for these latter potentialities, stressing that vigor is more sensitive to the environment than is viability. There is a direct analogy with vigor and viability in stored seed (Priestly, 1986): old seeds may often be viable, but produce weak seedlings.

A third group, population biologists and evolutionary ecologists, are primarily concerned with the role of pollen as it affects the genetic structure of populations and the operation of mating systems (see, for example, papers in Wyatt, 1992). Their experiments frequently compare the effects of different pollination treatments: supplemented vs. control, self vs. outcross, one outcross donor vs. another, single vs. multiple donors, etc. Arguably, scientists concerned with genetic influences on pollen performance should be equally concerned with the environmental effects that are studied by the physiologists. It is unusual for pollen viability or vigor to be mentioned specifically in papers of this sort, however, and rare for it to be measured. Instead, most experiments implicitly assume pollen to be sufficiently long-lived and robust that environmental

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deterioration during its collection and application can be ignored. For some species this is doubtless true, but this assumption is dangerous in others.

There are two dangers. First, results may be biased if the pollen from one donor is older than that of another donor. In that case, effects actually due to age will spuriously appear as effects of the donors. Second, although most experimenters will doubtless prevent such obvious biases by harvesting pollen from all donors at the same time, and treating it identically, a series of hand pollinations takes time. If all of the pollen is losing vigor during the experiment—and some grasses, for example, have half-lives for FCR viability of 30 min or less (Shivanna and Johri, 1985)—biased results should not arise, but the increased variability of replicate pollinations within donors will make true donor effects harder to detect.

In addition to influencing experimental results, pollen viability and vigor may constitute important constraints on the selection of floral phenotypes. In theoretical models, Harder and Thomson (1989) showed that selection for greater pollen donation should favor gradual presentation of pollen when pollinators are abundant, but simultaneous presentation when visits are rare. Thomson and Thomson (1992) added the additional constraint of a limited viability period to those models, and showed that gradual presentation is then favored by selection even if visitation is infrequent. Thomson and Thomson also used their model to calculate that in *Erythronium grandiflorum* (for which data were available on pollen presentation, donation efficiency, visitation rate, and FCR viability), the majority of pollen grains delivered to stigmas would arrive in an inviable state. However, the accuracy of these conclusions for *Erythronium grandiflorum* depends on a close concordance between FCR scores and seed-siring ability.

The present investigation attempted to test that concordance by, first, determining what effect aging, under field conditions, has on 1) FCR scores, 2) germination in vitro, and 3) ability to sire seeds in competitive pollinations, i.e., when a mixture of aged pollen from one donor and fresh pollen from another donor is applied to a stigma. Such experiments have not been done before. From the outset, we realized that we would need careful experimental designs because it has been shown many times in various species that certain outcross pollen donors are favored over others in competitive pollinations (e.g., Marshall and Ellstrand, 1986; Bertin, 1990; and other references therein). Such differences, which are presumably genetic in origin, could obscure the environmental effects that we sought. Therefore, we manipulated pollen age *within* donor-recipient pairs to hold the genetics constant. With this precaution, on the basis of preliminary FCR data (Thomson and Thomson, 1992), we expected that it would be simple to demonstrate strong effects of aging. In fact, our results are generally negative, and they cast doubt on the use of either FCR tests or in vitro growth assays to assess siring performance in competitive situations.

MATERIALS AND METHODS

Species and site—*Erythronium grandiflorum* Pursh (Liliaceae) is a long-lived perennial lily, extremely abundant at our principal study site (subalpine meadow) at Irwin, Colorado (107°06′00″W, 38°52′35″N, elevation 3,140 m). Flowering plants usually produce one or two (occasionally more) flowers within a few days of snowmelt. The anthers, which are typically 15–20 mm long and together contain ca. 10^5 grains, open gradually by splitting along longitudinal sutures. This "unzipping" process takes several hours in sunny weather, longer in wet (Thomson and Thomson, 1992); thus, grains at the proximal end of a fully dehisced anther may have had a considerably longer exposure to the elements than those at the distal end. Furthermore, the three anthers from the outer whorl generally complete all of their pollen presentation before any dehiscence begins in the inner whorl of three (for details and data, see Thomson and Thomson, 1992). In fair weather, the anther tissue becomes perceptibly dry and brittle within 1 d after dehiscence is complete. We used this knowledge of the pollen presentation schedule to obtain pollen with different exposure histories, but from the same plant. We used slightly different protocols in 1991 and 1992.

Assessment of viability and vigor by traditional methods—First, we examined the time courses of viability (as FCR scores) and vigor (as germination in vitro) in cut flowers held under laboratory conditions at the Rocky Mountain Biological Laboratory.

Three separate trials were undertaken during the 1992 field season. Two sites served as sources of flowers—Washington Gulch (WG) and Paradise Basin (PB). These meadows were several km northeast of Irwin, and slightly higher (WG: 3,350 m, PB: 3,440 m). Flowers for each trial were collected as buds from the field on the day preceding the laboratory trials by cutting flowering stems at their base (early WG collected 28 June; late WG collected 30 June; PB collected 4 July). Following collection, the cut ends were trimmed under water and the inflorescences were placed in a vase and stored overnight outdoors at the Rocky Mountain Biological Laboratory. On the morning of each viability trial, the cut flowers were brought indoors and placed in individual vases for the remainder of the trial. Pollen was collected from anthers after exposure periods of 0, 1, 2, 4, 8, and 24 hr. To assign pollen to these age groups, anther dehiscence was monitored throughout the morning, and entomologist's "minuten" pins were used to mark the location of freshly dehisced pollen. Individual anthers were assigned to a single age group and sampled only once for both the germination and FCR tests of viability. All anthers for a given age group were sampled at the same time. By staggering the starting time for each age group throughout the morning, the time separating the subsequent viability determination for the age groups of 0, 1, 2, 4, and 8 hr was minimized. Flowers were stored outdoors overnight for the 24-hr age group and scored on the second day. The number of anthers sampled for each age group was four for the early WG trial, six for the late WG trial, and five for the PB trial.

For each age group, pollen was taken from the marked location using an insect pin and both placed in a drop of FCR solution and spread on germination medium on a microscope slide. Samples averaged 179 pollen grains for the FCR tests and 218 pollen grains for the germination

scores over the course of the trials. The FCR solution was made fresh for each age group by diluting two drops of a stock solution (2 mg fluorescein diacetate/1 ml acetone, stored in a freezer) in 1 ml of a 12.5% sucrose solution. The pollen was incubated for 5 min in the reagent drop before scoring the FCR reaction. Each sample was scored twice and the values were averaged, once using UV only and once using UV in combination with low visible light. Brightly fluorescing grains were scored as viable, while dimly fluorescing and dark grains were scored as inviable.

The germination medium was made with 8% gelatin and 10% sucrose (by weight) in a standard Brewbaker's solution (Kearns and Inouye, 1993). The sampled pollen was spread over the medium and the slides were incubated at room temperature in a humid chamber for 2 hr prior to scoring germination. Grains were examined using a dissecting scope and scored as germinated if the grain possessed a pollen tube that was at least two grain diameters in length. Each sample was scored twice and the values were averaged.

Second, we assessed viability and germinability in a small sample of intact flowers in the field. On 17 and 18 June 1992, we located large-anthered flowers with anthers in the process of unzipping. At 0900 hours, we inserted a color-coded pin into the anther, at the cleft where it was splitting. We added differently colored pins at 1300 and 1700 hours. At 1730 hours, we plucked the anthers, brought them to the lab, and removed pollen from the vicinity of each of the three pins. The three lots of pollen had therefore been exposed for approximately 10.5, 4.5, and 0.5 hr. Part of each lot was scored by FCR, and part was placed on germination medium, as above. The germination tests were scored, as the fraction of grains that produced tubes, after approximately 16 hr.

Third, we sampled pollen in a way designed to indicate the viability and germinability of the pollen that would be delivered to stigmas by bees at various times of day. We prepared an "artificial bee" consisting of a 2 × 3-cm piece of velveteen fabric glued to the end of a stick. At 0930, 1230, 1530, and 1830 hours on 17 and 18 June 1992, an observer walked a fixed transect of about 100 m, brushing the velveteen against the anthers of 200 flowers. Flowers that were too old for bees to visit (i.e., faded, wilted) were passed by. Immediately after sampling, we scraped the pollen from the velveteen with a microscope slide, and performed FCR and germination tests as before. The germination tests for 18 June were spoiled by contamination. Fresh, clean velveteen was used for each sample.

Assessment of seed-siring ability—In June 1991, we located large, multiple-flowered plants of *E. grandiflorum* at Irwin and marked them with surveyor's pin flags. We removed approximately 1 cm of tip from one of the two large leaves on 400 plants and subjected the tissue samples to starch-gel electrophoresis, staining for the enzyme malate dehydrogenase (MDH). This population has two alleles at nearly equal frequency at one MDH locus that is easy to score (even in seeds). Homozygous plants served as both pollen donors and recipients. We also used heterozygous plants as recipients in experiments where we were not concerned with identifying sires.

When buds were large, yellow, and pendant—within a day of opening—we gently opened the tepals and slid a 2.5-cm length of plastic drinking straw, oval in cross section, over the gynoecium. A friction fit at the ovary keeps the straw in place. This arrangement protects the stigma from contaminating pollen but does not require emasculation of the flower. Therefore, its pollen remains available for making other crosses.

We performed hand pollinations when all six anthers of the recipients had dehisced. All pollen came from homozygous plants. In *mixed* pollinations, pollens from two opposite homozygotes (one fresh, one aged) were mixed and applied to the stigma of another homozygote. Subsequent recovery and electrophoresis of the seeds from the recipient identified which donor sired the seeds; thus, these trials indicate the relative competitive abilities of two pollen sources with different histories of exposure. In *pure* pollinations, pollen from a single homozygous donor was used to pollinate a heterozygote. Subsequent analysis of seed set assessed that pollen's ability to sire seeds in a noncompetitive environment; this information could then be used as a correction factor in analyzing the mixed pollinations.

In practice, each day we located homozygous donors that had pollen of two ages available. We used the following criteria to judge pollen age. *Fresh* pollen was taken only from anthers that were in mid-dehiscence, and was extracted, as much as possible, from the cleft where the suture had just given way. Usually, these anthers were from the second whorl. *Aged* pollen was usually taken from the first whorl of the same flower, from anthers that had completely dehisced but had not yet become brittle. In perhaps 25% of the cases, single flowers did not present pollen of both ages, so we used anthers in the appropriate stages from different flowers on the donor plant. Our observations of the usual course of pollen presentation (Thomson and Thomson, 1992) suggest that our aged pollen had usually been exposed to the atmosphere for between 3 and 15 hr, with 6–8 hr being most frequent. Although it would of course be preferable to know the precise ages and FCR viabilities of the pollens used in each trial, in 1991 we were unable to devise a procedure that was compatible with the other logistical constraints on performing the pollinations. It is certain, at least, that there was a *difference* in age of the pollens used in mixtures.

Having located a pair of suitable donors, we used their pollen on several recipients, as indicated in Fig. 1a. This design tested pollen of both ages from each donor in each pairing. To apply equal amounts of pollen, we plucked the anthers with forceps and placed them gently in labeled compartments of a plastic microtiter tray. We then picked up a clump of several hundred grains of the two desired donors, one on each point of a pair of fine forceps. We visually compared the sizes of the two pollen loads, adjusted them to apparent equality, and applied the pollen by bringing the points together and dabbing the stigma while turning the forceps to mix the load and distribute it evenly.

The number of flowers pollinated varied somewhat because we occasionally ran out of pollen or did not have enough homozygous recipients available, but we tried to pollinate six recipients per donor pair. Only one flower was pollinated on a donor plant; the other one or two flowers were removed.

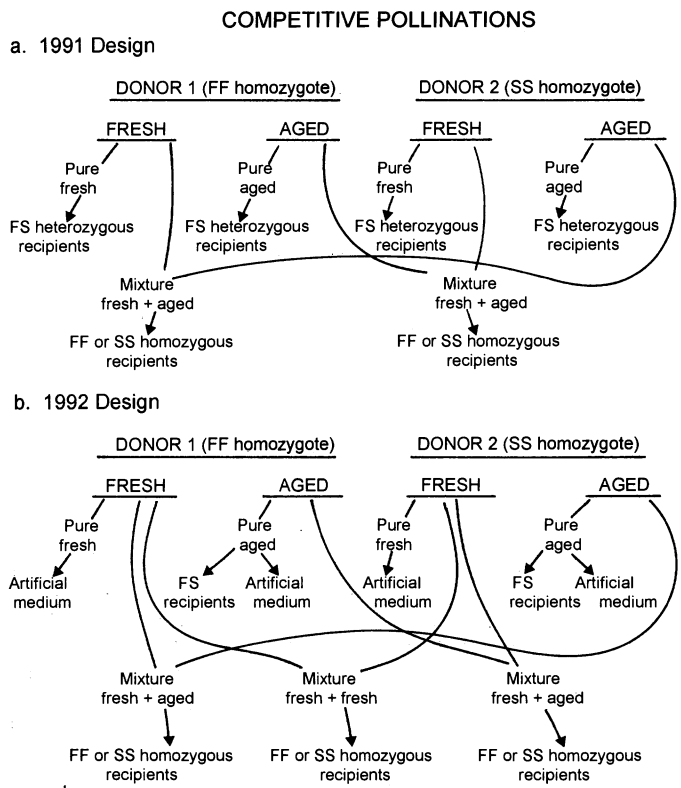

Fig. 1. Experimental design for the competitive pollinations and the ancillary tests of noncompetitive performance and of germinability. Fast and slow alleles used as markers are indicated by F and S. The arrows indicate pollen application. a. 1991 design. b. 1992 design, which differs by adding germinability tests and adding a mixed-donor treatment in which both donors were fresh.

Obviously, the forceps-point technique will not put exactly 50:50 mixtures on all stigmas. Variation from 50:50 should not bias the results, but it will tend to raise error variance, making treatment effects harder to discern. However, the technique has been successfully used in *Erythronium grandiflorum* to discriminate the performance of mixed outcross pollens from different distances (Rigney et al., 1993), so it is surely capable of detecting treatment effects, even fairly subtle ones.

At flowering, we measured the width of the larger of the two leaves to provide a surrogate measure of plant size (Wolfe, 1983). When the fruits were brown, dry, and beginning to dehisce, we harvested them, measured the lengths of the fruits, and then examined their contents. We counted the numbers of well-developed seeds, of partially developed but aborted seeds, and of undeveloped ovules. We interpret the last two categories as fertilized, then aborted, and unfertilized, respectively. The developed seeds from each fruit were weighed as a group. For the mixed pollinations, 20 seeds from each fruit were selected without regard to size or position in the ovary, and their MDH genotypes determined to identify which pollen parent had sired them. When fewer than 20 seeds were produced by a fruit, all were genotyped.

Because the 1991 data were equivocal, we repeated the experiment in 1992 with some changes in design. First, we determined the age of the old pollen more exactly, by finding potential donors with partly dehisced anthers and marking the position of the cleft with a minuten pin, as above. By subsequently taking pollen from the vicinity of the marking pin, we were able to apply pollen of a more closely controlled age. We pollinated from 10 to 18 June, only on sunny days.

Second, we established two age treatments. In addition to some pairings with 6 hr (same day) aged pollen, we conducted other trials where the aged pollen was 24 hr old, to provide a more extreme contrast between fresh and aged.

Third, immediately after performing a set of pollinations, we placed some of each pollen on the artificial growth medium as above, and scored percent germination after 16 hr. In the field, the gelatin mixture softened to such a consistency that some grains sank; we scored only those that stayed on the surface, where they had the necessary access to oxygen.

Fourth, we added a treatment in which both donors of a competing pair were fresh. This treatment let us assess intrinsic differences in competitive ability, without any age differential, allowing us to extract a cleaner age effect (Fig. 1b).

Fifth, on 19 and 21 June, we scored whether a fruit had been initiated or whether the ovary had suffered early abortion.

We initially genotyped 320 plants, but when many of those were lost to frost, we added 200 more. To reduce further losses to frost, on cold nights we protected the flowers with large Styrofoam drinking cups. By skewering the side of the cup with the shaft of a tall pin flag, we could insert the flag next to a plant and slide the cup up or down to expose or protect the plant as desired. We also used the cups to protect the flowers when occasional showers threatened. We harvested and genotyped seeds as in 1991, but we did not measure leaf widths or fruit lengths in 1992.

RESULTS

Viability vs. vigor comparisons—For cut flowers in lab conditions, patterns of FCR scores were consistent across the three samples, but germination in vitro was not (Fig. 2). The second sample from WG showed extremely low germination in the younger age classes (Fig. 2d, circles), which we are at a loss to explain. Only in the first sample from WG were viability and germinability significantly rank-correlated ($r_s = 0.86$, $N = 23$, $P < 0.0001$; for the second WG sample, $r_s = -0.09$, $N = 30$, $P = 0.63$, and for the PB sample, $r_s = 0.18$, $N = 30$, $P = 0.33$). Nevertheless, the correlation was so strong in the first sample that pooling the three samples still gives a significant, although weak, correlation ($r_s = 0.24$, $N = 83$, $P = 0.03$). In these conditions, FCR scores decline more rapidly than germination. Interestingly, overall germination scores tended to drop for 1-hr pollen, then rise again at 2–4 hr. This pattern still holds when the possibly anomalous second sample is removed from the data set.

For intact flowers under sunny, warm field conditions, FCR scores decline more rapidly than under lab conditions (Fig. 3; compare to Fig. 2). Germination is, again, less affected by exposure time. Here, viability and germinability are significantly correlated ($r_s = 0.53$, $N = 16$,

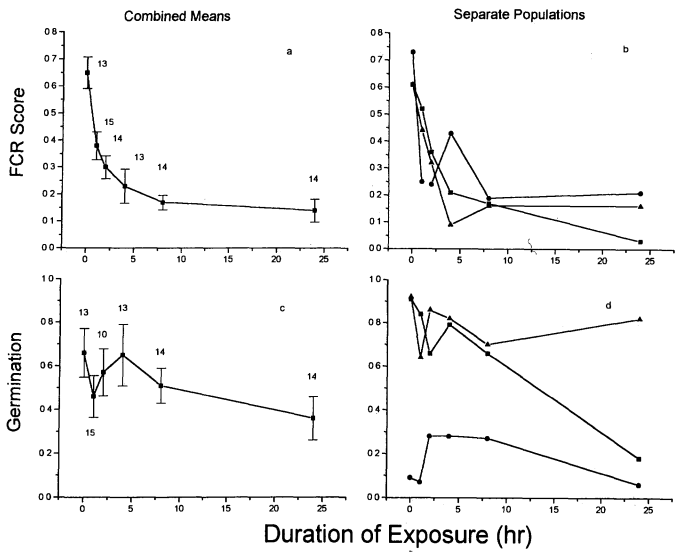

Fig. 2. Pollen capabilities as a function of pollen age, using cut flowers in lab conditions. a, b. Time course of responsiveness to the fluorochromatic reaction (FCR) test. In panel b, the different symbols refer to separate sets of tests using material from WG (early flowers, squares; late, circles) and PB (triangles). Sample sizes range from three to six flowers per datum; error bars are omitted for clarity. Panel a shows means and standard errors for the overall data pooled; the number of flowers sampled is shown next to each symbol. c, d. Time course of germinability of pollen from the same flowers on Brewbaker-Kwack medium in gelatin; symbols as in panels a, b. Note the heterogeneity among samples.

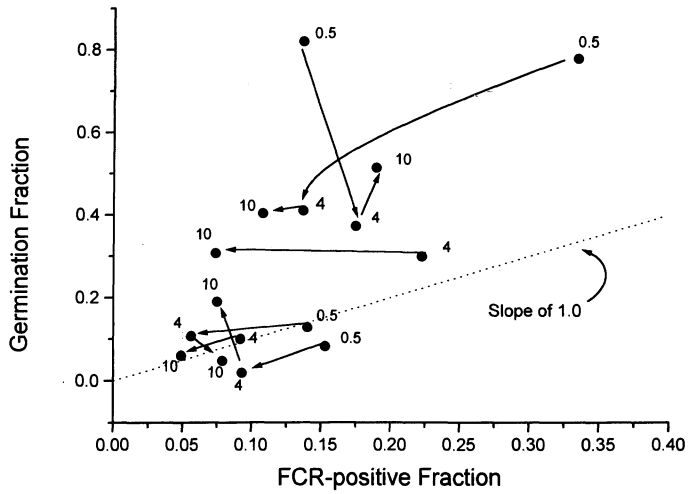

Fig. 3. Pollen capabilities as a function of pollen age for six intact flowers in sunny field conditions. Lines with arrows connect, in temporal sequence, points taken from single anthers. Pollen age in hr is indicated by the numerals 10, 4 and 0.5. Two of the flowers had data recorded for only two of the three time periods. If FCR score were a good predictor of germinability, the points should fall in a narrow band; if FCR and germinability were equivalent measures, the band should have a slope of 1.0 (dotted line). Sample sizes (grains tested per data point) range from 146 to 341 (mean = 253.9, SD = 59.1) for FCR scores, and from 28 to 309 (mean = 153.9, SD = 80.8) for germination.

$P < 0.01$), but there is considerable scatter (Fig. 3), and the progressive loss of viability through time is not consistent across plants (see arrows in Fig. 3).

A correlation of this magnitude indicates little more than that both viability and germinability decline with time; their declines are not tightly linked, and the value of one cannot accurately predict the other. Pollen collected by the "artificial bee" declined monotonically in FCR score through the day, but germination in vitro remained constant during this period (Fig. 4). That FCR scores declined more rapidly on 18 June than on 17 June may be attributable to the higher maximum temperature on 18 June (21.1 vs. 17.7 C). In summary, FCR scores are clearly affected by pollen exposure time, but the relationship between those scores and pollen vigor (as germinability) is weak, especially for mass collections of pollen exposed to field conditions. Such mass collections are exactly what pollinating bees would deliver. On this basis, FCR scores would not be expected to provide a suitable index of seed-siring ability in nature.

Seed-siring ability—1991 data—Although we performed 90 mixed pollinations, and tested all of the donors on at least one heterozygote each, a number of these flowers were lost to deer, elk, or rodents. Some others aborted spontaneously, some developing fruits were attacked by insects, and the genotypes of a few plants were discovered to be erroneous when their progeny were genotyped. Affected fruits were removed from the analyses as appropriate; remaining sample sizes are indicated below for each analysis.

To test for overall effects of pollen age-class on seed set, we performed analyses of covariance on various response variables. There were three treatments (pure aged pollen, pure fresh pollen, and mixed aged + fresh): the first two involved heterozygous recipients, and the third, homozygotes. Leaf width was included as a covariate indicating plant size. The response variables were: the number of seeds, the length of the fruit, the fraction of all

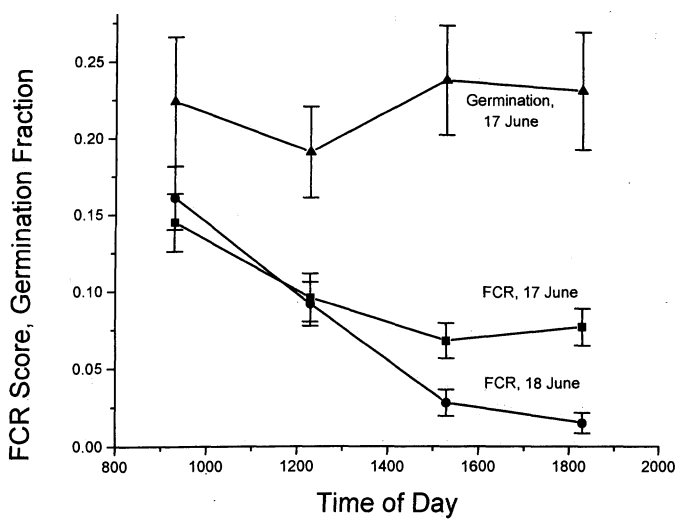

Fig. 4. Pollen viability (as FCR score) and germinability (on Brewbaker-Kwack gelatin) of mass collections of pollen from 200 flowers, in the field, at different times of day. FCR tests were performed on 17 and 18 June, germinability only on 17 June. Bars show standard deviations for the fractional scores (P) based on the binomial distribution, i.e., $\pm\sqrt{P(1-P)/N}$ where N is the number of grains scored, which ranges from 351 to 492 for FCR and from 98 to 143 for germination.

TABLE 1. Means (standard deviations) and *P* values from analyses of covariance for six response variables concerning development of *Erythronium grandiflorum* fruits after pollination with aged pollen, fresh pollen, or an equal mixture of the two types; 1991 data. Under "*P* values," the entries for number of aborted ovules and number of unfertilized ovules actually refer to analyses of the proportions of ovules falling into each of these categories. These proportions were angularly transformed, as was fractional seed set.

Response variable	Means: Pollination type — Fresh	Aged	Mixed	*P* values: Effects — Pollen type	Leaf width	Pollen × leaf width
Number of seeds	41.8 (22.5)	44.1 (16.2)	40.1 (20.3)	0.80	0.18	0.86
Number of aborted ovules	18.6 (16.3)	13.7 (9.6)	13.0 (11.1)	0.49	0.50	0.34
Number of unfertilized ovules	15.6 (12.8)	20.2 (17.6)	19.6 (13.6)	0.44	0.81	0.33
Fractional seed set (seed/ovules)	0.52 (0.25)	0.58 (0.20)	0.54 (0.23)	0.53	0.62	0.61
Mean seed weight	0.0075 (0.0013)	0.0077 (0.0016)	0.0074 (0.0014)	0.28	0.002**	0.24
Fruit length (mm)	39.54 (7.81)	41.64 (6.92)	37.43 (7.57)	0.68	0.18	0.86

ovules that developed into seeds, the fraction of ovules that aborted, the fraction of ovules that remained unfertilized, and the mean mass of a seed. We used angular transformation for the fractional variables. There were no significant treatment effects, and the only significant effects of the leaf width covariate were on fruit length and mean seed mass, which were both positively correlated with leaf size (Table 1). There is a hint in the data that fresh pollen might fertilize more ovules than aged pollen, but that these ovules are more likely to abort, as if seed number were regulated by maternal resources; this tendency is not significant, however.

The analyses above included only those plants that set fruits, so we separately examined whether pollen age affected fruit set. It did not (fresh pollen gave 35 fruits, 18 abortions; aged pollen, 35 fruits, 15 abortions).

To reduce the error variance contributed by interplant variation in intrinsic pollen vigor (independent of age), we also compared the performance of pure fresh vs. pure aged pollen *within donors,* using Wilcoxon signed-ranks tests. Fruits from both of these pollinations were recovered for 19 donors. Neither seed number, mean seed mass, nor percentage seed set showed significant treatment effects. (T_s values were 91.5, 93, and 78, respectively, $N = 19$.) We repeated the first and third of these analyses, including aborted fruits as having zero seeds, and zero percentage seed set: still no significance ($T_s = 231$, 227; $N = 30$).

Although these results suggested that fresh and aged pollen performed equally well, it remained possible that fresh pollen might still have an advantage in the competitive mixed pollinations. For example, fresh pollen might germinate more quickly. Several analyses investigated this possibility. First, we categorized mixed-treatment fruits according to whether the fresh or the aged pollen sired more seeds. Of the 64 fruits set, five were even ties, and seven were unusable because genotypes had been determined incorrectly. Of the remaining fruits, young pollen won in 30 and old pollen in 22, a nonsignificant departure from equality (goodness-of-fit $\chi^2 = 1.23$).

In our final attempt to discover an effect of pollen age, we asked whether, within a donor, fresh pollen did relatively better against aged pollen in competitive pollinations than it did in pure pollinations. For each donor we calculated the index PSFPURE, or the proportional success of fresh pollen in pure pollinations on heterozygous recipients, defined as $F/(F + A)$, where F = the mean number of seeds sired per fruit by fresh pollen of this donor, divided by the number of ovules, and A = the equivalent fraction for aged pollen. A second index, PSFCOMP, measured the proportional success of fresh pollen in mixed (competitive) pollinations on homozygous recipients, defined for each donor as the mean (across recipients) of the number of genotyped seeds sired by the young pollen, divided by the total number of seeds genotyped. Finally, the ratio PSFCOMP/PSFPURE gives a third index, COMPADVN, of the extent to which competition increases the relative advantage of fresh pollen: values near 1.0 indicate no additional effect of competition. Higher or lower values indicate that fresh or aged pollen, respectively, does relatively better in competition. Distributions of all three indices are given in Table 2. Only for PSFCOMP is there even a suggestion of a deviation from the null expectation, which is insignificant by a sign test. There is no evidence for an effect of competition, although the final index could be calculated for only a small set of donors.

TABLE 2. Stem-and-leaf representations of the distributions of the three indices of pollen performance for the 1991 seed-siring experiment. (The stem-and-leaf display [Tukey, 1977; Sokal and Rohlf, 1981, pp. 28–29] functions as a horizontal frequency histogram while also presenting the actual data values. The first five values of PSFPURE, for example, are 0.006, 0.19, 0.20, 0.24, and 0.25.) The indices are defined in the text. For PFSPURE and PFSCOMP, the null expectation is 0.5; for COMPADVN, it is 1.0. The horizontal lines indicate these null expectations. For none of the indices is there a significant departure from expectation (sign test on values above expectation vs. values below).

PSFPURE		PSFCOMP		COMPADVN	
0.0	6	0.0	0	$\underline{0}$	$\underline{0}$ $\underline{2}$ $\underline{8}$ $\underline{8}$ $\underline{9}$
0.1	9	0.1	0 8	$\overline{1}$	$\underline{4}$ $\underline{5}$
0.2	0 4 5	0.2		2	1 3
0.3	8	0.3	7 8	3	1 5
$\underline{0.4}$	$\underline{1}$ $\underline{2}$ $\underline{3}$ $\underline{7}$	$\underline{0.4}$	$\underline{4}$ $\underline{6}$ $\underline{7}$ $\underline{8}$		
$\overline{0.5}$	$\overline{1}$ $\overline{3}$ $\overline{6}$ $\overline{7}$	$\overline{0.5}$	$\overline{0}$ $\overline{4}$ $\overline{4}$ $\overline{5}$ 8 8 8 9		
0.6	3 5 7 9	0.6	2 6 6 6		
0.7	1	0.7	4		
		0.8	6		
		0.9	0 2		
		1.0	0		

Seed-siring ability—1992 data—As for 1991, our analysis began with an examination of the performance of

pure pollen loads on heterozygous recipients, but because we did not perform pure pollinations with fresh pollen in this year, our only treatment comparison is between 6-hr aged pollen and 24-hr aged pollen. We sought treatment effects on two response variables measured on fruits: proportional seed set (i.e., seeds/ovules, angularly transformed), and the total mass of all seeds. There were no significant treatment effects, whether or not the estimated vigor of the pollen (i.e., tubes/grain in artificial medium) was added as a covariate, nor did vigor itself have an effect on either response variable (Table 3).

We also asked whether pollen age affected fruit set. There was no effect on early fruit initiation vs. abortion (Table 4a), or on the probability of harvesting a fruit (Table 4b). However, some fruits that we scored as "initiated" on 19 or 21 June were not recovered. These may have been lost to herbivores or trampling, or they may have aborted. If all such lost fruits are scored as abortions, then 6-hr pollen appears to produce significantly more abortion than 24-hr pollen, a surprising result (Table 4c). Most of the excess in abortions with 6-hr pollen, however, occurred in pollinations done on the last 2 d, 15 and 18 June, all of which involved 6-hr delays. If we eliminated those days from the sample, the significant interaction disappears (Table 4d). The late pollinations, especially on 18 June, may have included recipients that had aged faster than expected, due to the rapidly warming weather. In any event, given the additional uncertainty about the true fates of unrecovered fruits, we conclude that 6- and 24-hr pollens do not differ in their ability to induce fruit set.

Pollen germinability vs. seed-siring ability—For 28 fruits on heterozygote recipients, we obtained data on the germination of the donor's pollen on artificial medium. Aging significantly reduced germination. Wilcoxon signed-ranks tests, which compare pollen ages within donors, showed that fresh pollen outperformed both 6-hr ($N = 10$, two-tailed $P = 0.023$) and 24-hr pollen ($N = 10$, $P = 0.017$). There was, however, no correlation between germination percentage and percent seed set (arcsine square-root transformed: Pearson $r = 0.26$, $P = 0.17$). Although germination percentage was more closely related to the total seed mass ($r = 0.35$, $P = 0.07$), pollen germination on Brewbaker-Kwack medium is hardly a useful predictor of that pollen's performance at producing seeds in this species.

Competitive pollinations—1992 data—To consider the success of fresh vs. aged pollen in competitive pollinations, we first entered data on siring success into 2 × 2 contingency tables, one table for each pairing of donors. All the seeds gathered from a particular pairing of donors were classified by 1) which donor sired them and 2) whether they were derived from an even-age pollination in which both donors were fresh, or a mixed-age pollination with one fresh and one aged donor. This involved pooling seeds from one to three fruits. The seed counts were thus cast into 2 × 2 contingency tables: ([donor A vs. donor B] × [fresh-fresh vs. fresh-aged pollination]). A significant interaction (contingency χ^2) would indicate that the aged pollen had a significant disadvantage (or advantage), relative to its performance, when fresh, against the same competitor. For 6-hr delays, there were eight pairings, of which two showed significant interactions; in both cases, the aged pollen outperformed the fresh. For 24-hr delays, three of 15 pairings were significant, with fresh pollen being favored twice, aged pollen once. The significant pairings suggest that batches of pollen can occasionally be disabled, but age does not seem to be a prominent cause.

Another approach to the same data involved calculating the *relative performances* of donors. For each mixed-age pairing, the fresh donor was chosen as the focal donor. We then calculated the focal donor's RELPERF(EVEN) as the fraction of all seeds that were sired by it in the competitive pollinations when both donors were fresh. We defined RELPERF(MIXED) as the fraction of all seeds sired by the focal donor in the pollinations that pitted fresh donors vs. aged ones. Thus these indices represent, respectively, the intrinsic competitive ability of the focal donor against its opponent when age is not a factor, and its competitive ability when it has the advantage of youth. We quantified this *advantage* as (RELPERF[MIXED])/(RELPERF[EVEN]). Values less than one indicate that aging gives pollen a competitive edge, and values greater than one indicate an advantage to freshness.

For both 6-hr and 24-hr delays, the distributions of

TABLE 3. Analyses of variance and covariance to determine effects of pollen age (6 hr vs. 24 hr) on proportional seed set (angularly transformed) and the total mass of seeds sired. $N = 28$ fruits. The ANCOVAs include the germinability in vitro of the pollen as a covariate. No effects are significant, although the effect of germinability on seed mass approaches significance.

	Source of variation	F	P
a. Response variable = proportional seed set = ($\sqrt{\arcsin(\text{seeds/ovules})}$)			
ANOVA:	Pollen age	1.14	0.30
ANCOVA:	Pollen age	1.07	0.31
	Germinability	1.83	0.19
b. Response variable = total seed mass			
ANOVA:	Pollen age	0.95	0.34
ANCOVA:	Pollen age	0.90	0.35
	Germinability	3.50	0.07

TABLE 4. Comparison of the fruit-siring ability of 6-hr vs. 24-hr pollen, 1992 data. The four tables reflect data on fruiting failure at different stages and different assumptions about the fates of missing fruits (see text).

Pollen age	Aborted	Initiated fruit	Fisher's exact probability
a. Initiation of fruits, recorded shortly after flowering			
6 hr	13	22	0.603
24 hr	9	22	
b. Fruits found at late-summer harvest			
6 hr	13	14	0.178
24 hr	9	22	
c. Assuming all fruits not found were aborted			
6 hr	21	14	0.015
24 hr	9	22	
d. As in c, but discounting late pollinations			
6 hr	11	12	0.254
24 hr	9	22	

TABLE 5. Stem-and-leaf representations of the distributions of the two indices of pollen competitive performance for the 1992 seed-siring experiment. The indices are defined in the text. The null expectation, indicated by the horizontal line, is 1.0. Larger or smaller values indicated an advantage to younger or older pollen, respectively.

Advantage, 24 hr age difference		Advantage, 6 hr age difference	
4	3		
2	6		
1	4	1	5
1	2	1	2 2 3
1	0 1		
0	8 8 9 9		
0	6	0	6 7 7
0	4 5		
0	2 3	0	2

advantage are centered squarely around unity (Table 5), providing no evidence for a consistent effect of pollen aging.

***Pollen vigor and seed siring in competitive pollinations*—** Our final analysis sought a relationship between germination in vitro and success in competitive pollinations. For each mixed-pollination pairing where germination data were available, we calculated relative germination scores (RELGERM, analogous to RELPERF above) as (VIGOR[focal plant])/(VIGOR[focal plant] + VIGOR[nonfocal plant]), where VIGOR = the number of tubes/grain in the in vitro growth trials. We then compared the RELGERM index to the RELPERF index that we derived above to indicate competitive seed-siring ability. There were no significant Spearman correlations: for fresh-fresh pairings, $r_s = 0.01$, $N = 9$; for 6-hr delays, $r_s = -0.37$, $N = 10$; for 24-hr delays, $r_s = 0.01$, $N = 9$; and for all pairings pooled, $r_s = -0.04$, $N = 28$. Not surprisingly, in view of the previous results, pollen germinability does not predict pollen competitive ability.

DISCUSSION

***Pollen viability and the FCR test*—** We undertook this work because theory suggested that viability was an important, but poorly understood, influence in the function and evolution of flowers. The rapid decline in FCR scores with exposure time ("pollen age"), which we saw in both the lab and the field, confirms the small pilot study presented by Thomson and Thomson (1992) in their models of optimal pollen presentation schedules. Using appropriate parameter estimates for *Erythronium grandiflorum,* those models predicted that most of the pollen grains being delivered to glacier lily stigmas would be dead on arrival. That prediction was here confirmed by our "artificial bee" samples, in the sense that 90% or more of the movable pollen is likely to test FCR-negative. Unfortunately, the evolutionary predictions of those models were based on the additional assumption that "inviable" grains could not sire seeds, or at least would be much less capable of doing so than "viable" grains. Although this assumption is hardly a radical proposition, after two seasons of testing it with genetic markers, we must view it as completely unsupported by evidence in our species.

Indeed, it appears that none of the three stages of pollen function that we have been able to probe—FCR viability, pollen germination in vitro, and seed-siring ability under realistic conditions in intact pistils—is closely related to any of the others. Furthermore, although pollen age clearly affects FCR scores, its effects on germination are much weaker, and its effects on seed-siring are undetectable. Admittedly, our germination trials seemed highly erratic (e.g., Fig. 2b, d), declining with age in some trials but not in others. Although we scored many pollen grains, our sample sizes are small in terms of flowers. We do not know whether the apparent variation among sites and dates reflects true differences in pollen capability, or whether subtle experimental artifacts (deterioration of medium, fluctuation in lab temperature, etc.) may have played a role. Possibly tighter control of conditions could produce more homogeneous results. On the other hand, what we really need is a simple indicator that can be used as an adjunct to studies involving pollinations in the field. So, although the interesting possibility of high site/date variation in pollen germinability will require further work with much larger samples and impeccable technique, we can conclude that germination on artificial medium does not succeed as a quick-and-dirty assay for seed-siring ability in *Erythronium grandiflorum.*

In contrast, the FCR test was well behaved and consistent in our trials. Unfortunately, it appears to mean very little about pollen's true competence, at least in the way we used it. This is disturbing, given the FCR test's prominence and its frequent recommendation as the most useful general test of pollen metabolism (e.g., Heslop-Harrison, Heslop-Harrison, and Shivanna, 1984; Knox, Williams, and Dumas, 1986; Shivanna and Johri, 1985; Dafni, 1992; Kearns and Inouye, 1993). Our conclusions are reminiscent of those of Stanley and Linskens (1974, pp. 82–86), who summarized the inconsistency of earlier, tetrazolium-based tests of viability in predicting other measures of pollen performance. Does the FCR test, then, really represent much improvement over previous methods that have largely been discarded?

It may well; our use of the FCR was unorthodox, as can be understood by considering the processes that cause grains to fail. A closer reading of the literature clarifies matters somewhat. Although a grain can fail the FCR test by lacking esterase activity or by having a disrupted, permeable plasmalemma, it seems that the latter is a more likely cause over short periods of exposure. Furthermore, although the mechanisms are imprecisely understood, desiccation is thought to be the most likely disrupter of membranes (Heslop-Harrison, 1979). Thus in some circumstances, the FCR test amounts to a test of desiccation status, and it seems likely that this is the case in our trials; we saw greater FCR declines in sunny field conditions than in the lab, and a greater decline on a warmer day. Desiccation is, however, reversible. On a moist stigma, membranes can be reconstituted. Most thorough discussions of the FCR test stress the likelihood of false negative indications due to pollen drying, and suggest letting the grains equilibrate in a humid atmosphere before testing (Heslop-Harrison, Heslop-Harrison, and Shivanna, 1984; Shivanna and Johri, 1985; Knox, Williams, and Dumas, 1986). Thus, by testing pollen without such pretreatment, we have not been using the recommended protocols that

Shivanna, Linskens, and Cresti (1991a) presumably had in mind when they defended the FCR test as an operational definition of "viability." We expect that we would see lower rates of FCR decline, and better correlation of FCR scores and germination, if we used the rehydration procedure. (Note that although we have followed Shivanna in using "viability" and "FCR score" interchangeably, not all authors agree. Dafni [1992, p. 66], for example, states flatly that FCR is "*not* a test of viability" [emphasis in original]. When the term "pollen viability" is used to describe FCR scores, it should be understood that this is an operational definition that may not correspond to the commonly understood definition of viability.)

We chose to bypass the recommended pretreatment for FCR tests not because of ignorance but because we felt that the time required for desiccated grains to reconstitute their membranes might be important in pollen competition in field conditions. That is, we specifically wanted a test that would register even temporary, reversible loss of function, because such loss might be critical in competitive situations. Although desiccated grains might not be dead, and might be capable of siring seed in a noncompetitive environment, it seemed reasonable that fresher grains, arriving simultaneously, might outcompete them for ovules. This logic assumes only that recovery from desiccation takes some time. For example, Shivanna, Linskens, and Cresti (1991a, b) reported that stressed pollen grains of *Nicotiana tabacum,* although they effected fertilization, took 40 hr longer than controls to reach the ovary. These stressed grains did not germinate in vitro, but were FCR positive. Although these grains were stressed by high temperature at high humidity, not by desiccation, the result nevertheless demonstrates that sublethal stresses might affect competitive ability, as Young and Stanton (1990) have also demonstrated for nutrient stress.

These arguments suggest that FCR viability might plausibly be linked to competitive seed siring, even without being correlated to germination in vitro. We wanted to add FCR tests, both with and without a rehydration period, to the design in Fig. 1b, but could not manage the logistics. Nevertheless, FCR score declines so drastically with pollen age that we would surely have found an effect of pollen age on siring ability if FCR and siring ability were closely linked. We found no such link, and tentatively conclude that pollen rehydration on *Erythronium grandiflorum* stigmas is effective and relatively rapid. The hydration and germination of a load of grains is gradual in any case in this species, even when the grains are of equal age, because of variation in grain position on the stigma (Thomson, 1989). Such variation would mask or weaken desiccation effects.

An alternative explanation for the lack of an age effect and for the lack of concordance between FCR and siring ability might be that *fresh* pollen, despite its high FCR score, might be immature with regard to siring ability. False FCR-positive results with very young pollen are known in other systems (Knox, Williams, and Dumas, 1986). Thus it is remotely possible that age does affect siring ability, but not monotonically: our young pollen and our old pollen might have similar, lowered capabilities because they straddle some optimum intermediate age. Although possible, this mechanism is hardly parsimonious, especially given that 6-hr pollen performed the same as 0-hr and 24-hr.

Pollen vigor—In erecting a dichotomy between pollen viability and pollen vigor, Shivanna, Linskens, and Cresti (1991a, b) considered "vigor" to include various components of pollen performance, including time to germination, pollen tube growth rate, and even seed-siring ability. Here, we have considered seed-siring ability separately from "pollen vigor," and in the absence of estimates of germination schedules or tube growth rates, our only measure related to "vigor" is the fraction of grains that germinate on Brewbaker-Kwack medium after ca. 16 hr. Not only is this definition more restricted than that of Shivanna et al., it is close to their concept of FCR-based viability, because they expect a close correspondence between FCR score and germinability. As explained above, such a correlation may exist when grains are pretreated, but we preferred to examine FCR scores on "raw" material from the field. Although one may argue about terminology, our separation of FCR-based "viability" from germination-based "vigor" is warranted by the generally poor correlation between the two: they clearly are different aspects of pollen performance in *E. grandiflorum.*

Why is pollen vigor, as measured by germinability, unrelated to seed-siring success? First, of course, there may be intrinsic differences in pollen quality—the other aspects of vigor considered by Shivanna, Linskens, and Cresti (1991a)—that may be more important. Second, there may be maternal-paternal interactions. In *E. grandiflorum,* Cruzan (1989, 1990) has shown that pollen-tube attrition rates depend on such interactions, and Rigney et al. (1993; L. Rigney, *Evolution,* in press) documented that differential success of pollen donors is also influenced by the differential abortion of fertilized ovules. Generally, *E. grandiflorum* has a complex mating system that includes weak self-compatibility, cryptic self-incompatibility, and effects of the physical distance from donor to recipient. Although we avoided using self pollen or nearby donors, it is likely that mating-system incongruities remained. Despite our attempts to minimize them through experimental designs that made within-donor comparisons whenever possible, such incongruities as remain would tend to obscure any effects of pollen age.

A paper so dominated by negative results necessarily concludes with more recommendations than discoveries. We continue to believe that evolutionary ecologists should be concerned with pollen aging, but we were unable to show any consistent effects of pollen age in a system where we expected them to be strong. Our expectation was based on a misguided interpretation of FCR tests, and our study joins others in warning against incautious extrapolation from such tests. Unfortunately, we found that the "next test down the line," germination in vitro, also failed to predict seed-siring ability.

What an evolutionary ecologist wants is a quick test that can be applied to a batch of pollen, at the same time as the pollen is being used in an experiment, that would indicate the extent to which the pollen's potential for seed siring has been reduced by environmental stress. We have tried the two most obvious tests and been disappointed. In the absence of technical breakthroughs, we recommend that one should use a battery of tests, looking at as many

components of pollen performance as possible. In communicating the results of such studies, we would suggest that broad terms such as viability and vigor should be clearly and operationally defined, or avoided entirely and replaced by more specific terms. We also advocate more interchange between pollen physiologists, who for obvious reasons study pollen under highly controlled conditions, and evolutionary ecologists, who need to know how pollen physiology responds to the uncontrollable variability of field conditions.

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