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The causes of selection on flowering time through male fitness in a hermaphroditic annual plant

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Flowering is a key life-history event whose timing almost certainly affects both male and female fitness, but tests of selection on flowering time through male fitness are few. Such selection may arise from direct effects of flowering time, and indirect effects through covariance between flowering time and the environment experienced during reproduction. To isolate these intrinsically correlated associations, we staggered planting dates of *Brassica rapa* families with known flowering times, creating populations in which age at flowering (i.e., flowering time genotype) and Julian date of flowering (i.e., flowering time environment) were positively, negatively, or uncorrelated. Genetic paternity analysis revealed that male fitness was not strongly influenced by seasonal environmental changes. Instead, when age and date were uncorrelated, selection through male fitness strongly favored young age at flowering. Strategic sampling offspring for paternity analysis rejected covariance between sire age at flowering and dam quality as the cause of this selection. Results instead suggest a negative association between age at flowering and pollen competitive ability. The manipulation also revealed that, at least in *B. rapa*, the often-observed correlation between flowering time and flowering duration is environmental, not genetic, in origin.

KEY WORDS: **Habitat choice, life history, mate fecundity, paternity analysis, phenology, social selection.**

Male reproductive success is determined by an individual's ability to acquire mates, and by the quality of those mates. Selection therefore favors phenotypes that improve a male's mating success, whether through competition (e.g., antler size influences male mating success in red deer, Kruuk et al. 2002) or passive encounter (e.g., rate of gamete release influences mating success in marine broadcast spawners, Levitan 1998). This principle is as true for plants as it is for animals, and for hermaphrodites as it is for diclinous species. Despite this universality, we know relatively little about selection through the male component of fitness in plants. Where male fitness is examined, it is usually in relation to traits associated with pollinator attraction and the efficiency of pollen transfer, such as floral form, reward production, and display size (Stanton et al. 1986; Broyles and Wyatt 1990; Morgan and Conner 2001; Benitez-Vieyra et al. 2006; Hodgins and Barrett 2008; Van Kleunen and Burczyk 2008). These traits fit neatly into the idea that attractiveness has a larger effect on male function than female function (Bell 1985; but see Wilson et al. 1994), but attractiveness is not the only trait to influence the male component of fitness.

Timing of reproduction has long been thought to affect male mating success (e.g., Darwin 1871; Charlesworth et al. 1987; Bertin 1988; Andersson 1994; Levitan 1998), but few have tested this hypothesis in plants. Of 87 studies reviewed in a recent meta-analysis of selection on flowering time, only five reported male fitness (Munguía-Rosas et al. 2011). Three of these were studies of flowering synchrony in rewardless orchids (O'Connell and Johnston 1998; Parra-Tabla 2004; Sun et al. 2009), which rely on naïve pollinators and bear pollinia; they are probably not representative of other taxa. In general, we would expect selection to favor plants that concentrate their pollen dispersal into the time of the season when recipients are both abundant and of high

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quality (Delph and Herlihy 2012; Austen et al. 2015). However, the flowering time optimum could also be affected by trade-offs with size at flowering (Kozłowski 1992) or other traits, such as pollen quantity or quality.

We define flowering time in annual plants as days from seedling emergence to first flowering ("age at flowering," AAF). To help describe the multiple ways this trait can affect male fitness, we partition male reproductive success into multiplicative fitness components: days in flower × flowers deployed per day in flower × mates acquired per flower deployed × ovules fertilized and matured per mate (Fig. 1; c.f. Arnold and Wade 1984; Conner 1996; Murphy 1998). Putative associations between AAF and these male fitness components can be categorized as follows.

1. Direct fitness effects of AAF (Fig. 1A). These arise if, for example, flower production (days in flower × flowers per day) is meristem limited in plants that flower young (Geber 1990) or old (Kudoh et al. 2002), or if pollen viability (and thus ovule fertilization, i.e., mates per flower × seeds per mate) decreases with meristem age, as it does in some perennials (Aizen and Rovere 1995; Bhattacharya 2005).
2. Indirect fitness effects of AAF through phenotypic covariance with condition at flowering onset (Fig. 1B). AAF frequently covaries with leaf number, plant mass, plant height, and other traits indicative of plant condition (e.g., King and Roughgarden 1983; Ollerton and Lack 1998; Shitaka and Hirose 1998; Colautti et al. 2010). Condition may in turn affect days in flower and flowers per day, attractiveness to pollinators, and/or the quantity or quality of pollen produced.
3. Indirect fitness effects of AAF through covariance with the environment experienced during flowering (Fig. 1C). Assuming low variance in emergence time, individuals that flower young begin flowering on an earlier Julian date (JD) than those that flower old. Pollinator service (Forrest et al. 2011), florivore and herbivore activity (Pilson 2000; Parachnowitsch and Caruso 2008; Austen and Weis 2015), and abiotic stress (Franks et al., 2007; Inouye 2008) can all vary temporally within a season, potentially leading to an environmentally mediated association between AAF and male fitness components.
4. Frequency-dependent fitness effects of AAF (Fig. 1D). An individual's AAF can influence the quantity and quality of conspecific interactions in which it participates. Assuming a unimodal AAF distribution and low variance in flowering duration, individuals that flower very young or old relative to the population mean should co-flower with fewer neighbors than those that flower closer to the mean. AAF can thereby affect mate availability and the intensity of pollen competition on stigmas (see Bartowska and Johnston 2014 for a female fitness analog), which can in turn affect mates per flower and seeds per mate. A sire's AAF may also affect the quality of mates with which it interacts. Plants mate assortatively by flowering time (Fox 2003; Weis et al. 2005), and female fecundity often varies with flowering time (Munguía-Rosas et al. 2011). Co-flowering with the most fecund dams ought to increase an individual's male fitness (Nakamura et al. 1989; Devlin and Ellstrand 1990; Delph and Herlihy 2012; Forrest 2014). The resulting selection on AAF is "mate fecundity selection" (Arnold 1994).

We can examine *how* selection acts on AAF by regressing seeds sired (total male fitness) on AAF and correlated traits (Lande and Arnold 1983). We can begin to understand *why* selection acts on AAF through path analysis of the associations between AAF and multiplicative fitness components (Conner 1996). Combining these approaches advances our understanding of selection on reproductive timing in plants. However, isolating the fitness effects of AAF through the phenotype–environment correlation remains problematic—plants blooming at a young age flower in an early season environment, whereas those blooming when old experience late-season conditions. Only experimental manipulation can separate the effects of age from the effects of season.

We aimed to describe the nature of selection on flowering time through male fitness in the annual plant *Brassica rapa* (Brassicaceae). Our goals were to (1) determine the direction of selection on AAF through male fitness, (2) examine the contribution of indirect selection on AAF via phenotypic correlation with condition, (3) directly test the contributions of phenotype–environment covariance to selection on AAF, and (4) directly test the contribution of mate fecundity selection.

To achieve these goals, we manipulated the direction and strength of covariance between AAF and seasonal environment, creating three experimental treatments (Fig. 1C, triple line). In the first, populations were designed so that AAF and environment (operationally encoded as JD of flowering, JDF) were positively correlated (Fig. 1C, solid line). In the second, AAF and JDF were negatively correlated (Fig. 1C, dashed line), and in the third, they were uncorrelated (Fig. 1C, gray dotted line). We coupled genetic paternity analysis with path analysis to estimate direct selection on AAF and indirect selection through covariance with condition (goals 1 and 2). We inferred the role of the phenotype–environment covariance in shaping selection on AAF (goal 3) by comparing selection across treatments: if covariance with environment were the main driver of selection, then the direction of selection on AAF should depend on the sign of the correlation between AAF and JDF. To evaluate the contribution of mate fecundity selection on AAF (goal 4), we conducted parallel paternity analyses for each treatment. The first analysis examined genotypes of offspring sampled *proportionately* across mothers according to their seed production. This sampling captured variance in male fitness arising from differential acquisition of

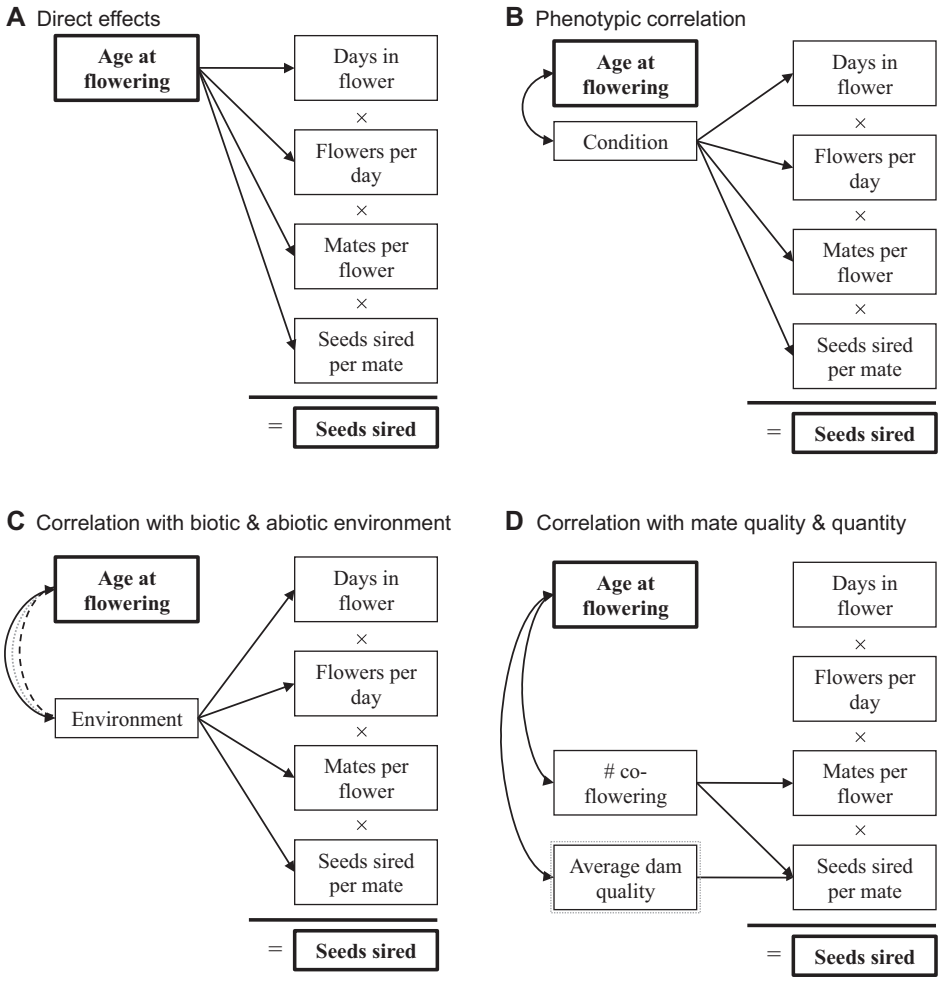

Figure 1. **Direct and indirect pathways through which a plant's age at flowering (AAF) may affect its male fitness. Double-headed arrows represent correlations, single-head arrows represent causal effects. (A) Direct effects of AAF on multiplicative male fitness components. (B) Indirect effects of AAF through phenotypic correlation with condition at flowering. (C) Indirect effects of AAF through correlation between AAF and the environment experienced during flowering. Triple line indicates this correlation altered by experimental manipulation. (D) Frequency-dependent effects of AAF: AAF may be correlated with the number of mates available or with the quality of those mates. Double box indicates variance in mate quality was manipulated by strategic sampling of offspring for genetic paternity analysis.**

high-quality mates (Fig. 1D, solid box around dam quality). The second examined offspring sampled *uniformly* across mothers, and thus revealed variance in male fitness holding female quality constant (Fig. 1D, gray dotted box).

The direction of selection on AAF through female fitness in this experiment depended on the sign of the AAF–JDF correlation (mean selection differential ± SE positive treatment: -0.328 ± 0.057; uncorrelated treatment: -0.028 ± 0.077; negative treatment: $+0.164 \pm 0.069$; $n = 5$ replicate populations per treatment across two seasons). The reversal in selection on AAF with reversal of its correlation to JDF suggested that the quality of the environment during flowering and fruit maturation was a strong determinant of female fitness (Austen and Weis 2015). Here, we report selection on flowering time through male fitness.

Methods

STUDY SYSTEM

Brassica rapa is a perfect-flowered, self-incompatible annual naturalized across much of North America (Gulden et al. 2008). In southeastern Canada, seeds germinate in the spring. Plants begin producing flower buds (bolt) ~25 days after emergence; first flowers open ~8 days later (EJA, pers. obs.). Individual flowers persist one or two days, and flower production continues long after first fruit have set. Flowers are generalist pollinated (Gulden et al. 2008); plants in this study were most visited by solitary bees, *Bombus* spp., Syrphidae and Lepidoptera (EJA, pers. obs.). Seeds used in this experiment were bulk collected from >200 plants sampled from a population of >5000 growing at the margins of a fallow field in the Eastern townships of Quebec, Canada, in

2009 (population BBF, 46.15N, 70.72W, voucher deposited at the Royal Ontario Museum [TRT]).

EXPERIMENTAL DESIGN

The experimental manipulation of covariance between AAF and JDF has been described elsewhere (Austen and Weis 2015); key details are provided here. The first (positive correlation) treatment mimicked the expected natural correlation: plants with a young AAF flowered on an early JDF, and those with an old AAF flowered on a late JDF. In the second (negative correlation) treatment, planting dates were staggered to reverse the correlation: plants with an old AAF flowered first, and those with a young AAF flowered last. In the final (uncorrelated) treatment, plantings were scheduled to eliminate the phenotype–environment correlation: AAF varied independently of JDF. If phenotype–environment covariance were the sole mechanistic cause of selection on AAF (Fig. 1C), we would expect a reversal of the direction of selection on AAF between the positive correlation and negative correlation treatments, and no effect of AAF on fitness in the uncorrelated treatment.

To accomplish the manipulation, we used seeds from a partially pedigreed population that had been subjected to two generations of perfect phenological assortative mating in the University of Toronto's rooftop glasshouse. During these two generations, individuals mated only with others that shared their exact bolting date (Austen and Weis 2015). This mating regime increased genetic variance in AAF (Fox 2003; Weis 2005), and provided approximate genetic values for AAF for ~500 maternal families. We then rearranged the target flowering times of these families, and back-calculated planting dates that would have plants of a required AAF coming into flower on the desired JDF to create planting schedules for the three treatments (Austen and Weis 2015).

To setup the experimental populations, we sowed the seeds derived from the glasshouse mating into pots according to the planting schedules, and reared plants in a poly-covered greenhouse at the University of Toronto's Koffler Scientific Reserve at Jokers Hill (KSR; 44.02N 79.52W) until bolting. As plants bolted, individuals likely to exhibit the required AAF on the desired JDF were transplanted into field plots at KSR; plants were thus transplanted after bolting but before first flowering. Transplants occurred on six days over a 25-day period, with 56 plants per plot in total (between 4 and 15 per transplant date, Austen and Weis 2015). Plants were assigned randomized positions on a 14 cm grid within their plot. Plots consisted of 1.32 m × 1.05 m × 0.30 m pine frames filled with locally sourced, nutrient poor sand, and were situated in old field habitat with > 250 m, and often a forested patch, between plots; plots were randomly assigned to treatment. *Brassica rapa* does not naturally occur at KSR. Given the large distance and complex topography between plots, we assume all mating occurred within plots (hereafter, populations). Temperature and photoperiod decreased with JD (Austen and Weis 2015), indicating that JDF was a reasonable proxy for environmental quality.

Treatments were replicated three times in 2010 and twice in 2011, and selection through female fitness was examined in all 15 populations (Austen and Weis 2015). Owing to resource constraints, male fitness is often reported for fewer populations than female fitness (e.g., Wright and Meagher 2004; Hodgins and Barrett 2008). Here, we report selection through male fitness for one population per treatment (positive correlation treatment: 2011 rep 2, uncorrelated treatment: 2011 rep 1, negative correlation treatment: 2011 rep 1, Austen and Weis 2015). The novel experimental manipulation and associated clear predictions nonetheless permit inference on the causes of selection on flowering time through male fitness.

PHENOTYPIC DATA COLLECTION

We recorded the JD each seed was sown, and observed each plant's JDF by visiting populations daily. AAF is the difference between these dates. Because mean days to emergence (approx. three to five days) is necessarily much less than mean days to flowering, we assume that the contribution of variance in emergence time to variance in AAF is negligible. We described condition at flowering by three traits: (1) number of leaves (including leaf scars) produced along the primary axis before flowering; (2) display height (distance from the soil level to the persistent pedicel of the first flower on the primary axis, measured at senescence); and (3) dry mass of the taproot at senescence. The taproot in *B. rapa* serves as a resource store for flowering and fruiting. Although resources will have been consumed by senescence, larger taproots presumably held a larger store.

We tagged all inflorescences with a date-marked Shark-Skin jeweler's tag immediately below the lowermost fresh flower every 10 days throughout the experiment. This tagging established flower production 'intervals' within each plant; fruit positioned between two tags must have been sired by another plant in flower during that interval. We counted fresh open flowers on each plant three times per interval. The sum of flower counts over a plant's life span estimates the total number of flowers it produced, and the number of days between its JDF and last nonzero flower count estimates its flowering duration. We estimated an individual's mate availability by the average number of co-flowering plants across its nonzero flower count days. We harvested and weighed fruit and seed per plant by flower production interval.

OFFSPRING SAMPLING

The contribution of mate fecundity selection to total selection on AAF (Fig. 1D) can be inferred by manipulating variance in dam

quality, which in turn can be achieved through strategic sampling of offspring for paternity analysis. Within each population, we drew two parallel offspring samples. The first, proportional, sample assumed natural variance in female reproductive success, such that mothers contributed seed to a total sample of 504 offspring in proportion to the total mass of seed they produced. The second, uniform, sample eliminated variance in female reproductive success by sampling exactly nine seeds from each of the 56 dams (504 seeds total). Estimates of selection on AAF through male fitness based on the proportional sample include effects of covariance between sire AAF and dam quality; those based on the uniform sample do not.

To create the uniform sample, we subsampled seeds from dams that contributed >9 seeds to the proportional sample, and sampled additional seeds for dams that contributed <9 seeds to the proportional sample. For each population, ~400 seeds were shared by the two samples, and ~200 were unique to one sample or the other. In eight cases, only eight seeds were available for the uniform sample (three dams in positive population; four in uncorrelated; one in negative). In a single case (one dam from positive population), just two seeds were available. For the remaining 159 of 168 dams, precisely nine seeds were sampled. By comparison, contributions to the proportional sample ranged from zero (one dam) to ≥20 seeds (eight dams, max = 26 seeds). In both samples, seeds were drawn proportionately across tagging intervals within each dam.

GENETIC DATA COLLECTION

Leaf tissue was sampled from parents as they were chosen for transplant into experimental populations. Offspring were sampled by planting seeds into plug trays and harvesting true leaves from seedlings. All tissue was dried on silica gel powder.

We extracted genomic DNA from the dried samples using an isopropanol precipitation method (Rogers et al. 1996), and characterized samples at eight microsatellite loci previously isolated from *B. rapa* or close relatives (Suwabe et al. 2002; Lowe et al. 2004; Supporting Information). Capillary gel fragment analysis of PCR product was performed by Centre for the Analysis of Genome Evolution and Function (University of Toronto). We scored genotypes using Peak Scanner version 1.0 (Applied Biosystems 2006).

Parents were amplified and scored twice at each locus. If scores disagreed across the two amplifications, we ran the sample a third time, and reexamined scores in Peak Scanner to assign a genotype. We repeated PCR and scoring for a subsample of 96 offspring, and compared genotype assignments across the two readings to estimate the allelic dropout rate (ε_1, also affected by null alleles) and the stochastic typing error rate (ε_2, includes mutation, miscalling, polymerase error, data entry error, etc.) for each locus (Wang 2004). We supplied these error rates (Table S1) to the paternity analysis (see below).

Parental genotype frequencies at two loci were inconsistent with Hardy–Weinburg equilibrium (Table S1). BRMS-042-2 exhibited a surplus of one heterozygote and deficit of another, indicating sampling error. BRMS-019 exhibited a surplus of homozygotes, suggesting a null allele may have been present (Hoffman and Amos 2005). Excluding BRMS-019 from genetic paternity analysis did not qualitatively affect results, and so we report results using all eight loci here. The combined exclusion probability was 0.91.

SELECTION GRADIENTS

We estimated selection gradients on AAF, root mass (cube-root transformed for linearity), leaves at flowering, and height using full probability paternity analysis models implemented in R package MasterBayes (Hadfield et al. 2006). Full probability models use genetic and nongenetic data (e.g., phenotypic traits) to identify the most likely father for each offspring–mother dyad (i.e., the pedigree) (Jones et al. 2010). Genetic probabilities of paternity are calculated by Mendelian transmission probabilities of microsatellite loci (Devlin et al. 1988), and effects of nongenetic information on male relative fitness are expressed in a multinomial log-linear model (Smouse et al. 1999). The log-linear model estimates effects of phenotypic traits on male reproductive success (expressed relative to the total success of the population), and can also estimate effects of pairwise distance (e.g., spatial distance) between dams and putative sires. MasterBayes uses a Bayesian framework to jointly estimate posterior distributions for the pedigree $\mathbf{P}$ and the vector $\boldsymbol{\beta}$ of log-linear model coefficients for the nongenetic data. Because the coefficients of the multinomial log-linear model are analogous to the coefficients of multiple regression, the coefficients for standardized phenotypic traits can be interpreted as selection gradients (Smouse et al. 1999; Morgan and Conner 2001; J. Hadfield, pers. comm.).

We ran separate analyses for uniform and proportional offspring samples from each of the three populations. Putative sires for each offspring were restricted to exclude self-fertilization and plants that were not flowering during the tagging interval in which the seed was produced. We allowed genotyping error at rates ε_1 and ε_2 (Table S1), but permitted no more than three mismatched loci between sire and offspring. Our model for estimating selection gradients ($\boldsymbol{\beta}$) included sire AAF, leaf number, height to first flower, cube root of taproot dry mass, and pairwise spatial distance between dams and putative sires (measured in 10 cm units) as nongenetic predictors of paternity. Including pairwise spatial distance in the paternity model accounts for some variation in fertility unrelated to the sire traits, and can therefore increase power to estimate selection (Morgan and Conner 2001). The four

phenotypic traits were standardized to mean zero and standard deviation one within each population. To estimate selection differentials, we ran additional paternity models that included only sire AAF and pairwise distance between dams and putative sires as predictors of male fertility.

We assumed uniform prior distributions for $\boldsymbol{\beta}$ and $\mathbf{P}$. We ran all models from three starting configurations for $\boldsymbol{\beta}$: first, from maximum-likelihood estimates (MasterBayes default); second, with all $\boldsymbol{\beta} = 0$; and third, with elements of $\boldsymbol{\beta}$ drawn from a normal distribution of mean = –0.1 or +0.1 (alternating along $\boldsymbol{\beta}$) and standard deviation = 0.05. Each chain was run for 100,000 iterations with a burn-in of 10,000 and thinning interval of 100. We verified convergence across chains using R package CODA (Plummer et al. 2006), and extracted posterior distributions for $\mathbf{P}$ and $\boldsymbol{\beta}$ from the first chain.

The posterior modes of $\boldsymbol{\beta}$ estimate selection gradients, and the 95% intervals of the posterior distributions provide 95% Bayesian credibility interval (BCI) for gradients. We considered a gradient to be different from zero if the BCI excluded 0. Because number of offspring sired is likely overdispersed, and MasterBayes cannot estimate overdispersion (J. Hadfield, pers. comm.), we verified the statistical significance of gradients using generalized linear models assuming a quasi-Poisson distribution. For this analysis, fitness was estimated by extracting from $\mathbf{P}$ the number of seeds sired by each individual. We do not present the results of this analysis because they agreed with conclusions based on 95% BCI.

Selection gradients and differentials on AAF describe direct (Fig. 1A) and total selection, respectively (goal 1). We inferred the contribution of phenotypic covariance between AAF and condition to total selection on AAF (Fig. 1B, goal 2) by comparing differentials and gradient. We evaluated the contribution of covariance between AAF and environment (JDF) to total selection on AAF (Fig. 1C, goal 3) by comparing gradients across populations in an overdispersed Poisson generalized linear model, with traits standardized across all three populations. We did not relativize fitness for this analysis. Because treatments were not replicated, we cautiously interpret results of this analysis in terms of the experimental manipulation. A difference in the direction of selection on AAF across populations in which the AAF–JDF correlation has been reversed (i.e., between the positive and negative experimental treatments), coupled with weak selection in the uncorrelated population, would suggest that most selection was due to covariance with environment. Finally, we inferred the contribution of covariance between sire AAF and dam quality to total selection on AAF (Fig. 1D, goal 4) by qualitatively comparing selection gradients estimated using proportional versus uniform offspring samples. If covariance with dam quality drives selection on AAF through male fitness, we would expect selection gradients in the proportional sample to be stronger than those in the uniform sample.

PATH ANALYSIS

Selection gradients describe how selection acts on AAF. We used path analysis to better understand the causes of selection. Paths incorporated sire AAF, two sire traits indicative of condition at flowering (height at flowering and cube root of taproot dry mass; we excluded leaves at flowering because we did not detect selection on this trait in any population, see Results), two frequency-dependent traits (mean daily number of co-flowering plants [i.e., synchrony], and average dam quality), four multiplicative fitness components (days in flower, flowers per day, mates per flower, and seeds sired per mate), and the estimate of total fitness (seeds sired). Effects of JDF are again subsumed into the direct effects of AAF. Synchrony, days in flower, and flowers per day (total flowers / flowering duration) were extracted from flower count data. We estimated mean dam quality, mates per flower, and seeds sired per mate using the pedigrees generated by paternity analysis of proportional offspring samples. From these pedigrees, we extracted for each individual the number of mates on which it sired seeds, the identity of its mates, and the total number of seeds it sired. Mean dam quality is the weighted mean total seed mass produced by an individual's mates, mates per flower is number of mates / total flowers on a focal individual, and seeds per mate is total seeds sired / number of mates.

Path structure was elaborated from the hypothesized associations in Figure 1, and analysis followed the three steps for path analysis on multiplicative fitness components outlined by Conner (1996). First, we calculated correlation coefficients between traits. Second, we calculated standardized coefficients for paths running from traits to fitness components by multiple linear regression. These models were as follows:

$$\text{days in flower}_i = \mu_A + \beta_{A1}\text{AAF}_i + \beta_{A2}\text{rootmass}^{(1/3)_i} + \varepsilon_{Ai}$$

$$\text{flowers per day}_i = \mu_B + \beta_{B1}\text{AAF}_i + \beta_{B2}\text{rootmass}^{(1/3)_i} + \varepsilon_{Bi}$$

$$\text{mates per flower}_i = \mu_C + \beta_{C1}\text{AAF}_i + \beta_{C2}\text{rootmass}^{(1/3)_i} + \beta_{C3}\text{height}_i + \beta_{C4}\text{co-flowering} + \varepsilon_{Ci}$$

$$\text{seeds per mate}_i = \mu_D + \beta_{D1}\text{AAF}_i + \beta_{D2}\text{rootmass}^{(1/3)_i} + \beta_{D3}\text{co-flowering} + \beta_{D4}\text{mean dam quality} + \varepsilon_{Di}$$

In each, μ is the mean of the response variable, β are model coefficients, and ε is a normally distributed error term. Subscripts A through D distinguish effects on the four multiplicative fitness components. Unexplained variance in fitness components is

estimated by $(1 - R^2)$ from the above models. Finally, we calculated the standardized coefficients of simple regressions of total fitness (seeds sired) on fitness components individually. Simple regression is appropriate because by definition, the fitness components completely describe fitness, making multiple regression invalid (Conner 1996). These simple regression coefficients are the net of direct fitness effects of components and their trade-offs with other components. When depicted in a path diagram, this three-step analysis provides an overall picture of the direct and indirect effects of AAF on seeds sired.

One or two individuals per population failed to sire any seed in the offspring sample; the mean dam AAF and seeds sired per mate were thus undefined for these few plants. Coefficients for paths involving either of these two variables were therefore calculated including only those individuals that sired at least one seed in the sample. All analyses were conducted in R (R Core Team 2015).

Results

TRAIT MEANS

As intended, the experimental manipulation altered the correlation between AAF and JDF, a proxy for the environment experienced during reproduction (Fig. 2). For the most part, the manipulation did not affect trait means or variances, but taproots were smaller in the negative treatment population (Table S2A). The weighted mean mass of seeds produced by a sire's dams also varied by population (Table S2B).

DIRECTION OF SELECTION ON AAF (GOAL 1)

Selection on AAF varied among populations (Table 1B). Both direct selection (Fig. 3A; Table 1B) and total selection (Fig. 5) favored young AAF in the positive and uncorrelated treatments, with the strongest selection detected in the uncorrelated population. Selection on AAF did not differ from zero in the negative correlation treatment (Figs. 3A, 5; Table 1B). We examined effects of AAF on multiplicative fitness components to better understand causes of selection. Flowers deployed per day decreased with older AAF in all three populations, although this association was not statistically significant in the positive population (Fig. 4; Table S5C). In the uncorrelated population only, older AAF had an additional direct, and strong, negative effect on the number of seeds sired per flower produced (mates per flower × seeds per mate, Fig. 4, Table S5D, E). Further, the fitness component mates per flower had the largest net effect on total seeds sired (Fig. 4, Table S5A). Collectively, these results suggest that the direct negative effect of AAF on mates per flower was the principal cause of selection for young AAF in the uncorrelated population.

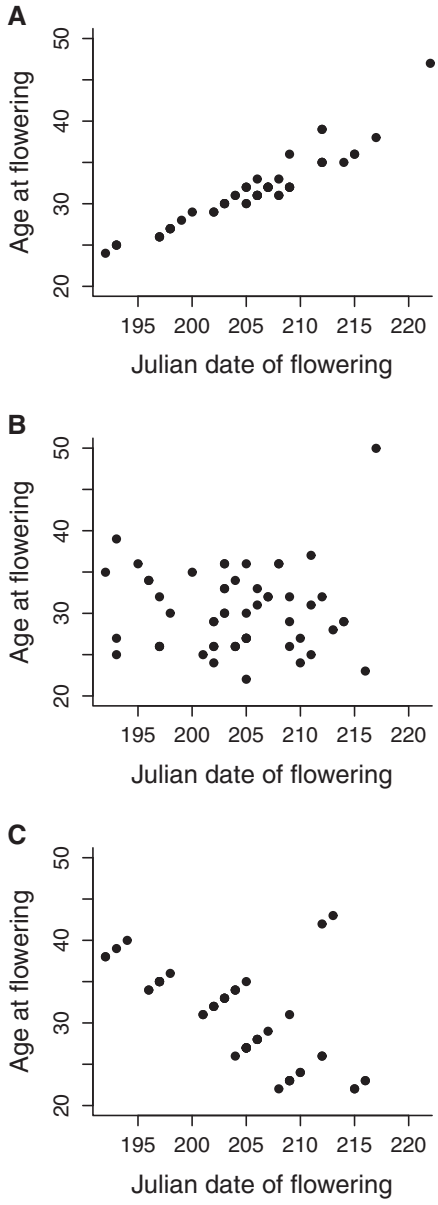

Figure 2. Association between age at flowering (days from planting to first flowering) and environment experienced during reproduction (flowering Julian date) in (A) positive correlation ($r = 0.94$), (B) uncorrelated ($r = 0.03$), and (C) negative correlation ($r = -0.66$) treatments. $N = 56$ individuals per treatment.

INDIRECT SELECTION THROUGH PHENOTYPIC COVARIANCE (GOAL 2)

In all populations, selection gradients on AAF (Fig. 3A) closely matched selection differentials (Fig. 5), indicating that phenotypic covariance with condition (Fig. 1B) contributed little to total selection on AAF. This is not because AAF did not covary with condition: both leaf number and the cube root of taproot dry mass tended to increase with AAF (Table S3A, B; correlation between AAF and root mass not statistically significant in positive population). Selection did not, however, act on leaf

Table 1. Coefficients (SE) of overdispersed Poisson generalized linear model to test for among population variation in association between traits and male fitness (number of seeds sired).

Coefficient	Estimate (SE)	*t*	*P*
(A) Intercepts			
Intercept (uncorrelated)	1.886 (0.106)	17.869	0
Intercept × positive	0.292 (0.134)	2.178	0.031
Intercept × negative	0.297 (0.133)	2.235	0.027
(B) Selection on AAF			
AAF (uncorrelated)	–0.811 (0.155)	–5.242	0
AAF × positive	0.464 (0.206)	2.251	0.026
AAF × negative	0.822 (0.194)	4.233	0
(C) Selection on number of leaves at first flowering			
Leaves (uncorrelated)	0.099 (0.133)	0.743	0.459
Leaves × positive	–0.027 (0.178)	–0.149	0.881
Leaves × negative	–0.102 (0.181)	–0.564	0.574
(D) Selection on taproot dry mass[1]			
Root mass (uncorrelated)	0.345 (0.106)	3.25	0.001
Root mass × positive	–0.26 (0.143)	–1.818	0.071
Root mass × negative	0.004 (0.135)	0.029	0.977
(E) Selection on height			
Height (uncorrelated)	0.057 (0.081)	0.704	0.483
Height × positive	–0.083 (0.129)	–0.642	0.522
Height × negative	0.268 (0.123)	2.18	0.031

Notes Dispersion parameter = 2.85. Population (positive correlation treatment, uncorrelated treatment, negative correlation treatment) coded as a dummy variable, with "uncorrelated treatment" taken as base. $n = 56$ plants in each of three populations. All traits standardized to mean = 0 and standard deviation = 1 before analysis. AAF = age at first flowering; height = distance from ground to first flower on primary axis.

[1]Root mass cube-root transformed prior to standardization.

number in any population (Fig. 3B, Table 1C), and so the lack of indirect selection on AAF through covariance with leaf number is not surprising. In contrast, selection favored greater root mass in the negative and uncorrelated treatments (Fig. 3C, Table 1D), seemingly because plants with larger roots produced more flowers (days in flower × flowers per day, Fig. 4, Table S5B, C). The fitness components days in flower and flowers per day were, however, relatively weak contributors to total variance in fitness (Fig. 4). Thus, the covariance between AAF and root mass contributed little to total selection on AAF.

Plant height was also measured as an indicator of condition, but its correlation with AAF varied among populations (Fig. 4, Table S3C). Consequently, height did not consistently contribute to indirect selection on AAF. In the negative population only, taller plants acquired more dams per flower (Fig. 4, Table S5D), leading to direct selection on height (Fig. 3D, Table 1E). However, AAF and height were uncorrelated in this population (Fig. 4, Table S3C), and so the selection on height did not affect total selection on AAF.

INDIRECT SELECTION THROUGH PHENOTYPE–ENVIRONMENT COVARIANCE (GOAL 3)

If genotype–environment covariance between AAF and JDF were the primary cause of selection on AAF (Fig. 1C), then the direction of selection on AAF in the negative population should be reversed relative to that in the positive population, and selection on AAF in the uncorrelated population should be intermediate to that in the other two. Selection favored young AAF in the positive population, and did not act on AAF in the negative (Fig. 3A). Moreover, JDF was negatively correlated with total seeds sired in these two populations (Table S4D). However, confidence intervals on selection gradients in the positive and negative populations were broadly overlapping (Fig. 3A), and, more importantly, selection was strongest in the uncorrelated population (Fig. 3A). We therefore must reject AAF–JDF covariance as a principal, universal driver of selection on AAF through male fitness.

Nonetheless, phenotype–environment covariance did affect the association between AAF and one multiplicative fitness component (Fig. 4, Table S5B). In all populations, individuals with the earliest JDF flowered longest, regardless of their AAF

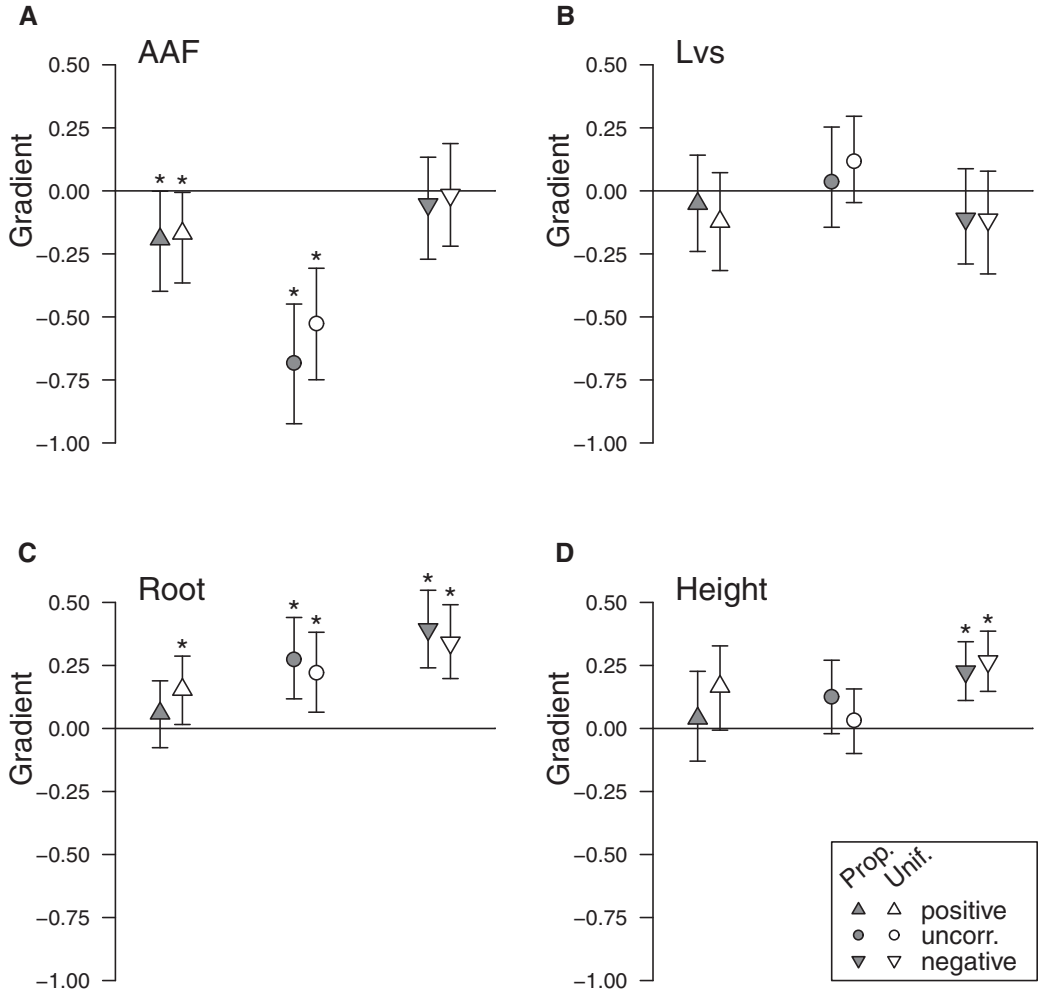

Figure 3. Selection gradients on (A) age at flowering, (B) number of leaves produced before flowering, (C) cube root of taproot dry mass, and (D) height of first flower in three experimental populations of *Brassica rapa*. Gradients and 95% Bayesian credibility intervals estimated through full probability paternity analysis models including pairwise spatial distance between dams and putative sires as a predictor of paternity in addition to the four phenotypic traits. $N = 56$ parents and ~500 offspring per population; closed symbols, offspring sampled across mothers in proportion to their total seed production; open symbols, offspring sampled uniformly across mothers. *, 95% Bayesian credibility interval excludes 0.

(Table S4C). The net effect of flowering duration on seeds sired was, however, weak or even negative (Fig. 4, Table S5A; negative net effect arises through negative correlation with other fitness components). Thus, although the experimental manipulation altered the association between AAF and this one fitness component, it did not strongly affect selection on AAF through male fitness.

The manipulation also affected the correlation between AAF and the social trait, mean number of co-flowering plants per day. In the positive and negative populations, plants with the latest JDF (old AAF and young AAF, respectively) co-flowered with the most neighbors per day (Fig. 4, Tables S3G, S4B). However, the number of co-flowering plants generally did not affect fitness components (Fig. 4, Tables S5C, D), and so again, there was no universal contribution to selection on AAF.

MATE FECUNDITY SELECTION (GOAL 4)

If sires with a younger AAF donated their pollen to more fecund mates than sires with an old AAF, or vice versa (Fig. 1D), then selection gradients on AAF estimated using the proportional offspring sample should be stronger than those estimated using the uniform offspring sample. Instead, we found no difference in selection on AAF across the two offspring samples (filled vs. open symbols, Fig. 3A). Thus, mate fecundity selection did not contribute to selection on AAF through male fitness.

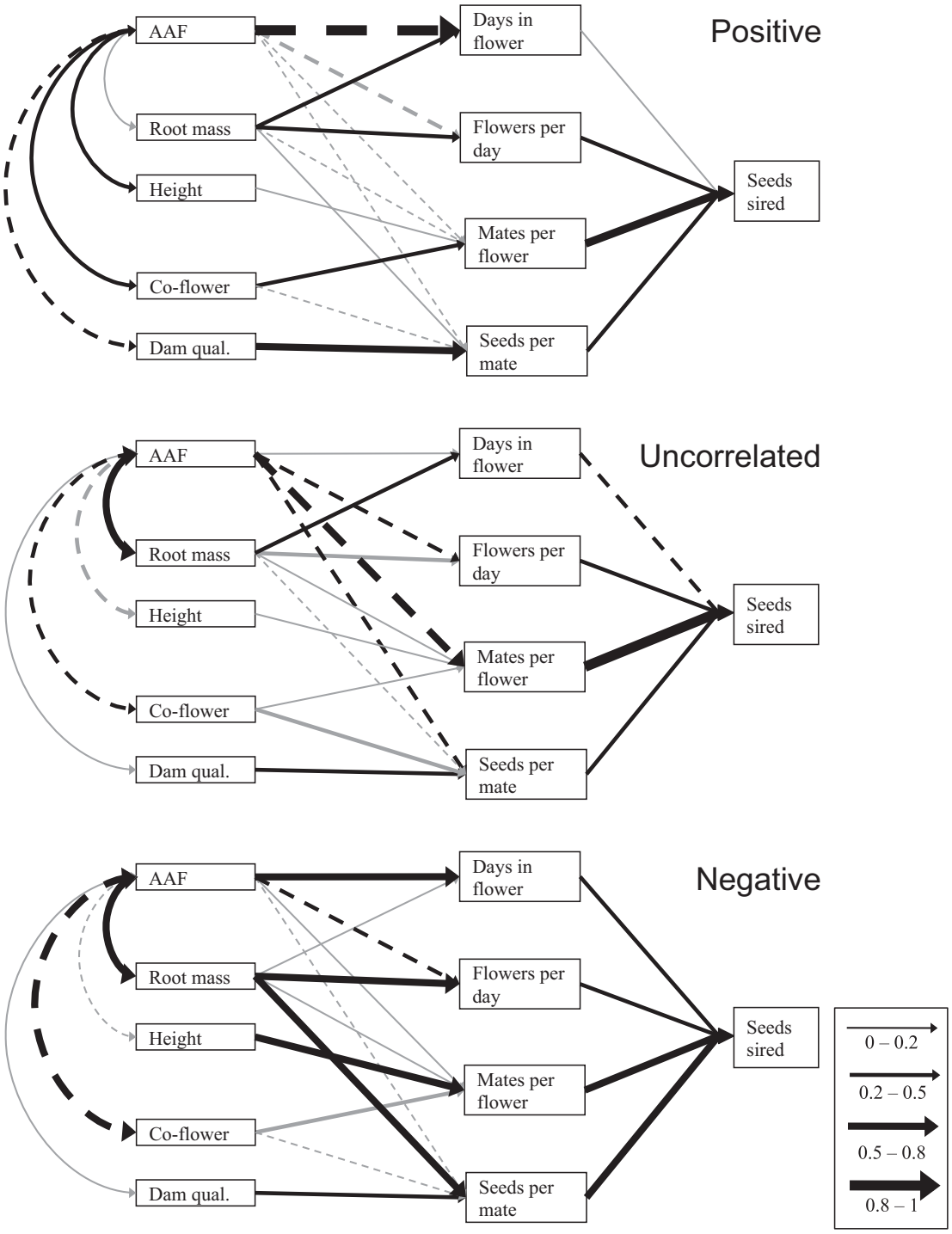

Figure 4. Path diagrams depicting direct and indirect effects of age at flowering on male fitness components in three experimental populations of *Brassica rapa*. Male fitness estimated by genetic paternity analysis in which offspring were sampled across dams in proportion to their total seed production. Double-headed arrows are correlations, and single-headed arrows are causal relationships. Unexplained variance not shown. Dashed lines represent negative coefficients; line width indicates magnitude of standardized coefficients (see legend); line color indicates statistical significance of association (black = $P < 0.05$; gray = $P \geq 0.05$). Correlations not involving age at flowering are omitted for clarity; leaves at flowering omitted because selection did not act on this trait. AAF, age at flowering; root mass, cube root of taproot dry mass; height, height to first flower on primary axis; co-flower, mean number of co-flowering plants per day in flower; dam qual., weighted mean seed production of dams with which a focal individual mated. $N = 56$ individuals per population.

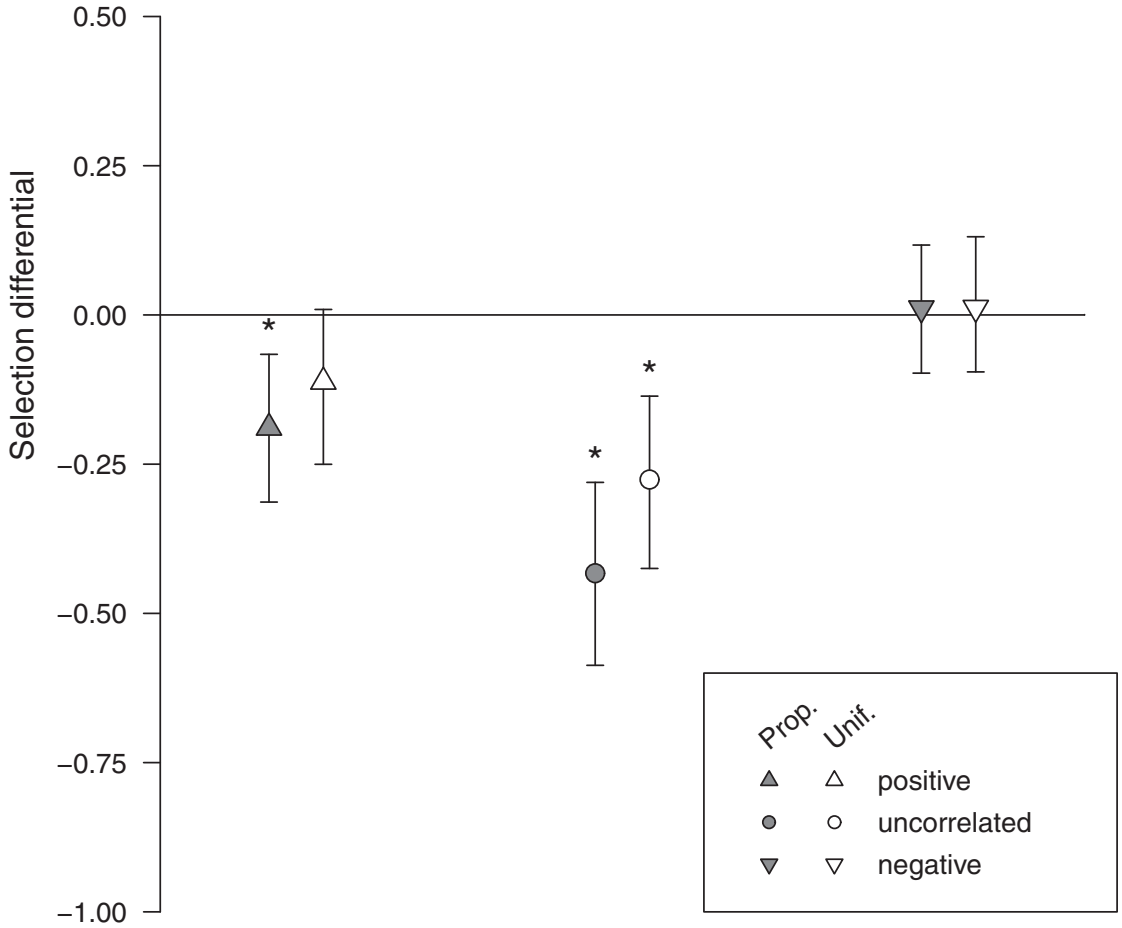

Figure 5. Selection differentials (95% Bayesian credibility interval) on age at first flowering (AAF) through male fitness in three experimental populations of *Brassica rapa*. Differentials estimated by genetic paternity analysis in a full probability model including AAF and pairwise spatial distance between dams and putative sires as predictors of siring probability. $N = 56$ parents and ~500 offspring per population; closed symbols, offspring sampled across mothers in proportion to their total seed production; open symbols, offspring sampled uniformly across mothers. *, 95% Bayesian credibility interval excludes 0.

Sires mating with higher quality dams did sire more seeds per mate in all populations (Fig. 4, Table S5E). However, sire AAF was not correlated with dam quality in the uncorrelated or negative population, precluding mate fecundity selection here. In the positive population, sire AAF was negatively correlated with dam quality (Fig. 4, Table S3H), but this did not translate to detectable mate fecundity selection (filled vs. open symbols, Fig. 3A).

OTHER FACTORS AFFECTING MALE FITNESS

In all populations, the probability of siring a seed decreased with increasing distance between dam and sire (Fig. S1). As noted above, plant condition also affected male fitness: plants with greater root mass sired more seeds in the uncorrelated and negative populations (Fig. 3C), and taller plants sired more in the negative population (Fig. 3D).

Discussion

SELECTION ON FLOWERING TIME THROUGH MALE FITNESS INVOLVES DIRECT EFFECTS AND ENVIRONMENTAL COVARIANCE

Selection through male fitness favored plants that flowered young in two of the three study populations, including the population in which AAF was uncorrelated with JDF (Fig. 3A). Before interpreting this result, we again note that treatments were not replicated. Studies estimating selection through the male component of fitness on other traits frequently find varying patterns of selection among populations (e.g., Elle and Meagher 2000; Morgan and Conner 2001; Hodgins and Barrett 2008), and in the experiment here, the strength (but not direction) of selection on AAF through the female component of fitness sometimes varied among replicates within treatments (Austen and Weis 2015). A

different picture of selection on AAF through male fitness may have emerged if it had been possible to examine more populations.

We tested three mechanisms of selection on AAF through male fitness. First, the similarity between selection gradients (Fig. 3A) and selection differentials (Fig. 5) on AAF rejected the hypothesis that selection on AAF was largely attributable to covariance between AAF and leaf number, root mass, or height (Fig. 1B). Second, the difference in strength of selection on AAF across the positive and negative populations (Fig. 3A), and negative correlation between JDF and male fitness in these two populations (Table S4D) suggested that covariance between AAF and environment during reproduction may have contributed to total selection on AAF in these populations (Fig. 1C). However, male fitness effects of AAF cannot be fully explained by AAF–JDF covariance, because the strongest selection occurred in the population where AAF varied independently of JDF (Fig. 3A). Third, the strong agreement between selection gradients estimated using proportional versus uniform offspring samples (Fig. 3A, filled vs. open symbols) rejected the hypothesis that covariance between sire AAF and dam quality drove selection on AAF (Fig. 1D). In sum, covariance between AAF and JDF (Fig. 1C) may have contributed to selection for young AAF in the positive population and to the absence of selection in the negative population, but a direct effect of AAF on male fitness (Fig. 1A) is required to explain the strong selection for young AAF in the uncorrelated population.

Few other studies have examined selection on flowering time through male fitness, and none have experimentally decoupled direct effects of the flowering time phenotype from those of the environment or mate quality. Some studies have estimated male fitness by pollen or pollinia removal; two of these found no effect of timing of flowering onset or peak flowering (Bertin and Sholes 1993; Maad 2000), and two found greater pollen removal with earlier flowering (O'Connell and Johnston 1998; Sun et al. 2009). However, pollen removal does not necessarily correspond to total male fitness (Broyles and Wyatt 1990; Devlin and Ellstrand 1990). Moreover, it is unclear whether selection occurred because AAF correlated with attractiveness, or because pollinator service varied temporally (although the rewardless nature of two species suggests decreasing pollinator attraction with later JDF). In a study using genetic paternity analysis, *Raphanus raphistrum* individuals whose peak flowering occurred early or late in the season sired more seeds than those peaking mid-season (Devlin and Ellstrand 1990). Temporal variation in dam quality was seemingly responsible—peak-flowering dams produced the fewest fruit and therefore provided the least siring opportunity—but this hypothesis was not directly tested through uniform offspring sampling. Our study design is unique for its capacity to test causal mechanisms responsible for selection on flowering time through male fitness. Below, we discuss implications of three principal findings: (1) environmental covariance between AAF and days in flower; (2) the absence of mate fecundity selection; and (3) the strong negative effect of AAF on seeds sired in the uncorrelated treatment only.

COVARIANCE BETWEEN FLOWERING TIME AND DURATION IS ENVIRONMENTAL, NOT GENETIC

Covariance between AAF and JDF affected the association between AAF and the fitness component days in flower (Fig. 4). In all populations, individuals flowering on the earliest JDF flowered longest, regardless of their AAF (Table S4C). Effects of JDF on flowering duration may have contributed to the difference in selection gradients on AAF between the positive and negative populations.

Flowering duration frequently decreases with later flowering onset (e.g., Hendry and Day 2005; Weis et al. 2014a), but the nature of this correlation has not been examined. Some have suggested a genetic basis, hypothesizing that this association could arise through adaptation to temporally varying conditions within the season, that is, through correlational selection on timing of onset and duration (Hendry and Day 2005). Our experiment tested this hypothesis by decoupling AAF from the environment experienced during reproduction, and convincingly demonstrated a purely environmental correlation: the sign of the association between AAF and duration (days in flower) depended on the sign of the correlation between AAF and JDF, and not on AAF itself (Fig. 4; Table S4C). At least in *B. rapa,* genetic correlation between flowering onset and flowering duration is unlikely.

UNCERTAIN IMPORTANCE OF SOCIAL SELECTION

Social selection—the notion that an individual's fitness may be influenced by the phenotypes of its social partners (Wolf et al. 1999; Okasha 2004)—is gaining traction in evolutionary biology (see, e.g., Christakis and Fowler 2014 for a discussion in a human context), but empirical tests are few. Mate fecundity selection, such as we examined here, arises from among-individual variance in the mean traits of social partners, and is therefore a form of social selection. In this experiment, social selection was directly tested through strategic offspring sampling (Fig. 3), and social selection gradients were estimated through the regression of seeds sired per mate onto dam quality (Fig. 4). These tests revealed social selection to be unimportant relative to other drivers of selection on AAF.

Few other studies have estimated selection owing to social partners. Stevens et al. (1995) found that viability selection in *Impatiens capensis* favored large individual size, and membership in a group with a small mean plant size. Formica et al. (2011) found opposing sexual selection on individual and group traits in demes of the beetle *Bolitotherus cornutus*: a male's expected copulation success increased with his own body size, but decreased

as he competed with larger males. Because large males tended to interact with smaller males, both individual and group selection contributed positively to total selection on male size in this case. We detected negligible social selection. With so few data available, the overall importance of social selection in influencing evolutionary trajectories remains unclear.

VARIATION IN COMPETITIVE ABILITY EXPOSED?

The strong selection for young AAF in the uncorrelated population raises the interesting possibility that pollen competitive ability is genetically correlated to AAF. Theory suggests that genetic variance in gamete competitive ability is quickly exhausted by rapid fixation of the superior alleles (Haldane 1932; Charlesworth et al. 1987). However, under normal circumstances, pollen produced by plants that begin flowering at a young age will not often compete with that produced by plants that begin flowering when old. Plants mate assortatively by JDF ("phenological assortative mating"; Fox 2003). By extension, pollen grains landing on a stigma *compete* assortatively by JDF. In the positive and negative correlation treatments, JDF and AAF were correlated. Direct assortative mating by JDF therefore led to indirect assortative mating (and assortative competition) by AAF (Weis 2005): young AAF pollen should have competed primarily with young, and old with old. In contrast, in the uncorrelated treatment, mating and competition were random with respect to AAF: pollen produced by young AAF and old AAF plants circulated at every date, and so the two extremes should have regularly competed on the same stigma. If pollen quality declines with AAF, the superiority of pollen from young AAF plants would be most evident in the uncorrelated treatment, and would manifest as (1) a direct negative effect of old AAF on mate acquisition (Fig. 4), and (2) selection for young AAF through male function (Fig. 3A). In natural populations, assortative competition should mask this effect, and thereby ease selection on AAF through this avenue. Although conclusions drawn from a single replicate population per treatment are necessarily tentative, the possibility of a negative direct effect of AAF on pollen quality merits closer study.

SELECTION ACTS VIA DIFFERENT MECHANISMS THROUGH MALE AND FEMALE FITNESS

Studies examining selection through only one gender role risk misrepresenting total selection acting on a trait (Conner 1996). Weak mate fecundity selection on AAF in this experiment suggests that selection through male fitness was not tightly constrained by selection through female fitness. We previously reported that selection through female fitness tended to favor early JDF, regardless of AAF (Austen and Weis 2015). Taking the positive correlation treatment to be the closest approximation of a natural population, we infer that early flowering likely enhances both male and female function in *B. rapa*. Delph and Ashman (2006) term this agreement "harmonious" selection, and find that selection on other plant traits is often harmonious, too.

Despite harmony in direction, the mechanistic cause of selection of selection on AAF may differ for male and female fitness. Early flowering increased female fitness through its correlation with the temporal slice of the seasonal environment experienced: early plants benefited from favorable conditions for seed maturation and, in one year, escape of seed predation (Austen and Weis 2015). In contrast, from a male fitness perspective, covariance with environment seemed to contribute only weakly to selection on AAF (positive and negative populations), and AAF had a strong, direct, negative effect on male fitness in at least one population (uncorrelated treatment). This mechanistic difference means that selection to maintain optimal AAF for male function could conceivably slow response to selection for earlier or later JDF through female function.

Experimental manipulation helps to isolate the pathways through which traits affect fitness (Mitchell-Olds and Shaw 1987; Wade and Kalisz 1990; Weis et al. 2014b). Through such manipulation, we have shown that the tendency of selection to favor early flowering through male fitness in *B. rapa* is not a result of early flowering plants flowering at a larger size or accessing higher quality mates. Instead, association between AAF and environment seems to have weakly contributed to selection through male fitness in the positive and negative populations, and a strong, negative effect of AAF on mate acquisition was detected when plants mated, and competed, randomly with respect to AAF (uncorrelated population). We cannot at present fully resolve the precise effects of AAF on mating success, but our experiment has served to identify the pathways of greatest effect, and thus of greatest interest for future study.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.5g5rp.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Text. PCR protocol.
Table S1. Microsatellite loci and PCR conditions.
Table S2. Trait means and standard deviations by population.
Table S3. Phenotypic correlations between traits by population.
Table S4. Correlations between JDF and phenotypic traits, frequency-dependent traits, and fitness components.
Table S5. Path analysis coefficients.
Figure S1. Association between pairwise distance between plants and probability of paternity.