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GENETIC VARIATION IN FLOWERING TIME INDUCES PHENOLOGICAL ASSORTATIVE MATING: QUANTITATIVE GENETIC METHODS APPLIED TO *BRASSICA RAPA*[1]

ARTHUR E. WEIS[2] AND TANYA M. KOSSLER

Department of Ecology and Evolutionary Biology, University of California–Irvine, Irvine, California 92687 USA

It has been argued from first principles that plants mate assortatively by flowering time. However, there have been very few studies of phenological assortative mating, perhaps because current methods to infer paternal phenotype are difficult to apply to natural populations. Two methods are presented to estimate the phenotypic correlation between mates—the quantitative genetic metric for assortative mating—for phenological traits. The first method uses individual flowering schedules to estimate mating probabilities for every potential pairing in a sample. These probabilities are then incorporated into a weighted phenotypic correlation between all potential mates and thus yield a *prospective* estimate based on mating opportunities. The correlation between mates can also be estimated *retrospectively* by comparing the regression of offspring phenotype over one parent, which is inflated by assortative mating, to the regression over mid-parent, which is not. In a demonstration experiment with *Brassica rapa*, the prospective correlation between flowering times (days from germination to anthesis) of pollen recipients and their potential donors was 0.58. The retrospective estimate of this correlation strongly agreed with the prospective estimate. The prospective method is easily employed in field studies that explore the effect of phenological assortative mating on selection response and population differentiation.

Key words: flowering phenology; functional gender; genetic correlations; natural selection; nonrandom mating; pollination; quantitative genetics.

It has been argued from first principles that plants will assortatively mate by flowering time (Breese, 1956; Wagner, 1976; Jain, 1979; Hartl and Clark, 1989; Lyons and Mulley, 1992; Lynch and Walsh, 1998; Hedrick, 2000; Kirkpatrick, 2000; Fox, 2003). Early bloomers will mate disproportionately with other early flowering plants, while late individuals will mate disproportionately with late bloomers. There is no doubt that phenotypic variation in phenology is the rule in natural populations (Augspurger, 1981; Rathke and Lacey, 1985), and many studies have confirmed a genetic cause to this variation (e.g., Pors and Werner, 1989; Fox, 1990; Dorn and Mitchell-Olds, 1991; Kelly, 1992; Conner and Via, 1993; O'Neil, 1997; Quinn and Weitherington, 2002). Given this widespread variation, Fox (2003) remarked that phenological assortative mating may be ubiquitous, with potentially important consequences in plant population biology. For instance, phenological assortative mating may promote local adaptation (Antonovics and Bradshaw, 1970; Kirkpatrick, 2000) and structure hybrid zones (Cruzan and Arnold, 1994). And because herbivory and competition alter flowering phenology, phenological assortative mating may have a role in the evolution of plant defense (Strauss et al., 2002) and competitive ability (Lyons and Mulley, 1992).

Genetic variation in flowering schedules creates temporal population structure similar to spatial structure (Fox, 2003). In so doing, it inflates the additive genetic variance in phenological traits by bringing together alleles with similar phenotypic effect (Wright, 1921; Breese, 1956; Crow and Kimura, 1970; Felsenstein, 1981; Barton and Turelli, 1991; Lynch and Walsh, 1998), a condition termed gametic phase disequilibrium. This variance inflation can make flowering time—and any traits genetically correlated to flowering time—more responsive to selection (Breese, 1956; Lyons and Mulley, 1992; Strauss et al., 2002; Fox, 2003).

Despite the potential importance of assortative mating for phenology, we know virtually nothing of its strength in natural populations. Assortment by flower color or inflorescence structure is well studied (e.g., Levin and Watkins, 1984; Jones, 2001; Jones and Reithel, 2001), but we are aware of only three papers reporting tests for nonrandom mating by flowering phenology. Gutierrez and Sprague (1959) planted several strains of maize, each with a distinctive visible recessive marker trait, and let them freely cross-pollinate. Segregation of markers in F_2 progeny revealed that phenologically similar strains were more likely to cross. Ennos and Dodson (1987) found similar results by analyzing the frequency of isozyme markers in the progeny from open-pollinated experimental plantings comprised of five clones of the self-incompatible grass *Cynosurus cristatus*. Stanton et al. (1997) found no evidence for phenological assortment from allele frequencies at three isozyme loci in a natural population of *Ranunculus adoneus*. The marker-based approach can be powerful in a small, genetically characterized population where paternity of each seed can be established. To achieve this level of resolution in natural populations, however, many pollen donors (thousands?) would have to be characterized at many loci, as would numerous offspring from each seed parent. Difficulties such as like these could be why phenological assortment remains unexplored. Clearly, less cumbersome methods to study assortative mating in natural populations are needed.

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[2] E-mail: aeweis@uci.edu.

An alternative method is suggested by the work of Lyons and Mulley (1992) who explored the *potential* for assortative mating in *Nicotiana* by measuring temporal overlap in their flowering schedules. Large plants flowered longer than small ones and so had more within-type mating opportunities. Fox (2003) further explored this approach by showing that variation in any component of the flowering schedule (e.g., day for first flower, peak flowering, last flowering, and the skew and kurtosis of the schedule) could induce nonrandom mating.

The present paper takes the schedule-based approach to its logical conclusion by deriving a method to estimate the phenotypic correlation between mates—the standard quantitative genetic metric for assortative mating—from individual flowering schedules. The method first calculates the mating probability between all possible mating pairs in a sample and then uses these probabilities to calculate a weighted correlation between the phenotypes of pollen recipients and the phenotypes of their potential pollen donors. The resulting correlation coefficient is symbolized by ρ. This is a *prospective* estimate of the correlation between mates because it is based on mating opportunities.

We then validate the schedule-based method by comparing its prediction to the results of a second method based on established quantitative genetic principles. This second method is *retrospective* because it calculates the phenotypic correlation between mates using information from the offspring generation. By standard theory (Falconer and Mackay, 1996), for a polygenetic trait, the slope of the regression of offspring phenotype over maternal phenotype equals half the heritability, provided mating is random. When mating is assortative, however, the regression of offspring phenotype over maternal phenotype is inflated by a factor of $1 + \rho$ (Fisher, 1918; Wright, 1969; Lynch and Walsh, 1998). Thus, deviation of the offspring-maternal regression from the random mating expectation measures the correlation between the mother and her mates. The prospective method is easily applied in natural populations, and so, if valid, it can become an important tool in plant population biology.

Flowering phenology, the shifting mating pool, and assortative mating—Variation in the flowering schedule causes a shift in the composition of the mating pool as the flowering season progresses (Ennos and Dodson, 1987; Fox, 2003). This shift is the fundamental cause of phenological assortative mating. Say, for instance, that individuals vary in flowering time (days from germination to first flowering). The mating pool can be characterized on day d of the flowering season by a weighted mean flowering time, $\bar{z}_d$, of the contributors to the pool on that day:

$$\bar{z}_d = \frac{\sum_{j=1}^{N} n_{jd} z_j}{\sum_{j=1}^{N} n_{jd}} \quad (1)$$

where z_j is the flowering time for contributor j and n_{jd} is the number of its flowers open on day d, and N is the number of contributors in the sample. Any trait can be substituted for z in Eq. 1; if it is correlated to phenology, its daily mean also will shift across the season.

How can we test if the phenotypic shift in $\bar{z}_d$ over the season reflects a genetic shift in the mating pool? Ennos and Dodson (1987) argued that if flowering time is heritable, the flowers produced by a mother at the beginning of her flowering period receive pollen from ''genetically earlier'' fathers than those she produces at the end. Thus, a significant difference in the flowering times of first- and last-produced offspring by individual mothers demonstrates that the fundamental condition for phenological assortative mating pertains. In essence, flowering time itself is the genetic marker for paternal phenotype. However, the validity of this interpretation rests squarely on the assumption that maternal age at the time a flower is pollinated has no effect on flowering time of the resulting offspring. Later we present an experimental protocol to test this assumption.

Although we used the day of first flowering as our example, variation in other components of the flowering schedule can cause seasonal shifts in their means. When individuals vary in duration of the flowering period or the day within the period for peak flower production, components of schedule ''shape,'' they will not contribute equally to the mating pool across the mating season, and their daily averages will shift (see Fox, 2003). Correlations of the ''temporal position'' or ''shape'' components of the flowering schedule with flower production, the ''size'' component, will accentuate shifts in their daily means.

A prospective estimate for the intensity of phenological assortative mating—How strong is assortative mating? The potential for phenological assortative mating can be characterized by the phenotypic correlation between potential mates, ρ. For hermaphrodites,

$$\rho = \frac{\mathrm{Cov}(z_m, z_f)}{\sigma^2(z)} = \frac{\sum_m \sum_f [\Phi_{mf}(z_m - \bar{z})(z_f - \bar{z})]}{\sum_m X_m (z_m - \bar{z})^2}, \quad (2)$$

where z is flowering time, m and f denote the mother and father in a mating pair, and X_m is the proportion of all flowers from sampled plants that were produced by mother m. Because they are hermaphrodites, the phenotypic variance of mothers and fathers is the same. Φ_{mf} is the proportion of all mating opportunities for which m is the mother and f is the expected father, estimated as

$$\Phi_{mf} = \sum_{d=1}^{D} \theta_{md} \left(\frac{n_{fd}}{\sum_{f=1}^{N} n_{fd}} \right), \quad (3)$$

where n_{fd} is the number of open flowers on potential father f on day d, and θ_{md} is the proportion of all the flowers produced by the population over the season that were open on mother m on day d. The Φ_{mf}'s sum to 1, $m \neq f$ for self-incompatible species, and Φ_{ij} need not equal Φ_{ji} when individuals exchange roles as mother and father. When all Φ_{mf} are equal, the numerator in Eq. 2 becomes zero.

When $\sigma^2(z)$ is the same for both sexes, ρ is also the regression slope of the expected paternal phenotype over the observed maternal phenotype. The fathers contributing pollen to mother m will have the expected phenotype

$$\bar{z}_{f|m} = \sum_{d=1}^{D} \sum_{f=1}^{N} \theta_{md} \left(\frac{n_{fd} z_f}{\sum_{f=1}^{N} n_{fd}} \right). \quad (4)$$

In summary, $\bar{z}_{f|m}$ is the average phenotype of the fathers available to mother m, weighted by the proportion of mating opportunities they share with her. Rearranging the numerator of

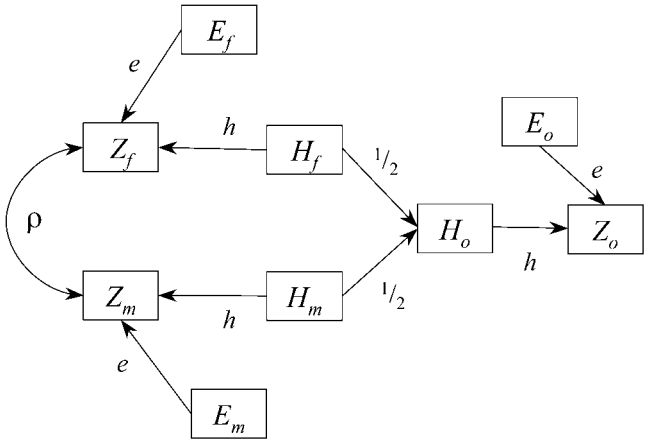

Fig. 1. Path diagram depicting the correlation structure between phenotype of mother and father (Z_m and Z_f, respectively) and offspring (Z_o). Variables H_i and E_i denote genetic and environmental effects on phenotype, respectively.

Eq. 2 shows that $\text{Cov}(z_m, \bar{z}_{f|m}) = \text{Cov}(z_m, z_f)$. Thus, the slope of the regression of $\bar{z}_{f|m}$ over z_m is also equal to ρ. It is easy to estimate $\bar{z}_{f|m}$ on a spreadsheet, making Eq. 4 more practical than Eq. 2.

Age-specific variation in fecundity can bias estimates of ρ. In a later section we show how θ_{id} can be adjusted to account for the lower rate of fruit set typically seen toward the end of the flowering period (e.g., Stephenson et al., 1988).

Estimates of ρ by Eqs. 2, 3, and 4 are based on the contributions of individuals to the mating pool, rather than their representation in the population generally. Thus, ρ values are affected by selection (see Lewontin et al., 1968). Suppose late-flowering plants produce more flowers (late flowering favored). The frequency of early × late mating will increase (as the early plants approach the end of their flowering period), which commensurately reduces the frequency of early × early. As a result, ρ is lower than if all individuals had equal flower production. It may be desirable for some purposes to know how selection, acting through variation in the ''size'' of the mating schedule, changes the potential for assortment, which acts through ''temporal position'' or ''shape.'' We define ρ* as the correlation between mates assuming equal flower production. This statistic is calculated with Eqs. 2 and 3 or by Eq. 4, substituting θ_{jd} (proportion of father *j*'s flowers on day *d*) for n_{jd} (number of flowers on day *d*).

A retrospective estimate for the intensity of assortative mating—Flowering schedules provide circumstantial evidence for the phenotype, and genotype, of the father for an offspring produced at a given time. Direct evidence can be gained from the phenotypic correlations between parents and offspring. Drawing on Wright (1921, 1969), consider the path diagram in Fig. 1. The regression of phenotype, *Z*, over genetic value, *H*, has a coefficient of *h*. Under standard assumptions, this is true for both of the parents and for the offspring. The regression of an offspring's genetic value over the genetic value of one parent has a coefficient of one-half, assuming equilibrium (Li, 1975; Lynch and Walsh, 1998). Under random mating ($\rho = 0$), the regression of offspring over maternal phenotype is the product of the path coefficients directly connecting Z_m to Z_o (Fig. 1): $b_{o,m} = h \times \frac{1}{2} \times h = \frac{1}{2} h^2$. Assortative mating ($\rho \neq 0$) changes the regression by adding the path going through paternal phenotype:

$$b_{o,m} = \frac{1}{2}h^2 + \rho\frac{1}{2}h^2 = \frac{1}{2}h^2(1 + \rho). \quad (5)$$

The correlation between paternal and offspring phenotypes also is inflated by $1 + \rho$.

The regression of offspring phenotype over the mid-parent phenotype, $b_{o,\bar{p}}$, equals h^2 and is *not* biased by assortative mating (the covariance between offspring and mid-parent is inflated, but so is the variance of the mid-parent phenotype; Fisher, 1918; Wright, 1969; Lynch and Walsh, 1998). Thus the phenotypic correlation between mates is

$$\rho' = \frac{2b_{o,m}}{b_{o,\bar{p}}} - 1 = \frac{2b_{o,f}}{b_{o,\bar{p}}} - 1, \quad (6)$$

where the prime distinguishes it from the prospective estimate. Later we present a regression method based on Eq. 5 to give both a point estimate and standard error for ρ′.

MATERIALS AND METHODS

Starting in December of 2001, we performed two coordinated experiments with *Brassica rapa* in the University of California-Irvine Greenhouse. Both experiments covered two plant generations. The first experiment used controlled crosses to estimate both the heritability of flowering time by parent-offspring regression and the environmental maternal effects, including those caused by maternal aging. The second experiment used open pollination to explore phenological assortative mating by testing for within-season genetic shifts in the mating pool and by prospective and retrospective estimates of the phenotypic correlation between mates.

Plants descended from a naturalized population of *B. rapa* found along the Back Bay, Newport Beach, California. This species is a self-incompatible winter annual found in mesic or frequently disturbed sites. To ensure adequate genetic variation in flowering time (to avoid type II errors) we created a synthetic population by combining genetic lines provided by Dr. Gretchen LeBuhn (San Francisco State University). Seeds collected in 1999 from the Back Bay were divided into three selection lines: accelerated flowering, delayed flowering, and a nonselected control. Two generations of selection moved mean flowering times approximately 0.75 standard deviations below and above the control. To establish the parental generation for the two experiments we randomly drew seed from a pool comprised of equal proportions of the three lines.

The parental generations were planted in 12 × 12 × 14 cm pots filled with a 67 : 33 mixture of soil-less mix and coarse sand. Parents were fertilized every 2 wk with 20 : 20 : 20 soluble fertilizer. The offspring generations were planted in ConeTainer pots (Steuwe and Sons, Corvallis, Oregon, USA) with the same soil mix and fertilized every 2 wk until flowering.

Controlled pollination experiment—The retrospective method for estimating ρ requires that maternal effects be negligibly small (maternal effects also inflate the regression of offspring phenotype over maternal phenotype). It also requires an unbiased estimate of h^2 for flowering time. This experiment addressed these points. It also established whether maternal age at pollination had an effect on offspring flowering time. The test for a maternal age effect was important: if siblings from a mother's first-produced flowers were different from those in the last-produced flowers because of maternal age, genetic differences among the pollen donors to those flowers would be obscured, and with them, the within-season shift in the pollen pool.

In the parental generation, 16 plants were assigned to eight monogamous pairs. Plants within a pair were reciprocally pollinated every 3 d during the flowering period. Maternal age at each pollination was calculated as days since first flowering. Seeds were collected at senescence. These crosses gave rise to eight reciprocal full-sibships (half the full sibs having one parent as

mother and the reciprocal having the other parent as mother). All offspring within a cross had the same genetic relatedness, so maternal (or paternal) age effects could be attributed to environmental causes. We tested the effects of cross, reciprocal mothers within cross, and maternal age at pollination, plus the two interactions by analysis of covariance (ANCOVA). We approximated variance explained as the proportion of the type I sum of squares associated with each effect.

To ensure that each individual had early- and late-produced offspring sired by the same pollen donor, it was necessary to reciprocally mate the first-flowering plant with the second, the third with the fourth, and so on. Thus, the parents were assortatively mated. As stated earlier, assortative mating does not bias the regression of offspring on mid-parent (Falconer and Mackay, 1996; Lynch and Walsh, 1998). It also greatly increased statistical power for estimating the regression slope (Lynch and Walsh, 1998). The significance of the heritability estimate was tested by the regression of sibship means over the mid-parent value (df = 7). However, when sibship sizes are unequal, the resulting regression slope can be biased (Lynch and Walsh, 1998), and so we used the slope for the regression of individual offspring over their parents as our point estimate of heritability.

Open pollination experiment—Flowering schedules were recorded for 48 individuals in the parental generation. Here we define our terms to describe flowering schedules. We use ''day'' to denote the number of days since seed sowing. ''Germination time'' is the number of days from sowing to seedling emergence. ''Flowering time'' is the number of days between emergence and first flowering (a plant emerging on day 8 and flowering on day 50 had a flowering time of 42 d). ''Flowering period'' is the number of days from first flowering to last. We calculated ''flowering period symmetry'' by subtracting one-half of the flowering period (number of days) from the number of days into the flowering period that 50% of the eventual total flower production was reached and then divided by flowering period. The symmetry index is negative when a plant's flower production is concentrated near the end of its flowering period and positive when flowering is concentrated toward the beginning. Traits were defined to avoid artificial correlations (e.g., ''flowering time'' is potentially independent from ''germination time,'' whereas ''day of first flowering'' is not). However some traits are composites, and so measurement errors can be correlated (e.g., ''flowering time'' = ''day of first flowering'' − ''germination time,'' and so if germination is mistakenly recorded a day late, flowering time will be mistakenly calculated a day shorter). Such errors are unlikely to be large.

The days of germination and of first flowering were recorded for each plant. We censused the number of open, pre-senescent flowers for each plant every third day. Plants were pollinated at random after each census. We followed a computer-generated, random path through the population, picking up and depositing pollen on every open flower on each plant (starting at the bottom of the inflorescence) with a camel hair brush. A new path was generated for each day. If a plant was not in flower, we moved to the next on the list.

Flowering schedules were used to calculate $\bar{z}_d$ the mean flowering time of the plants contributing to the mating pool on day d (Eq. 1) and the prospective correlation between mates (Eqs. 2 and 3). The corresponding calculations were also performed for germination time, flowering period, flowering period symmetry, and total number of flowers produced. Confidence intervals (95%) were based on a sample size of 48.

To get the retrospective estimate of the correlation between mates, ρ', we examined offspring produced by 17 of the plants: the five earliest plants (rank order 1–5), five intermediates (ranks 21–25), and the seven latest (ranks 42–48). On each census day we marked the two newest flowers on each plant and collected resulting fruits at the end of the season. Thus, for the offspring from each marked flower, we knew maternal phenotype and the day on which it was produced. Based on flowering schedules, we knew the expected paternal phenotype on that day.

In May of 2002, we planted 1371 offspring from the 17 maternal plants. We attempted to raise 15 offspring per mother per census day. The total number of offspring per maternal plant varied according to the length of her flowering period and her success on each pollination day. Germination and flowering times were recorded as described. It was not feasible to record the other elements of the flowering schedule for that many plants. The offspring were grown under longer photoperiods than the parents and this resulted in a compressed flowering season. When comparing parents to offspring, we standardized data to $\bar{z} = 0$ and $\sigma^2 = 1$ to control for generational differences in environmental variance. Because of the environmental differences between generations, the estimated heritability may be lower than that under the natural photoperiod (Lynch and Walsh, 1998), but this should not seriously affect our comparison of prospective and retrospective estimates of the correlation between mates.

In the next section we show how the open pollination experiment was analyzed to estimate the correlation between mates. We also analyzed the experiment to determine if the fundamental condition for assortative mating pertains, namely, that the genetic composition of the mating pool changes over the course of the season. We used ANCOVA to determine if offspring flowering time could be explained by maternal flowering group (early, intermediate, or late), and day of the season the offspring was produced (day of pollination), or their interaction. A significant ''day of season'' term would show that after holding maternal contributions constant (including genetic contributions), first-produced offspring flower earlier than last produced offspring and therefore must have had ''genetically early'' fathers. Maternal age at pollination was entered as a covariate. Not all mothers produced flowers on every census day, and so type IV sums of squares were calculated with PROC GLM of SAS (1990).

Prospective and retrospective estimates of ρ—Flowering schedules were used to construct prospective estimates of the correlation between mates, ρ, for all measured traits. All 2256 Φ_{mf}'s, pair-wise mating probabilities, were calculated by Eq. 3 and inserted into Eq 2. We also calculated ρ as the regression of $\bar{z}_{f|m}$ (Eq. 4) over maternal phenotype for each trait. Later-produced flowers on a mother are unlikely to set fruit, so we weighted the contribution of each flower to the ovule pool by the age-dependent probability of fruit-set seen among the 17-plant subset, which we determined by logistic regression of fruit-set on maternal age.

The retrospective estimate of ρ could be based only on the 17 marked maternal plants, thus we revised the prospective estimate accordingly. Only the two marked flowers per census day were included in the revised maternal flowering schedules.

Next, we combined information from both the controlled and open pollination experiments to estimate the retrospective correlation between mates, ρ'. Drawing on Eq. 5 for the slope of the regression of offspring over mother, we used the NLIN procedure of SAS (1990) to fit the model

$$z_o = z_m \frac{1}{2} h^2 (1 + \rho'), \tag{7}$$

where z_o is offspring flowering time, z_m is the flowering time of its mother, and h^2 was held constant to the value obtained in the controlled pollination experiment. We used the same method to estimate ρ' in the controlled pollination experiment.

RESULTS

Controlled pollination experiment—Both cross and maternal age had significant effects on offspring flowering time (Table 1). The regression of family mean flowering time over mid-parent flowering time was significant ($R^2 = 0.84$, $F_{1,7} = 33.5$, $P = 0.0012$). Heritability, estimated from regression on the mid-parent, was 0.71 (Fig. 2A). The term for reciprocal mothers within crosses was not significant, which indicates no general maternal (or paternal) effect on offspring flowering time. Although maternal age at pollination had a significant effect on offspring flowering time, it accounted for less than 2% of the variance (Table 1, Fig. 2B).

Open pollination experiment—A total of 34 151 flowers were counted over the 76 d flowering season of the parental

TABLE 1. Analysis of covariance for offspring flowering time in the controlled pollination experiment on *Brassica rapa* in the greenhouse. The variance explained by each source was approximated by partitioning the sum of squares.

Source	df	Mean square	*F*	*P*	Partial R^2
Cross[a]	7	675	45.74	<0.0001	0.626
Mother (Cross)	8	14	0.53	0.85	0.003
Maternal age	1	354	13.25	<0.0003	0.014
Cross × Age	7	27	1.01	0.42	0.006
Mother (Cross) × Age	8	28	1.04	0.40	0.006
Error	503	26			

[a] Tested over synthetic mean square (0.9595 × MS(Mother(Cross)) + 0.0405 × MS(Error)), with denominator df = 9.32.

generation. The first plant flowered at 36 d (post-germination) and the last at 72 d (Fig. 3). Mean time to first flower was 51.8 d (SD = 10.7). On average, plants produced flowers for 36.5 d (SD = 6.4), and the average plant produced 711.5 flowers (SD = 230.9). The mean of the schedule asymmetry index was 0.85 (SD = 0.87), indicating that plants concentrated flower production in their later days.

Temporal shift in the mating pool—The weighted phenotypic mean flowering time for the ''standing crop'' of reproducing plants shifted to later dates as the flowering season progressed (Fig. 4). This within-season phenotypic shift in the potential fathers resulted in a within-season genetic shift in the pollen pool. ANCOVA (Table 2) shows that offspring sired at the beginning of the parental season had significantly earlier flowering times than those sired near the end (Fig. 5). Maternal class (early, intermediate, or late) and maternal age at pollination are statistically held constant in this analysis, and so the difference between early- and late-produced offspring can be explained by genetic differences in their fathers. The significant interaction between maternal class and day was expected because the shift in the pollen pool was sigmoidal (Fig. 4): early, intermediate, and late mothers were exposed to different segments of the curve.

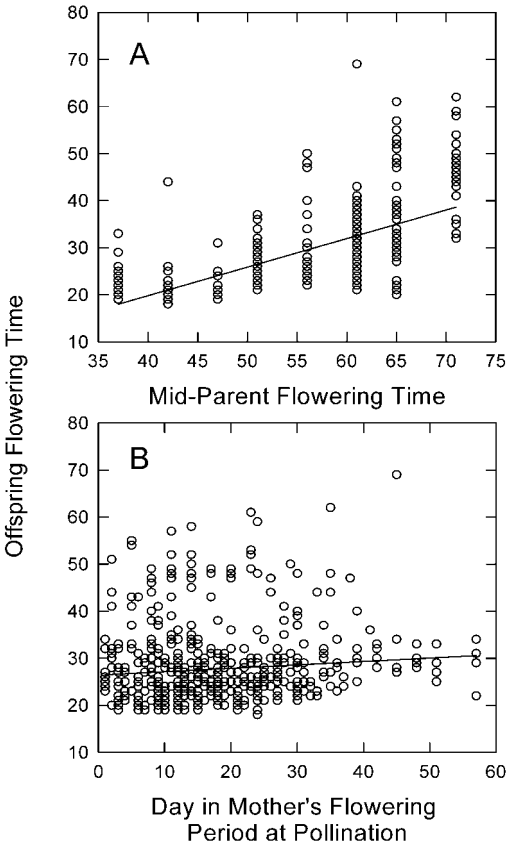

Fig 2. Heritable and environmental effects on flowering time in the controlled pollination experiment on *Brassica rapa* in the greenhouse. (A) Offspring flowering time vs. mid-parent flowering time. Heritability, from regression of offspring over mid-parent: h^2 = 0.71, 95% CI = 0.654–0.754. (B) Offspring flowering time vs. maternal age (days since first flowering) at time of pollination. ANCOVA indicates maternal age explains only 1.6% of the variance in offspring flowering time (see Table 1).

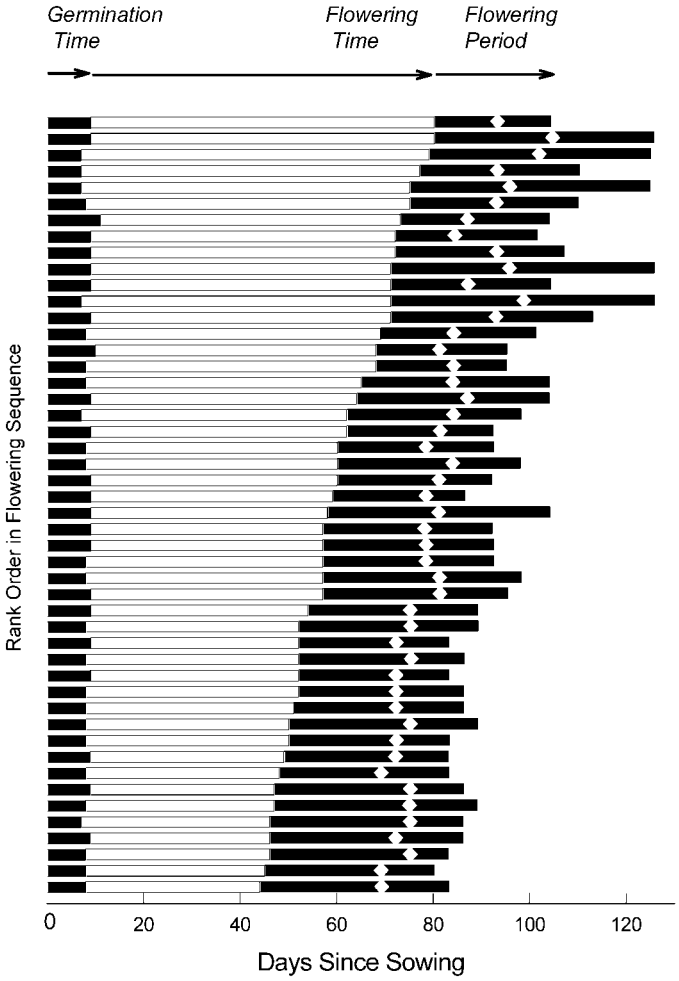

Fig. 3. Flowering schedules for the 48 plants in the parental generation of the open pollination experiment on *Brassica rapa* in the greenhouse. White diamonds inside the flowering period portion of the bars indicate the median flowering date. See also Fig. 8B.

The prospective correlation between mates—Estimates of ρ based on flower counts could be biased if not all flowers are equally likely to set fruit. Figure 6A shows that flowers produced late in a mother's flowering period are much less likely to set seed than earlier flowers. As a result, flowering schedules for maternal function differ from those for paternal function (Fig. 6B). We used these adjusted schedules to calculate the correlation between mates.

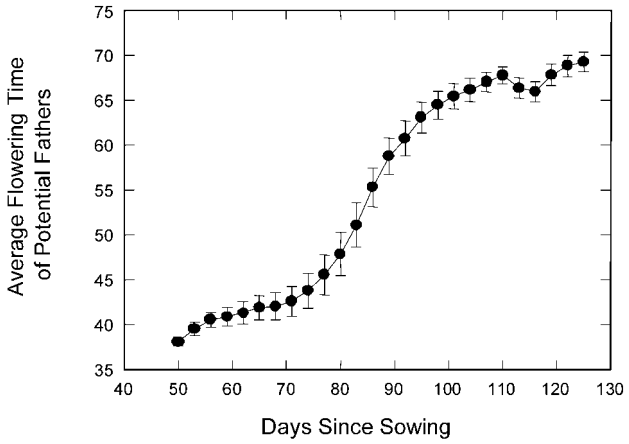

Fig. 4. Average flowering time of potential fathers across the flowering season in the open pollination experiment on *Brassica rapa* in the greenhouse. Each point is the average flowering time of all 48 plants, weighted for the number of open flowers on each on that day. Error bars indicate 95% confidence intervals.

The shifting mean flowering time in the mating pool (Fig. 4) led to a correlation of $\rho = 0.58$ between flowering times of maternal plants and their potential mates (Fig. 7A). The area of the each symbol in the bivaraite plot is proportional to Φ_{mf}, the probability of mating between the corresponding mother and father. For instance, the first plant to flower had a 4.28% chance of receiving pollen from the second plant to flower, but less than a 0.01% chance of receiving pollen from the 48th (Fig. 7A). The 48th plant to flower had a 0.02% chance of receiving pollen from the first, but a 3.47% chance of receiving it from the 47th. Under panmixia, all plants would have had a 2.12% chance of mating with any other plant. The regression of the expected father phenotype, $\bar{z}_{f|m}$, over maternal phenotype was also 0.58 (95% CI: 0.53–0.63) (Fig. 7B). Issues related to significance testing for ρ are raised in the Discussion.

When flowering schedules based on the number of flowers per father per day (n_{fd}), ρ is affected by differences in flower production. To determine the correlation between mates based purely on assortment, we calculated ρ^*, which substitutes θ_{fd} (the proportion of seasonal flower production for father f realized on day d) for n_{fd}. The resulting correlation coefficient was 0.59 (95% CI: 0.54–0.64), which is indistinguishable from ρ.

We expected the degree of assortment for the other measured traits to vary in proportion to their correlation with flowering time. Germination time was not correlated with flowering time (Table 3) and as expected, we observed no potential for assortative mating for this trait (Fig. 8A, B). Symmetry of the flowering schedule and total flower production were both positively correlated with flowering time (Table 3) and both showed significant potential for assortment (Fig. 8E–H). Although duration of the flowering period was not correlated with flowering time (Table 3), duration did show a significant potential for assortment (Fig. 8C, D). This is a reasonable result; two plants that produce flowers over many days are likely to share more mating opportunities than two plants with short flowering periods. Some of the assortment in flower production may have been caused by its correlation to flowering period duration (Table 3). Assortment for schedule symmetry may have been reduced by its negative correlation with duration.

TABLE 2. Test for genetic change in mating pool over the mating season in the open pollination experiment on *Brassica rapa* in the greenhouse. The significance of the main terms show that after correcting for maternal genetic contributions to offspring flowering time, offspring produced at the beginning of the season were sired by fathers with genetically earlier flowering times than those produced at season's end. The significant interaction indicates that the genetic shift in the mating pool was not linear. See Figs. 4 and 5.

Source	df	Mean square	*F*	*P*
Maternal age	1	86.5	5.59	0.018
Maternal class	2	75.7	4.90	0.008
Day of season	18	80.9	5.23	<0.0001
Class × Day	9	41.3	2.67	0.005
Error	1324	15.4		

Comparing prospective and retrospective estimates of the correlation between mate—We used data from the controlled pollination experiment (both maternal and paternal phenotypes known) to evaluate the estimate of assortative mating made in the open pollination experiment (maternal phenotypes known but paternal phenotypes inferred).

Table 4 shows that the correlation between maternal and paternal plants in the controlled pollination experiment was $\rho = 0.93$. Recalling that the estimated heritability of flowering time was $h^2 = 0.71$ and that no general maternal effects were detected, the regression of offspring over mother is expected to be $b_{o,m} = 0.355$, assuming panmixia and equilibrium. Assortative mating inflates the regression of offspring over one parent by a factor of $1 + \rho$, and so given the prospective estimate of ρ in this experiment, the expected offspring–mother regression is $b_{o,m} = 0.355 \times 1.93 = 0.685$. This is close to the observed value, $b_{o,m} = 0.70$ (Table 4). Fitting the observed maternal and offspring flowering times to Eq. 7 yields a retrospective estimate of $\rho' = 0.97$. The prospective and retrospective estimates lie within each other's 95% confidence intervals (Table 4).

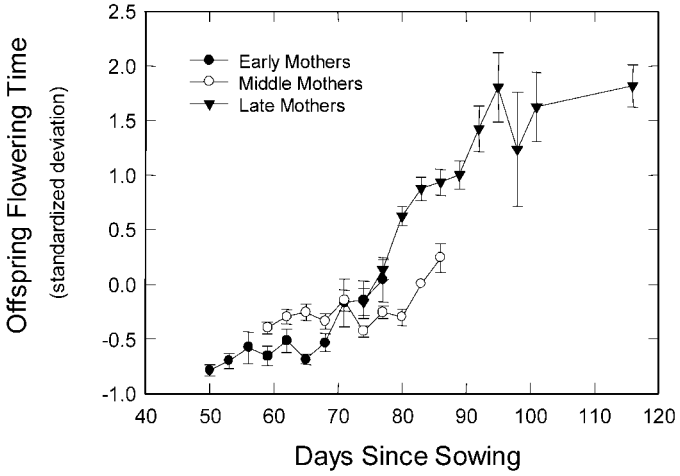

Fig. 5. Average offspring flowering time for offspring produced at progressively later days during the parental generation in the open pollination experiment on *Brassica rapa* in the greenhouse. ANCOVA (Table 3) showed that, when maternal genotype and age are held statistically constant, offspring produced later in the season of the parental generation flowered later in their own generation. These results support the hypothesis that the phenotypic shift among the potential fathers (Fig. 4) led to a genetic shift in the pollen pool. Error bars indicate one standard error.

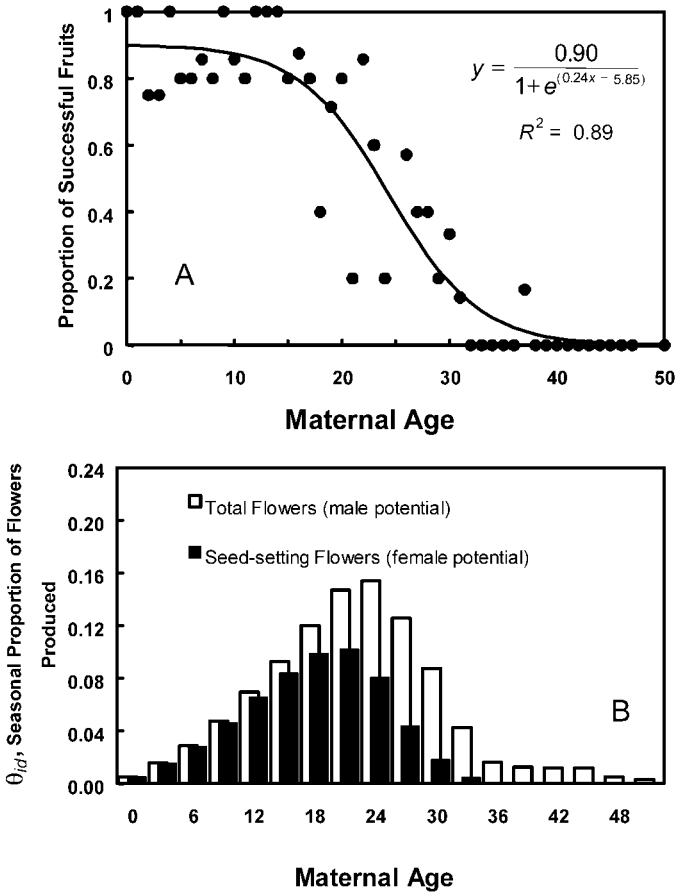

Fig. 6. Effect of maternal age on fruit set for *Brassica rapa* in the greenhouse. (A) Proportion of pollinated flowers successfully setting seed across the flowering period. Data from marked flowers on the 17 selected plants in the open pollination experiment. (B) Open bars, flower production over the flowering period for the 'average' plant. Closed bars, production of flowers that set fruit over the flowering period for the 'average' plant. Pollen could be donated in proportion to the height of the open bars and seed in proportion to the closed bars.

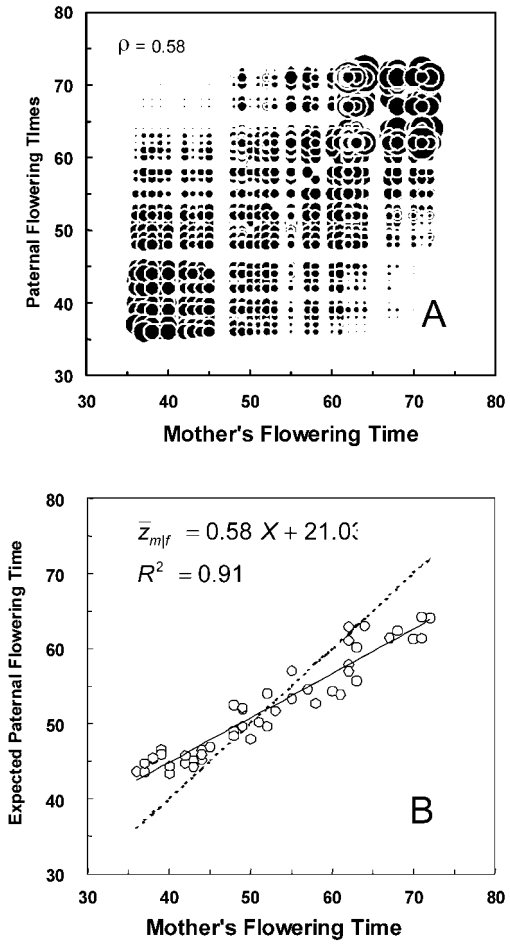

Fig. 7. Correlation between mates for flowering time in the open pollination experiment on *Brassica rapa* in the greenhouse. (A) Correlation based on Eq. 2. Size of the symbols are proportional to Φ_{ij}, the proportion of all mating opportunities for mother *i* shared with potential father *f* (Eq. 3). (B) Regression of the flowering time for the average potential father for each mother ($\bar{z}_{m|f}$, Eq. 4) over mother's flowering time. Dashed diagonal indicates expected line under perfect correspondence between maternal and paternal phenotype.

Before performing this analysis on the open pollination experiment, we revised our prospective estimate for the correlation between mates to include only the marked flowers on the 17 designated maternal plants. After making this adjustment, ρ was 0.43 (Fig. 9). We note that the intercept for the regression of expected paternal over maternal flowering time was similar to that for the full data set, whereas the slope was 28% lower. In the discussion we will argue that this pattern indicates selection on flowering time through male function.

Assuming the heritability of flowering time was the same in the two experiments, the expected slope for the regression of offspring over maternal flowering time is $b_{o,m} = 0.355 \times 1.43 = 0.507$. This lies within the 95% confidence interval for the observed regression coefficient of 0.48 (Table 4). Fitting the observed maternal and offspring flowering times to Eq. 7 yields a retrospective estimate of $\rho' = 0.39$ for this experiment (Table 4). The prospective and retrospective estimates lay within each other's 95% confidence intervals.

DISCUSSION

Variation in flowering time caused a seasonal shift in the phenotypic and genetic composition of the mating pool (Figs.

TABLE 3. Phenotypic correlations among phenological traits and flower production for *Brassica rapa* in the greenhouse.

Trait	Days to germination	Days to first flower	Flowering period duration	Schedule symmetry
Days to first flower	0.00^{NS}			
Flowering period duration	-0.34*	0.10^{NS}		
Flowering schedule symmetry	-0.08^{NS}	0.61***	-0.71***	
Total flower production	-0.30*	0.50***	0.66***	-0.44**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; ^{NS} not significant.

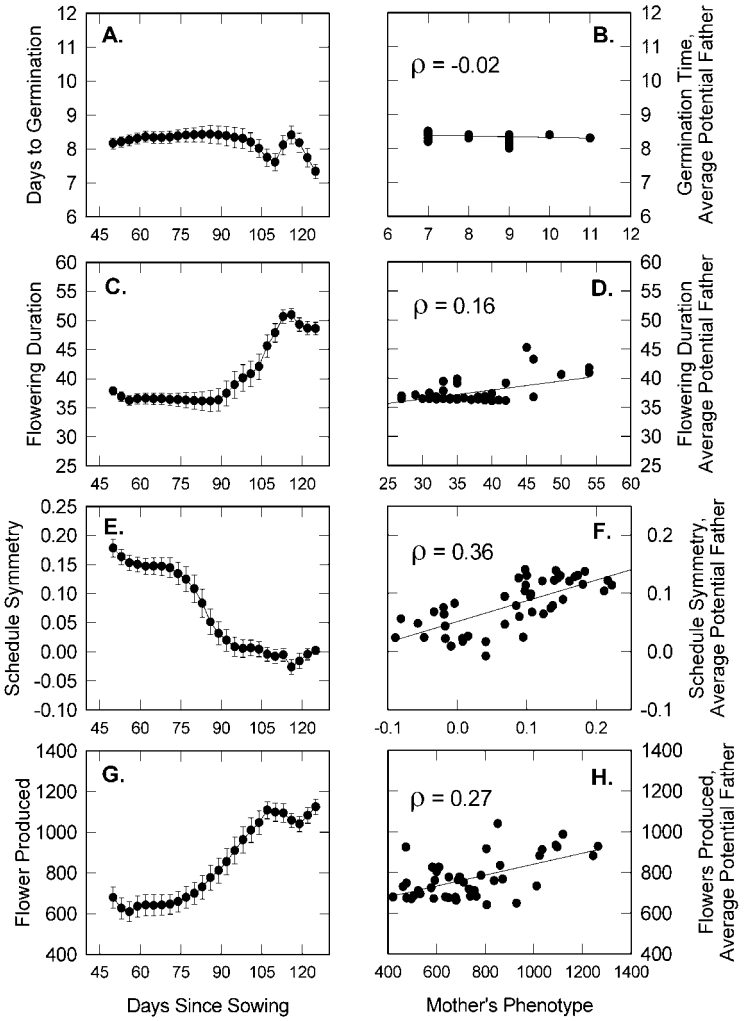

Fig. 8. Average phenotypes of potential father across the season, and the regression of phenotypes for the average potential father for each mother ($\bar{z}_{m|f}$) over the mother's phenotype in the open pollination experiment on *Brassica rapa* in the greenhouse. (A, B) Germination time (in days from sowing to emergence). (C, D) Duration of the flowering period (in days). (E, F) Symmetry of the flowering schedule (zero value indicates that 50% of flowers were produced before and after the mid-date of the flowering period). (G, H) Flower production (total flowers produced over flowering period). Error bars indicate 95% confidence intervals.

5, 6). Phenologically similar individuals shared more mating opportunities than expected under random mating. The prospective estimate of the phenotypic correlation between mates, ρ, indicated a strong potential for phenological assortative mating (Fig. 7). This was confirmed by the retrospective estimate of assortative mating, ρ′, which we measured by the inflation in the offspring–mother regression (Table 4).

Statistical issues—The method for prospectively estimating ρ can be easily applied to natural plant populations. However, testing the null hypothesis, that ρ is significantly different from zero, remains problematic. For each point in the regression of expected paternal phenotype, $\bar{z}_{f|m}$, on maternal phenotype (Fig. 7B), one of the plants is contrasted to its 47 potential mates. Thus each point relies on the information for the same 48 plants, and so confidence intervals associated with our prospective estimate of ρ will be artifactually narrow. This makes the test that $\rho \neq 0$ overly liberal. However, it also makes the test that $\rho = \rho'$ conservative—confidence intervals for prospective and retrospective estimates are less likely to overlap.

TABLE 4. Parent offspring regressions and the correlation between mates for the controlled and open pollination experiments on *Brassica rapa* in the greenhouse.

Statistic	Controlled pollination		Open pollination	
	Estimate	95% CI	Estimate	95% CI
Correlation between mates, prospective	0.93	0.884–0.982	0.43	0.334–0.562
Regression on mid-parent	0.71	0.654–0.754	—	—
Regression on mother (panmictic expectation = 0.355)	0.70	0.645–0.767	0.48	0.436–0.524
Correlation between mates, retrospective	0.97	0.794–1.132	0.39	0.288–0.489

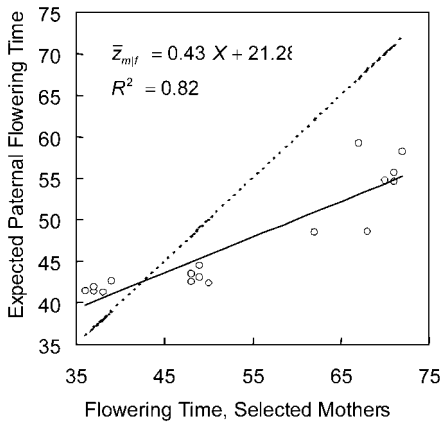

Fig. 9. Regression of the flowering time for the average potential father for each of 17 selected mothers ($\bar{z}_{m|f}$, Eq. 4) over the mother's flowering time in the open pollination experiment on *Brassica rapa* in the greenhouse. Calculations of $\bar{z}_{m|f}$ account for the female flowering schedule imposed by our marking procedures. Dashed diagonal indicates expected line under perfect correspondence between maternal and paternal phenotype.

We are developing resampling methods to derive unbiased confidence intervals.

A caveat: gametic phase disequilibrium in the synthetic population—We found close agreement between the prospective and retrospective estimates of ρ in both experiments. However, there was reason to expect that the retrospective estimate, ρ′, would be larger that the prospective estimate. Our decision to construct the experimental population from selected lines increased both the potential for assortative mating and our ability to detect a seasonal genetic shift in the pollen pool. However, it may have biased our estimate of ρ′.

Lush (1937) noted that additive genetic variance can be partitioned into two components. First is the *genic* variance, which is the variance expected under equilibrium, given observed allele frequencies. The remaining portion is caused by gametic phase disequilibrium, that is, the tendency of individual genotypes to contain an excess of alleles at different loci with similar phenotypic effect (e.g., *AB* and *ab* gametes occur more frequently than *Ab* and *aB* gametes). Gametic phase disequilibrium causes the variance inflation under assortative mating. Disruptive selection also induces gametic phase disequilibrium (Walsh and Lynch, in press). Our synthetic population, composed of divergently selected lines, was unlikely to be in Hardy-Weinberg equilibrium. It is conceivable that even if we mated plants randomly, the regression of offspring over a single parent could have been greater than $\frac{1}{2}h^2$ (Walsh and Lynch, in press). However, if the inflated offspring-maternal regression is caused only by gametic phase disequilibrium in the parental generation, we would not have expected such close agreement between the prospective and retrospective estimates of ρ in both of the experiments (Table 4).

Assortative and selective mating—A considerable body of theory connects selection and assortative mating under various mate choice scenarios in animals (O'Donald, 1960; Karlin and Scudo, 1968; Scudo and Karlin, 1968; Karlin, 1969; Burley, 1983; Gimmelfarb, 1988). We will bypass the controversy over mate choice in plants (Wilson and Burley, 1983; Charlesworth et al., 1987; Marshall, 1998) and focus on the interplay of assortative and selective mating caused by differences in male and female flowering schedules.

Our analysis of the open pollination experiment included three prospective estimates of ρ, each with different assumptions about male and female flowering schedules. The first of these estimates, ρ* = 0.59, assumed all individuals contributed equally, and with equal success, to the mating pool through both genders. Thus, ρ* reflects the potential for pure phenological assortative mating. The mean paternal phenotype in this regression (mean of $\bar{z}_{f|m}$) was the same as the population mean, 51.8 d, and this indicates no selection through male function.

The second calculation, ρ = 0.58, accounted for individual variation in flower production and for the reduced fruit set in last-produced flowers (Fig. 6). Flower production was positively correlated with flowering time, and because male success is often associated with flower production (e.g., Devlin and Ellstrand, 1990; Broyles and Wyatt, 1995), late flowering should be favored through male function. However, we found that the average paternal phenotype fell to 49.8 d; thus there was a selection differential on flowering time of −2.0 d through male function (−0.19 standard deviation units). Fruit set declined as mothers reached the end of their flowering period (Fig. 6), and this imposed selection for earlier flowering time through male function. Consider the siring prospects of early- and late-flowering individuals, remembering that flowering time is approximately normally distributed. Over the season, high-quality flowers are most abundant when the plants with close-to-average flowering times approach the midpoint of their flowering periods (Figs. 3, 6). Pollen from an early-flowering plant will have access to other early plants, plus the most successful flowers on average plants. In contrast, when a late plant comes into flower its pollen has only brief access to the high-quality flowers on average plants. Its prospects deteriorate at the end of the flowering period when only the last flowers on late plants are available as recipients and these are almost sure to fail. Even if late plants make more pollen, low availability of high-quality recipients lowers male fitness.

When we restricted the analysis for female function to the marked flowers, ρ = 0.43, high-quality pollen recipients were even more limited. Because our design allowed only two flowers per maternal plant per census date to succeed, the female flowering schedule was more or less flat (cf. Fig. 6B). This damps the peak in abundance of high-quality flowers, and so late-flowering plants have even greater mate limitations on male function. This gave the regression for the revised estimate of ρ a lower slope, but similar intercept, than the first two estimates. The average paternal flowering time was lowered to 45.9 d, resulting in a selection differential of −5.9 d (−0.55 standard deviations). These calculations also indicate that functional gender correlates with flowering time (Ennos and Dodson, 1987; Campbell, 1989).

Applications—The last 25 yr have seen great progress in understanding the many factors that can impose selection on the size, shape, and temporal position of the flowering schedule. The selective factors acting on phenology are diverse and include direct effects of seasonal change in physical resources (Torres et al., 2002), seasonal change mediated by competition (Schemske, 1977; Schmitt, 1983), pollinator availability (Schemske, 1977; Gross and Werner, 1983; Torres et al., 2002), and by flower or seed predation (English-Loeb and Karban, 1983; Gross and Werner, 1983; Biere and Honders, 1996;

Bishop and Schemske, 1998; Pilson, 2000; Mahoro, 2002). Mate limitation may typically select on phenology through male function (Campbell, 1989; Devlin and Ellstrand, 1990) but Ollerton and Diaz (1999) showed it can also act through female function. Of course, selection on phenology is variable (Ollerton and Lack, 1992): in these cited studies, not all aspects of the flowering schedule were under selection in every population or generation. However, very few papers (e.g., Buide et al., 2002) report no relationship between reproductive success and some feature of flowering phenology.

We find it surprising that in this wealth of information on flowering phenology, the role of assortative mating has been so seldom considered (e.g., Ennos and Dodson, 1987; Lyons and Mulley, 1992; Fox, 2003). Studies cited in the Introduction indicate that flowering schedule components are often heritable, and in some cases the intensity of maternal or paternal environmental effects on offspring phenology may also vary genetically (Purrington, 1993; Lacey, 1996). We concur with Fox (2003) that phenotypic assortative mating on flowering phenology should be ubiquitous, and because phenological traits are often heritable, genetic assortative mating should be common. Perhaps phenological assortative mating has not received more scrutiny because of the lack of a quantitative method. Augspurger (1981) developed a synchrony index based on the overlap in individual flowering periods, but it does not account for the number of flowers produced across the period. Recently, others (Meagher and Delph, 2001; Mallo, 2002) have fitted flowering schedules to algebraic functions for descriptive purposes. However, these methods do not by themselves relate phenotype to mating probability.

More intensive studies of phenological assortative mating can contribute to our understanding of some long-standing questions in plant population biology. First among these is the contribution of nonrandom mating to speciation. Many plant species have parapatric, locally adapted subpopulations; frequently these subpopulations differ in phenology (e.g., Antonovics and Bradshaw, 1970; Schmitt, 1983; see also Mazur and LeBuhn, 1999). Kirkpatrick and Ravigne (2002) emphasize the power of disruptive selection acting directly on assorting traits to create reproductive isolation among lineages and cite flowering time as a prime example. Bridle and Ritchie (2001) argue the importance of assortative mating in shaping the adaptive landscape, saying ''An important challenge for the future is . . . to understand the ecological and genetic changes that cause divergence in quantitative traits contributing to assortative mating.'' Our methods can be used to investigate the three-way balance among selection, gene-flow, and phenological assortative mating during the initial stages of speciation.

Our methods may be also useful in addressing a very different question: why do natural plant populations maintain genetic variance in resistance to insects and pathogens (Rausher, 1996)? The fitness costs of resistance have been invoked as a constraint on defense evolution, but empirical evidence suggest that resistance costs are not often strong, compared to its benefits (Simms, 1992; Bergelson and Purrington, 1996). Recent work shows that highly defended plants flower late (Agrawal et al., 2002; Traw, 2002), suggesting that populations assortatively mate by defensive ability (Lyons and Mulley, 1992; Strauss et al., 2002), possibly inflating the genetic variance in resistance. This raises an interesting possibility. During periods of low herbivory, selection against resistance (through costs) acts on genetic variance inflated by assortative mating, which in turn drives resistance to lower levels. However, herbivore attack often delays flowering (Junger and Bergleson, 2000; Krupnick et al., 1999) which could bring susceptible (damaged) individuals into synchrony with resistant (undamaged) individuals. Several successive generations of attack would select for greater resistance, but the concomitant mating synchrony could reduce the genetic variance available for an evolutionary response. A model by Winterer and Weis (in press) suggests that the asymmetry in genetic variance between periods of no herbivory and high herbivory could allow susceptibility to persist even in resistance benefits exceed costs. Our methods could be useful to study damage-induced mating delays and the evolution of resistance.

Although our method to estimate potential phenological assortative mating is easy to apply to natural populations, results must be interpreted in light of underlying assumptions. The calculation for ρ assumes that on each day, every open flower has an equal chance of donating pollen to every other open flower (except self-flowers). Of course, close neighbors are more likely to mate than distant ones (Campbell, 1989; Devlin and Ellstrand, 1990). Low mate density in the neighborhood will cause ''sampling error,'' such that the mean phenotype of the locally available pollen donors may deviate from the populations-wide expectation (the mean of $\bar{z}_{f|m}$).

We tailored our methods to deal with hermaphrodites that mate promiscuously and repeatedly. However, there should be no great difficulty adjusting them to other mating systems. Examination of mating schedules can yield estimates of the potential for phenological assortative mating in a variety of species to address a variety of questions.

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