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What drives selection on flowering time? An experimental manipulation of the inherent correlation between genotype and environment

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The optimal timing of the seasonal switch from somatic growth to reproduction can depend on an individual's condition at reproduction, the quality of the environment in which it will reproduce, or both. In annual plants, vegetative size (a function of age at flowering) affects resources available for seed production, whereas exposure to mutualists, antagonists, and abiotic stresses in the environment (functions of Julian date of flowering) influences success in converting resources into offspring. The inherent tight correlation between age, size, and environment obscures their independent fitness contributions. We isolated the fitness effects of these factors by experimentally manipulating the correlation between age at flowering and date of flowering in *Brassica rapa*. We staggered the planting dates of families with differing ages at flowering to produce experimental populations in which age at flowering and date of flowering were positively, negatively, or uncorrelated. In all populations, plants with an early date of flowering produced more seed than those flowering late, regardless of age or size at flowering onset. The temporal environment was thus the principal driver of selection on flowering time, but its importance relative to that of age and size varied with the presence/absence of herbivores and seed predators.

KEY WORDS: *Brassica rapa*, herbivory, life history, natural selection, phenology, seed predation.

Timing of reproduction, condition at reproduction, and fitness are fundamentally related. Among animals, individuals that breed early during a season tend to be in better condition, and to achieve greater reproductive success, than those that breed late (e.g., birds, Verhulst et al. 1995; fish, Schultz et al. 1991; small mammals, Réale et al. 2003). Similarly, in plants, flowering time within the growing season usually covaries with fitness (reviewed in Munguía-Rosas et al. 2011) and with size at flowering (King and Roughgarden 1983; Dieringer 1991; Kelly 1992; Mitchell-Olds 1996; Ollerton and Lack 1998; Shitaka and Hirose 1998). These associations raise the question, what governs the optimal timing of reproduction: size at the start of reproduction, the environment during reproduction, or both?

Life-history theory uses optimal control models to investigate breeding time as a trade-off between investment in growth versus reproduction (Fox 1992). In these models, organisms produce energy at some production rate, and allocate the produced energy to growth *versus* reproduction according to a temporally varying control function. The model is optimized by finding the control function that maximizes some measure of fitness, typically reproductive biomass (Kozłowski 1992). The timing of the switch to reproduction in this function is the optimal breeding time.

Several variations on optimal control models are possible, each emphasizing different aspects of the size–time trade-off. Some model the energy production rate as a function of size (e.g., King and Roughgarden 1983; Ejsmond et al. 2010; Weis et al. 2014), and some as a function of temporally varying environmental conditions (Johansson et al. 2013). Others incorporate environmental variation by discounting the value of reproductive investment (i.e., offspring production) later in the season (Ejsmond et al. 2010). However, one axis of the size–time trade-off is not captured: optimal control models do not consider

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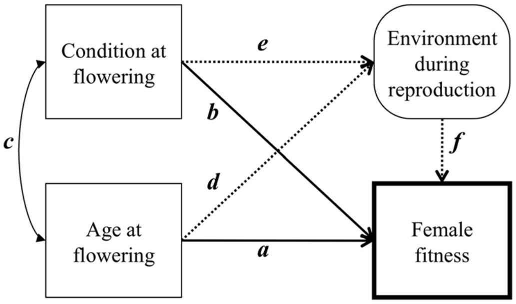

Figure 1. Path diagram tracing association between age at flowering and female fitness through its correlation with condition at flowering onset and with the environment experienced during flowering (operationally encoded in our study as Julian date of flowering). Phenotypes, phenotypic correlations, and phenotypic effects on fitness are indicated by square boxes and solid arrows; environment, phenotype–environment associations, and environmental effects on fitness are indicated by rounded boxes and dashed arrows.

that the modular nature of plant development may impose direct constraints on reproductive output (Fox 1992). Growth and reproduction in plants occurs at meristems, which can take one of three states: vegetative differentiation, reproductive differentiation, or quiescence. Direct constraints arise if flowering time affects meristem number, or the probability of meristem quiescence (Geber 1990; Duffy et al. 1999; Kudoh et al. 2002).

Of course, models are meant to be simplified representations of the natural world, and attempting to bring all possible effects into a single model would make for ugly, and perhaps intractable, mathematics. Experiments aimed at determining which paths dominate the association between reproductive timing and reproductive output under which conditions can help guide model development. We stress that many paths are possible (cf., Ehrlén 2015). First, flowering time (defined here as days from germination to flowering in an annual plant, "age at flowering" or AAF) may directly affect reproductive output through developmental constraints and meristem limitation (Fig. 1, path *a*). Second, AAF may indirectly affect fitness through phenotypic correlation with size and condition at the onset of reproduction if larger plants make a greater absolute reproductive investment (Fig. 1, compound path *cb*). Indirect effects of AAF owing to correlation with condition could also arise if condition affects the environment experienced during reproduction (Fig. 1, path *e*; e.g., if large plants attract more pollinators), but we do not distinguish such selection on AAF (Fig. 1, path *cef*) from the direct effects of condition (Fig. 1, path *cb*). Third, AAF may indirectly influence fitness through phenotype–environment covariance (Fig. 1, compound path *df*): assuming all else is equal (i.e., low variance in germination time), AAF determines the Julian date (JD) on which a plant begins flowering (Julian date of flowering, JDF), and therefore partially determines which interval of the seasonally shifting environment the plant occupies during reproduction (Fig. 1, path *d*). JDF can influence phenological matching with effective pollinators (e.g., Rafferty and Ives 2012), seed predators and herbivores (e.g., Pilson 2000), and abiotic environmental stress (e.g., drought, Franks et al. 2007; frost, Inouye 2008), and can thereby affect reproductive output (Fig. 1, path *f*). The inherent correlation between breeding time and condition on the one hand, and the temporal environment experienced during reproduction on the other, presents a major challenge to isolating the independent fitness effects of flowering time.

Experiments in animals, especially birds, have manipulated the association between condition at breeding and breeding JD to isolate the independent effects of these variables (reviewed by Verhulst and Nilsson 2008; see Einum and Fleming 2000 for an example in fish). Analogous experiments in plants are lacking. This is surprising, because manipulating a plant's flowering time is probably easier than manipulating an animal's breeding time (e.g., Augspurger 1981; Griffith and Watson 2006; Galloway and Burgess 2009). Some investigators have planted seeds in two or more cohorts to extend the range of the temporally varying environment experienced by a study population (e.g., Pilson 2000; Parachnowitsch and Caruso 2008), but such studies do not fully manipulate the AAF–JDF genotype–environment correlation: plants flowering earliest are still those that are genetically inclined to flower young, and those flowering last are those that are inclined to flower old.

We aimed to isolate the direct fitness effects of AAF, condition at flowering, and the slice of the temporal environment experienced during reproduction (which we operationally encode as JDF) by manipulating the genotype–environment correlation between AAF and JDF (Fig. 1, path *d*). In effect, we displaced AAF genotypes across a temporal JDF gradient, making the experiment analogous to a reciprocal transplant across a spatial gradient. To achieve this manipulation, we staggered the planting dates of families of the annual plant *Brassica rapa* (Brassicaceae) with known approximate genotypic values for AAF. This created three experimental treatments in which the correlation between AAF and JDF was positive (the presumed natural condition), negative, and zero.

We asked three questions. First, is the relationship between flowering time and fitness attributable to a direct effect of AAF, to an indirect effect through its phenotypic correlation with condition, or to its correlation with the environment? If a direct effect, we expect selection on AAF to be unaffected by its correlation with JDF, that is, to be uniform across treatments. If condition, we expect direct selection on AAF to be weak, and selection on condition to be similar across treatments. Finally, if environment, we expect the direction and strength of selection on AAF to depend on its association with JDF, that is, to reverse direction with the reversal of the genotype–environment correlation. Second, does the relative importance of age, condition, and environment depend on the presence/absence of antagonists? Herbivore and seed predator abundance varied greatly between the two years of our study. Thus, if antagonist presence altered the relative importance of direct and indirect effects, we would expect the contribution of various fitness effects to vary by year. Third, did antagonists act as agents of selection on flowering time during the year of strong herbivory and seed predation? If yes, we would expect damage to correlate with flowering time, and plants to be unable to fully compensate for this damage. The fitness effects of enemies can be complex. Herbivores consume vegetative biomass, which intuitively should reduce reproductive output. However, plants may respond to herbivore (or other) damage by activating quiescent meristems and producing new tissue (Strauss and Agrawal 1999). If compensation is sufficient, herbivores may have no net effect on reproduction.

Our experiment moves beyond asking *whether* selection acts on flowering time, and begins to ask *why*. The timing of transition to reproduction may intrinsically impact fitness. Alternatively, fitness effects may arise because this timing determines which slice of the temporal environment is experienced, making flowering time analogous to habitat choice (Bazzaz 1991; Donohue 2003; Hendry and Day 2005). We examined an annual plant. Fitness effects may differ in perennials because (1) perennials vary not only in flowering time within a season, but also in years to first flowering, and (2) flowering time–size trade-offs within a season may differ in perennials (Forrest and Miller-Rushing 2010; Ehrlén 2015). Nonetheless, our main premise—that understanding causes of selection on flowering time requires dissection of the multiple pathways by which this trait affects fitness—will still apply. This article reports selection through female fitness; we will present selection through male fitness elsewhere.

Methods

STUDY SYSTEM

Brassica rapa is a perfect-flowered, self-incompatible annual naturalized in North America (Gulden et al. 2008). In southeastern Canada, seeds germinate in the spring. Plants grow initially as rosettes; flower buds form at the apical meristem approximately 25 days after emergence and plants then rapidly elongate (bolting). Additional inflorescences may form at axillary meristems. Flowering begins approximately eight days after bolting (E. J. Austen, pers. obs.). Plants continue producing buds long after the first flowers have matured into dry, dehiscent fruit. Flowers persist one or two days, and are pollinated by generalist bees, small flies, and occasional butterflies.

Enemies of *B. rapa* in Canada include diamondback moth caterpillars (*Plutella xylostella* Lepidoptera: Plutellidae) and seed weevil larvae (*Ceutorhynchus obstrictus* Coloeptera: Curculionidae) (Gulden et al. 2008). Adult *P. xylostella* lay eggs on leaves. First instar larvae feed as leaf miners; later instars consume many tissues, including leaves, flower buds, immature fruit, and the outer layers of mature fruit walls (Brown et al. 1999). Affected gynoecia are fully consumed, or mature into pods lacking surface tissue. In contrast, *C. obstrictus* lays eggs directly inside maturing fruit. Larvae consume approximately five seeds before chewing an exit hole and dropping to the soil (Cárcamo et al. 2001). The damaged, distorted fruit bear oviposition and exit holes.

Seeds used in this experiment were bulk collected from >200 plants in a naturalized population of >5000 growing at the margins of a fallow field in the Eastern townships of Quebec, Canada, during 2009 (population BBF, 46.15N, 70.72W, voucher specimen deposited at the Royal Ontario Museum [TRT]).

EXPERIMENTAL DESIGN

To manipulate the association between AAF and the environment experienced during reproduction (Fig. 1, path *d*), we staggered planting dates of *B. rapa* families with approximately known genetic values for AAF. This created three experimental treatments, with three replicate populations per treatment during 2010, and two per treatment during 2011. In the positive correlation treatment, plants flowering at the youngest age began flowering on the earliest JD, and those flowering at the oldest age began on the latest JD. Selection on AAF owing to its correlation with environment (Fig. 1, path *df*) is subsumed into direct selection on AAF (Fig. 1, path *a*), as it would be in a natural population. In the negative correlation treatment, plants flowering at the oldest (youngest) age flowered first (last), thereby reversing the sign of age–environment covariance (Fig. 1, path *d*). Selection on AAF owing to its correlation with environment (Fig. 1, path *df*) is again subsumed into direct selection on AAF (Fig. 1, path *a*), but its contribution is reversed relative to that in the positive correlation treatment. In the uncorrelated treatment, AAF varied independently of JDF (Fig. 1, path $d = 0$): selection gradients on AAF reflect direct effects of AAF (Fig. 1, path *a*) only. The experiment was run at the University of Toronto's Koffler Scientific Reserve at Jokers Hill (KSR, 44.02N, 79.52W).

The experimental design required prior knowledge of genetic value for AAF. Therefore, in advance of the experiment, we developed genetic lines that varied in AAF by rearing the descendants of approximately 500 bulk-collected field seeds in a rooftop glasshouse for two generations. During September 2009, we planted three seeds per pot (approximately 1500 total) into 164 mL "Cone-tainer" pots (Stewe & Sons, Corvallis, OR) filled with a 3:1 mixture of ProMix BX potting medium (PremierTech Horticulture, Riviere-du-Loup, Canada) to concrete sand, supplemented with a 100-day slow release 14:14:14 Nutricote fertilizer

(Sun Gro Horticulture) at 600 mL per 100 L soil. Emergence began three days after planting; we thinned to a single seedling two days later. Plants were watered daily and fertilized with 15 mL of a 0.5 g/L 20:20:20 formula 10 and 17 days after planting. All plants bolting on a given day were moved as a group to separate bench space. We pollinated roughly three times per week by brushing a clean crafts feather against all inflorescences within a bolting group; individuals mated randomly with those others sharing their exact bolting time. Every plant acted as both dam and sire, and dams received pollen from multiple sires. This perfect phenological assortative mating enhanced genetic variance in AAF (Fox 2003; Weis 2005). We harvested fruit by maternal family, and planted three seed per maternal family in January 2010. Plants were thinned, fertilized, and pollinated as above, and fruit was again harvested by maternal family. After these two generations, a maternal plant's age at bolting was a strong predictor of the offspring mean (Fig. S1; standardized midparent–offspring regression, $h^2 = 0.73$).

To create the experimental populations, we divided the range of AAF observed in the glasshouse generations into cohorts (nine cohorts in 2010, six in 2011, Tables S1, S2, and S3). We rearranged the temporal sequence of cohorts to achieve positive, negative, and no correlation between AAF and JDF, and back-calculated the planting dates that would result in plants of the required AAF coming into flower on the desired JD (Tables S2 and S3). The resulting planting schedule was staggered over several weeks. For each required plant, we sowed four to 10 seeds from maternal families representing the appropriate cohort singly into Cone-tainer pots filled as above (replacing ProMix with Sunshine Mix, Sun Gro Horticulture, in 2011). Plants were fertilized as above, and reared under a polycovered greenhouse at KSR until bolting. The number of maternal families within glasshouse bolting groups exceeded the number of plants required in experimental populations (Table S1). Thus, with few exceptions, maternal families were not used more than once within populations (24 of the 423 experimental plants during 2010, and 20 of the 336 experimental plants during 2011 were doubles of the same maternal family within a population).

As plants bolted, we selected individuals exhibiting the target AAF for transplant into experimental populations; plants were transplanted after bolting but before first flowering. Transplanting occurred on nine days over a 32-day period in 2010, and on six days over a 25-day period in 2011. Figure 2 presents the achieved association between AAF and JDF. Subsets of individuals exhibiting a perfect positive correlation (Fig. 2A, C, D, F) are plants of the same AAF cohort, transplanted on the same JD. In 2010, accelerated plant development under summer conditions (compare Fig. S1 x to y range) forced us to begin transplants for the negative correlation and uncorrelated treatments on JD 165 (June 14), one week before beginning transplants for the positive correlation treatment (JD 173, June 22; Fig. 2). We adjusted planting schedules in 2011 so that transplants in all populations began JD 190 (July 9; Fig. 2).

The chosen individuals were transplanted with all soil from their pots into randomized positions in 1.32 m × 1.05 m × 0.30 m pine frames. We filled frames with a locally sourced, nutrient-poor sand to ensure that early-transplanted individuals could not capitalize on local soil resources. During 2010, each population (three replicates per treatment) comprised 47 plants in a hexagonal grid with 15 cm spacing between nearest neighbors. During 2011, populations (two replicates per treatment) were composed of 56 plants on a square grid with 14 cm spacing. We watered as required. Populations were situated >250 m from one another (Fig. S2), and were open pollinated. Each was surrounded by old field vegetation (grasses and forbs). *Brassica rapa* does not naturally occur in the study area.

DATA COLLECTION

We recorded the JD of seed planting and JDF (observed during daily site visits). AAF is the difference between these dates. At transplant, we counted leaves (including leaf scars) along the primary axis and measured the length and width of the largest leaf. We counted fresh open flowers per plant every four days in 2010, and three times during each 10-day period in 2011. The sum of flower counts estimates the total number of flowers produced, and days between the JDF and last nonzero flower count estimates flowering duration.

We harvested fruit by maternal plant as they matured, beginning on JD 203 (July 22) during 2010 and on JD 222 (August 10) during 2011. Fruits of individual plants were divided among several envelopes from harvests on different days. We counted and weighed all fruits per envelope, and cleaned seeds from fruits to weigh seeds. At senescence, we measured plant height as distance from the soil level to the tip of the tallest branch (2010). During 2011, we instead measured height as distance from soil to the display height of the first flower (i.e., persistent pedicel of the first flower on the primary axis) because the 2010 measure was likely influenced by growth following flowering as well as size at flowering. We weighed shoots (excluding fruit) and roots after drying them at 70°C for at least three days. Thus, in total, we examined three proxies for condition at flowering: leaf number, which determines axial meristem availability and photosynthetic area (leaf size and number correlation: Pearsons r [mean ± SD] $= 0.35 \pm 0.14$, $N = 15$ populations); cube root of taproot dry mass, which is a linear dimension of stored resources at flowering (Mitchell-Olds 1996); and height, which may affect perception by pollinators (Conner and Rush 1996; Donnelly et al. 1998).

During 2010, populations experienced intense herbivory by *P. xylostella* caterpillars and seed predation by *C. obstrictus*

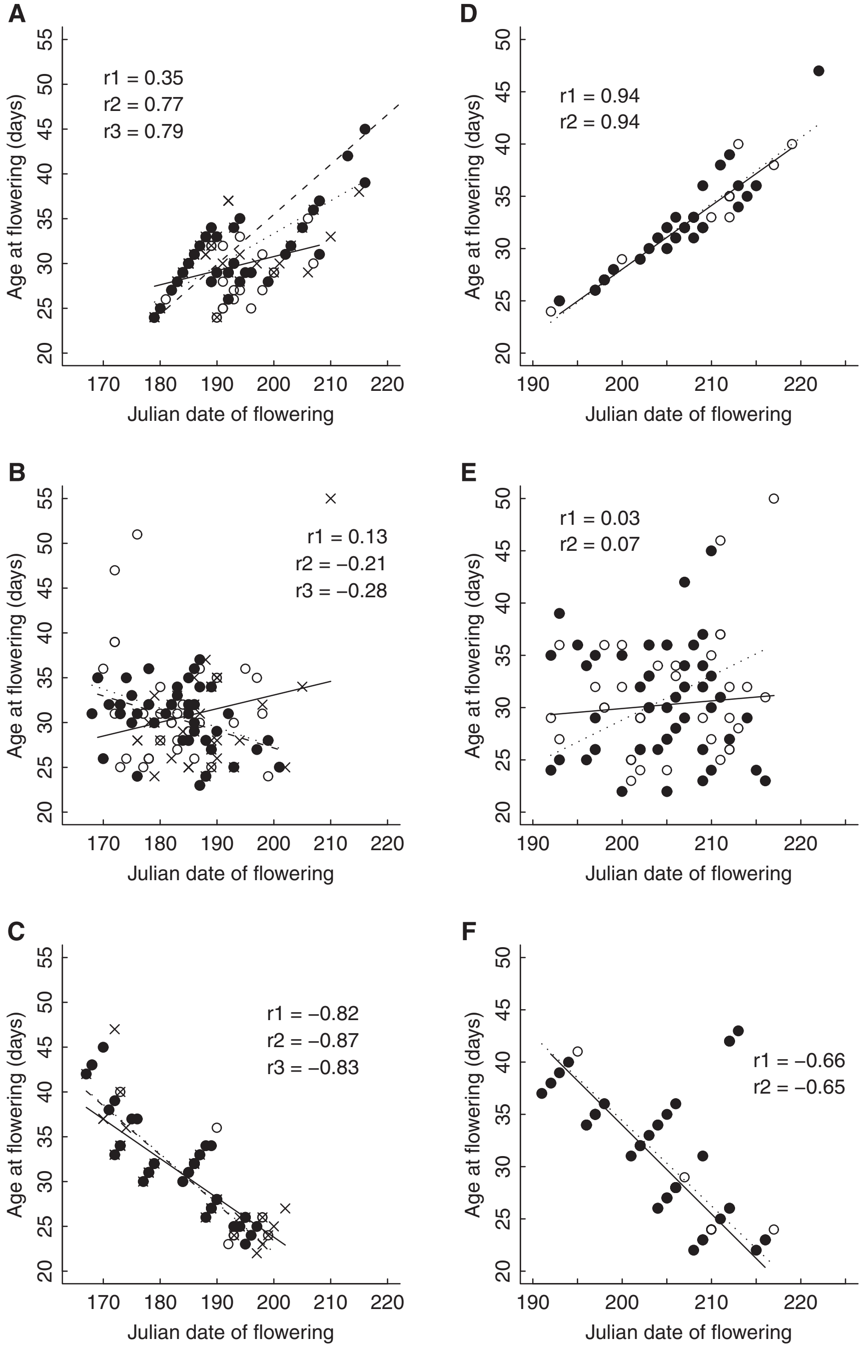

Figure 2. Associations between age at flowering (days from planting to first flowering) and environment experienced during reproduction (JDF) in positive correlation (A, D), uncorrelated (B, E), and negative correlation (C, F) treatments during 2010 (A–C, three replicate populations per treatment, 47 individuals per replicate) and 2011 (D–F, two replicates per treatment, 56 individuals per replicate). Within each panel, replicate populations are plotted by solid points and lines (rep1), open points and dotted lines (rep2), and crosses and dashed lines (rep3, 2010 only). r1 through r3 report Pearson correlation coefficients by replicate. Axes in (A) exclude one individual in rep3 that flowered on Julian date 242 at age 71 days; this individual was included when calculating the correlation coefficient.

larvae. These enemies were nearly absent during 2011, and when they did occur, we controlled their populations using *Bacillus thuringiensis* var. *kurstaki* (Safer's, Woodstream Canada Corporation, Brampton, Ontario), and Malathion 50 EC (Wilson, Nu-Gro IP, Inc., Brantford, Ontario), respectively, mixed to label specifications and sprayed during late afternoon to minimize harm to pollinators. The 2010 caterpillar infestation peaked on JD 194 (July 13). We visually assessed damage on JD 202 using a 5-point scale for loss of female reproductive effort (i.e., consumed or damaged flowers and fruit): 1 = 0–10% loss, 2 = 11–39%, 3 = 40–60%, 4 = 61–89%, and 5 = 90–100% loss. During 2010, the density of adult seed weevils peaked around JD 222 (August 10); we quantified the damage caused by weevils while cleaning fruit and weighing seeds. We examined fruit for distortion, holes, and frass, and estimated the percentage of seed lost. We calculated weevil damage to a plant as the weighted mean percentage of seed loss across the fruit envelopes of that plant.

SELECTION ANALYSIS

We estimated selection via female success on AAF and three traits that indicate condition at reproduction: leaf number, cube root of taproot dry mass, and height. We did not estimate selection on JDF because (1) we consider JDF to be a measure of the environment experienced during reproduction, not a characteristic of the plant; and (2) JDF is tightly correlated with AAF in the positive and negative treatments (Fig. 2), precluding its inclusion in the same model. Effects of JDF (Fig. 1, path *df*) are subsumed into the direct effects of AAF in the positive and negative treatments, and so the contribution of environment (JDF) to selection on AAF can be inferred by comparing gradients on AAF across the three treatments.

Traits were z-transformed within populations before analysis, and relative fitness was calculated as a plant's total seed mass divided by the population mean. We calculated total selection differentials on AAF through regression of relative fitness on standardized AAF only, and calculated directional selection gradients through multiple regression of relative fitness on all four standardized traits (Lande and Arnold 1983). Because the fitness measure (total seed mass) was left-skewed, we tested the statistical significance of associations between fitness and traits (i.e., differentials and gradients) in generalized linear models with a gamma distribution and log link. These models cannot accommodate values of 0 in the response variable, so when testing statistical significance of gradients, we replaced fitness observations of 0 g with the smallest measurable value (0.0001 g). This affected just six of the 759 plants in the experiment. These analyses were conducted independently for each population.

To test for stabilizing or disruptive selection, we repeated the multiple regression for each population, including all quadratic and correlational terms (Lande and Arnold 1983). We doubled the partial regression coefficient estimates for the four quadratic terms (Stinchcombe et al. 2008). Because nonlinear selection may act on combinations of traits other than those we defined, we also examined curvilinear selection on orthogonal axes derived through canonical rotation of the gamma matrix. Briefly, this analysis rotates the matrix of quadratic and correlational selection coefficients to find the axes (eigenvectors) of greatest curvature, which may correspond to combinations of the measured traits (Blows and Brooks 2003). We conducted a permutation test for statistically significant curvilinear selection on canonical axes, using an R script (R Development Core Team 2012) developed by Reynolds et al. (2010).

YEAR AND TREATMENT EFFECTS ON SELECTION

We tested for year and treatment effects on the association between traits and fitness in a generalized linear model using a gamma distribution with a log link. Because neither multiple regression with quadratic terms, nor canonical selection analysis suggested strong curvilinear selection (see below), we tested for year and treatment effects on directional selection gradients only. To ensure that any detected effects reflected true differences in fitness–trait covariances (and not, e.g., differences in trait distribution across populations), we standardized traits across all 15 populations for this analysis. We again replaced observations of 0 g seed mass with 0.0001 g, and did not mean-relativize fitness for this analysis. Treatment and year effects were evaluated through analysis of deviance on nested models. We began with a full model that included the four traits and their interactions with year and treatment. Working with one trait at a time, we sequentially removed the three-way interaction, the two-way interaction with year, and the two-way interaction with treatment.

Ideally, this analysis would include replicate populations nested within the year-by-treatment effects, but this caused convergence failure. Results of the simpler analysis of year and treatment effects were consistent with expectations based on selection gradients (see below), and so we are confident that although including population may have improved statistical power to detect fixed effects, it is unlikely that it would have changed interpretation. To further verify that variation among replicate populations did not drive or obscure treatment effects, we ran additional analyses to test population-by-trait interactions for each treatment-by-year separately. This revealed five instances (of 24 total) in which associations between a trait and fitness varied significantly among replicate populations (Table S4). In two instances, selection on AAF varied among replicate populations (Table S4), but these were both cases where selection varied in strength, not direction.

EFFECTS OF HERBIVORES AND SEED PREDATORS IN 2010

Plutella xylostella and *C. obstrictus* may have acted as agents of selection on AAF in 2010. To investigate this possibility, we first examined whether exposure to these antagonists varied with JDF. We tested for a temporal trend in *P. xylostella* damage score with proportional ordered logistic regression with population and centered JDF as predictors (Venables and Ripley 2002). We examined temporal trends in *C. obstrictus* damage with logistic multiple regression models using estimated proportion of seeds lost (weighted by the total number of fruit produced) as a response variable, and using centered JDF, population, and their interaction

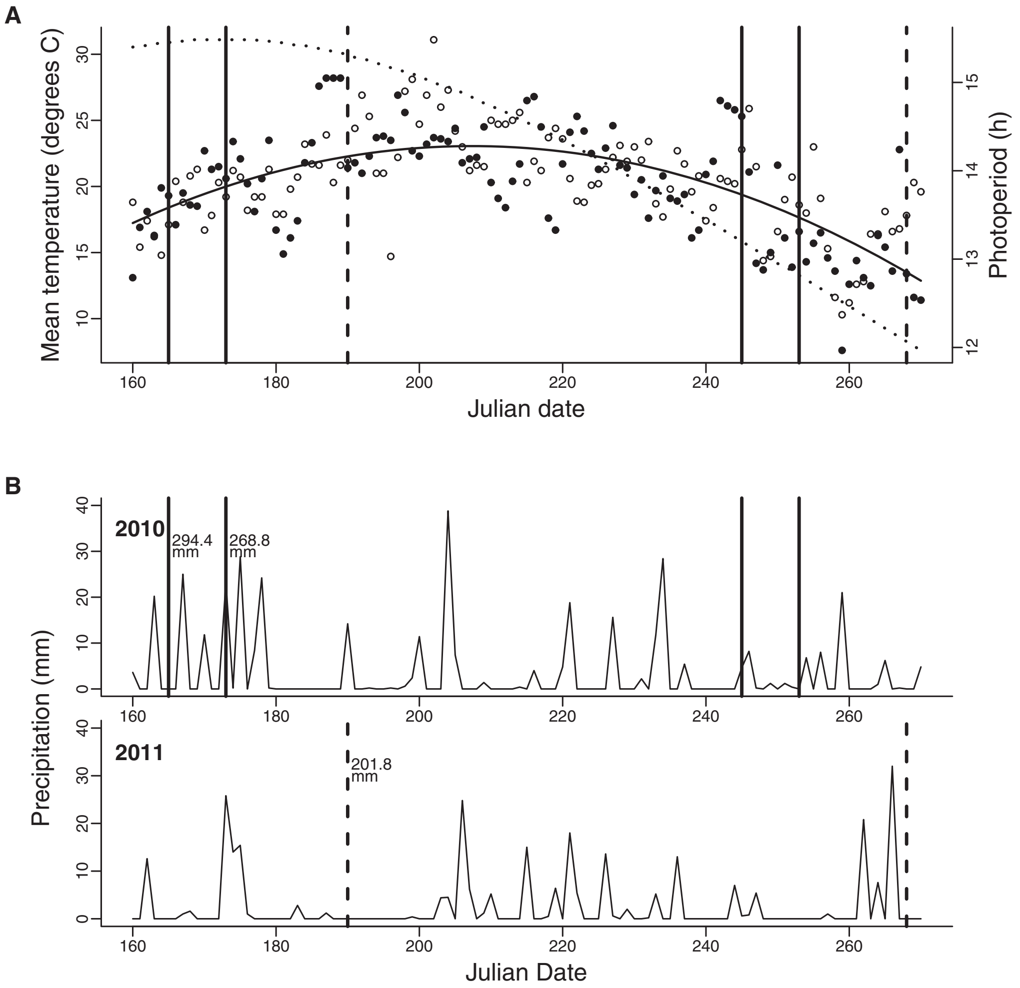

Figure 3. (A) Mean daily temperature (filled symbols: 2010; open symbols: 2011) with photoperiod overlay (dotted line), and (B) daily precipitation from first transplant to last harvest during 2010 and 2011. Data for Buttonville airport, Ontario (43.86 N, 79.37 W, Environment Canada 2013). Solid trend line in A is fitted daily temperature: $y = -88.57 + 1.08x - 0.03x^2$, $R^2 = 0.45$, including year and its interactions with Julian date did not improve model fit. In both panels, solid vertical lines indicate dates of first transplant and last plant harvest during 2010 (two lines for each event because planting of positive treatment populations was delayed during this year), and dashed vertical lines indicate dates of first transplant and last harvest during 2011. Values to right of first transplant indicators in (B): total precipitation from first transplant to last harvest.

as predictors. By weighting the proportion lost by the total number of fruit produced, we effectively modeled the number of "successful" fruit equivalents (after accounting for damage) relative to the total number of fruit "attempts." An association between damage and JDF would suggest that antagonists contributed to temporal variation in the environment during 2010.

We next investigated whether plants compensated for *P. xylostella* damage. Because this damage occurred before most fruits were mature, resources not spent on the maturation of lost gynoecia may have been reallocated to the production of new flowers and fruit (Williams and Free 1979). We therefore evaluated the effect of *P. xylostella* damage score on flower number, flowering duration, fruit number, and fruit mass. We limited these analyses to the five populations in which all five damage scores were observed, and included population, JDF, and their interaction in the models to account for spatial/temporal variation in flower and fruit production. Flower and fruit number were modeled by multiple Poisson regression. Because flowering duration was bimodally distributed, we reduced this variable to "long" or "short" duration using 35 days (the central value between modes) as the cut-off point, and proceeded with multiple logistic regression. We log-transformed fruit mass to improve normality prior to multiple linear regression.

Finally, because we expected that *C. obstrictus* would affect seed mass more than fruit mass, we examined its role as an agent of selection by re-estimating directional selection gradients using total fruit mass (including seeds and frass), instead of mass of seeds only, as the measure of fitness. If *C. obstrictus* contributed to selection on AAF during 2010, selection through total seed mass should be stronger than that through total fruit mass during 2010, but not during 2011. All analyses were performed in R (R Development Core Team 2012).

Results

TEMPORAL VARIATION IN ENVIRONMENTAL QUALITY

As planned, plants with different JDF were exposed to different temporal environments. Summer solstice coincided with (2010) or preceded (2011) the start of the experiment, and day length decreased approximately 2.5 h from first transplant to final harvest. Mean daily temperatures varied approximately 10°C during both years, and the effect of JD on mean temperature did not vary between years (analysis of variance [ANOVA], $F_{216,\,219} = 1.58$, $P = 0.19$; Fig. 3A). Temperature peaked midway through the experiment during 2010, and closer to the beginning of the experiment during 2011. Populations received more precipitation during 2010 than during 2011 (Fig. 3B), but regular watering likely minimized differences in water availability. There was no temporal trend in probability of precipitation during 2010 or 2011 (Table S5).

Herbivory also varied over the 2010 season. Both the odds of a higher *P. xylostella* damage score (Fig. 4A; Table S6) and the proportion of seeds lost to weevil larvae (Fig. 4B; Table S7) increased with later JDF in all uncorrelated and negative correlation populations. These temporal trends were more variable among the positive correlation populations, possibly because the later start date of these populations affected the probability and timing of their discovery by enemies.

PLANT TRAITS AND PRODUCTIVITY

As intended, AAF did not vary across populations (Table S8). All other traits varied at some level of the experimental design (S8). This variation likely reflects annual and spatial variation in the environment, and the different start dates of treatments during 2010. With the exception of height, which varied over years due to differences in the way this trait was measured (see above), trait distributions overlapped broadly across all populations. Leaf number and root mass were almost always positively correlated with AAF (Table S9).

Seed production and fruit production also varied among populations, though distributions were again broadly overlapping. Both total seed mass ($F_{1,\,753} = 104.78$, $P < 0.001$, Table S8) and total fruit mass ($F_{1,\,753} = 15.47$, $P < 0.001$) were greater during 2011 than during 2010, as expected given stronger enemy pressure during 2010. A significant year-by-treatment interaction effect for these two fitness measures (seed mass: $F_{2,\,753} = 4.54$, $P < 0.05$; fruit mass: $F_{2,\,753} = 3.76$, $P < 0.05$, Table S8) reflected variation among treatments in the magnitude (not direction) of difference between years.

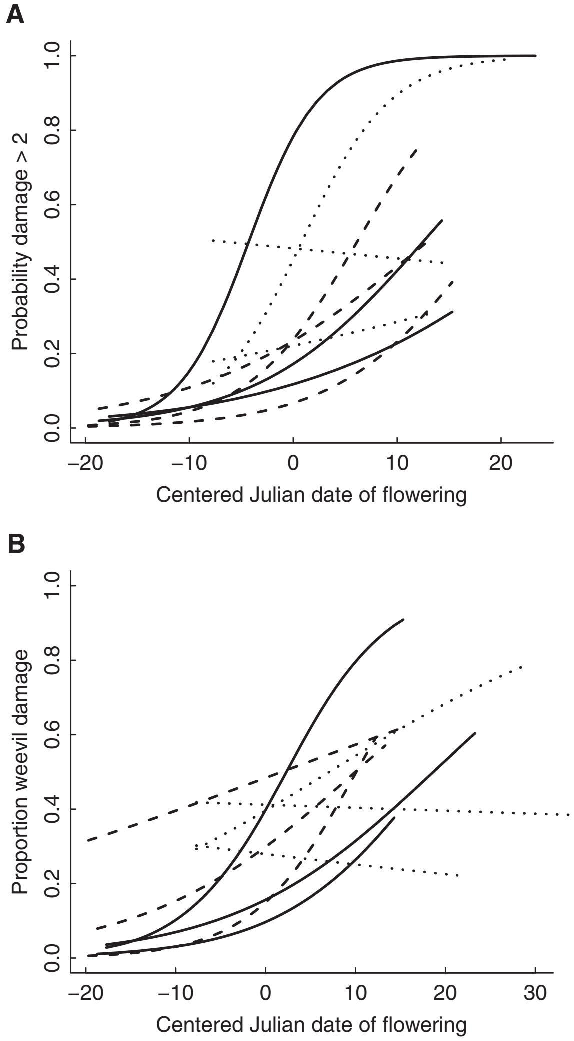

Figure 4. Variation in damage caused by (A) caterpillars and (B) seed weevil larvae by population and Julian date of flowering (JDF) during 2010. Dotted, solid, and dashed lines depict trends for populations in the positive correlation, uncorrelated, and negative correlation treatments, respectively. (A) Probability of losing >40% of flowers or fruits to consumption by *Plutella xylostella* (i.e., damage score >2 on Julian date 202) as a function of mean-centered JDF. (B) Proportional loss of fruit equivalents to predation by *Ceutorhynchus obstrictus* as a function of mean-centered JDF. Abscissa range excludes one plant with centered JDF = 55 (positive treatment); this plant was included in model fit.

SELECTION

The strength and direction of direct selection on AAF changed with the strength and direction of the correlation between AAF and JDF. Not all of the individual selection gradients on AAF differed significantly from zero (Fig. 5A). However, over all

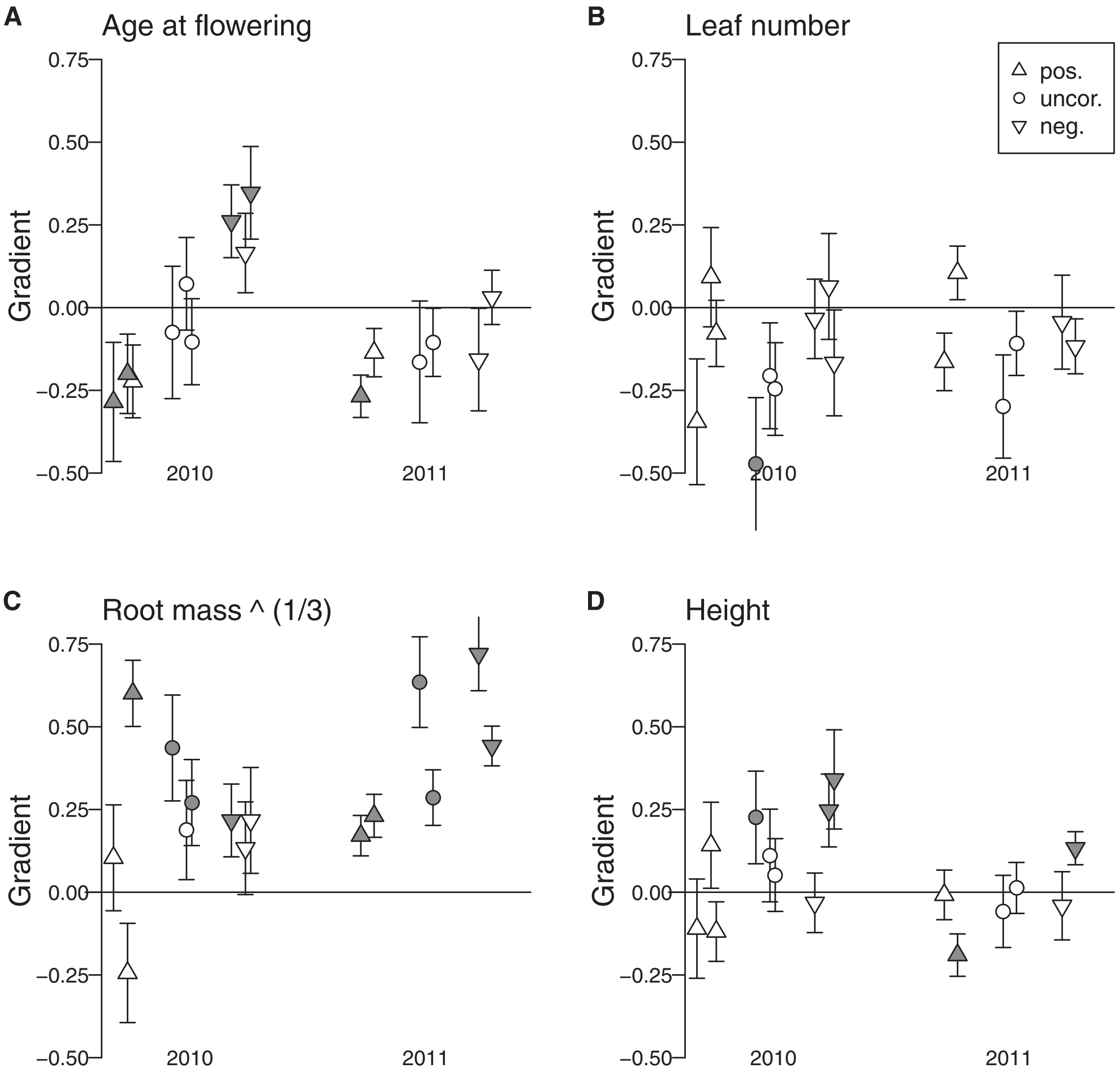

Figure 5. Selection gradients (± SE) on (A) age at flowering onset, (B) leaves at flowering onset, (C) cube root of taproot dry mass, and (D) height from ground to tip of tallest branch (2010) or to first flower on primary axis (2011) for 15 experimental populations of *Brassica rapa*. Populations are grouped by study year and treatment. 2010: Three replicates for each of positive correlation treatment (upwards pointing triangles), negative correlation treatment (downwards pointing triangles), and uncorrelated treatment (circles), $N = 47$ plants per population. 2011: Two replicate populations per treatment, $N = 56$ plants per population. Filled symbols indicate that selection gradient is statistically different from zero ($P < 0.05$, generalized linear model with gamma distribution and log link).

populations, direct selection tended to act in favor of *young* AAF in the positive correlation treatment (Fig. 5A; recall that in the positive correlation treatment, a young AAF corresponds to an early seasonal environment). In contrast, selection favored *older* AAF in the negative correlation treatment during 2010, when herbivory was stronger (recall that in the negative correlation treatment, an old AAF corresponds to an early seasonal environment). In 2011, direct selection on AAF was relaxed in the negative correlation treatment (Fig. 5A). The magnitude of the difference in selection between treatments thus varied by year (Table 1). During both years, selection on AAF in the uncorrelated treatment was roughly intermediate to that in the other treatments. The distribution of gradients on AAF was approximately centered on zero in 2010, and on a value slightly less than zero in 2011.

Strikingly, the overall pattern of total selection on AAF largely matched the pattern of direct selection (Figs. 5A and 6), though during 2011, selection differentials tended to be more positive than selection gradients. The agreement between selection differentials and selection gradients on AAF suggests that the environmental component of selection on AAF (Fig. 1, path *df*) tended to overwhelm indirect selection on AAF through the association with condition (Fig. 1, path *cb*), particularly during 2010.

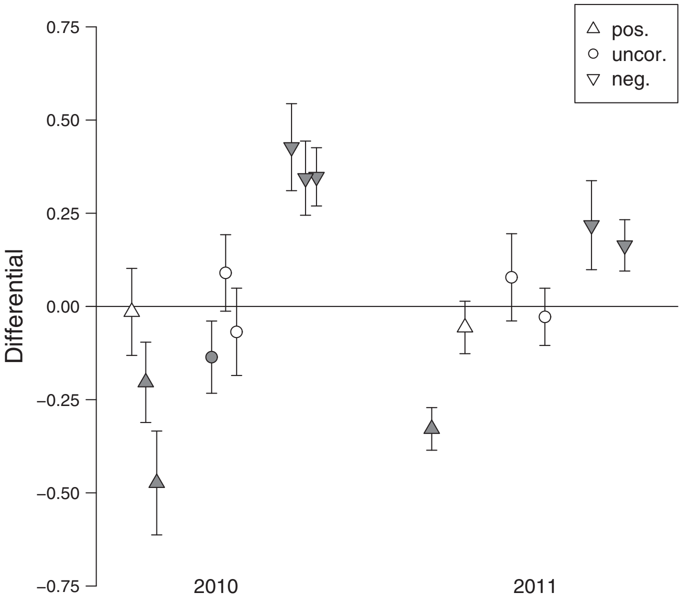

Figure 6. Selection differentials (± SE) on age at flowering onset through female fitness (total seed mass per plant) for 15 experimental populations of *Brassica rapa*. Populations are grouped by study year and treatment. 2010: Three replicates for each of positive correlation treatment (upwards pointing triangles), negative correlation treatment (downwards pointing triangles), and uncorrelated treatment (circles), $N = 47$ plants per population. 2011: Two replicate populations per treatment, $N = 56$ plants per population. Filled symbols indicate association between AAF and female fitness (total seed mass per plant) is statistically different from zero ($P < 0.05$, generalized linear model with gamma distribution and log link).

Table 1. Treatment and year interaction effects on the association between standardized traits and fitness (total mass of seeds produced) in experimental populations of *Brassica rapa*.

Dropped term	df	ΔDeviance	*P*
Age × treatment × year	**2**	**4.281**	**0.010**
Age × year	1	NA	NA
Age × treatment	2	NA	NA
Age	1	NA	NA
Lvs × treatment × year	2	0.022	0.977
Lvs × year	1	1.003	0.142
Lvs × treatment	2	1.898	0.129
Lvs	**1**	**4.277**	**0.002**
Root × treatment × year	2	0.116	0.880
Root × year	1	1.221	0.106
Root × treatment	2	2.249	0.088
Root	**1**	**61.896**	**<0.001**
Height × treatment × year	2	0.334	0.699
Height × year	1	1.766	0.051
Height × treatment	**2**	**5.694**	**0.002**
Height	1	NA	NA

Effects tested through analyses of deviance of nested generalized linear models (gamma distribution with log link). For each trait, analysis always began with a full model, including three-way interactions for all traits × treatment × year. Interaction terms for the trait were dropped sequentially in the order listed, and deviance compared to the previous model. Bold text indicates statistically significant association between term and fitness at $\alpha = 0.05$; statistical significance determined using a chi-square test.

Selection also acted directly on condition at flowering onset. Across all populations ($N = 759$ plants), selection favored individuals with fewer leaves (Table 1), though this selection was rarely statistically significant within populations ($N = 47$ or 56 plants; Fig. 5B). Selection also favored plants with larger taproots in nearly every population (Fig. 5C, Table 1). In both study years, the nature of selection on plant height varied by treatment (Table 1), with selection tending to favor shorter plants in positive correlation populations, and taller plants in negative correlation populations (Fig. 5D).

Nonlinear selection was weak, and it varied among populations (Table S10). Canonical analyses similarly revealed little curvature of the fitness surface. Just six of 60 total canonical axes (from four of 15 populations) exhibited significant quadratic selection; four of these six were negative (Table S11). This suggests a convex fitness surface, if it is curved at all.

COMPENSATION FOR *P. xylostella* DAMAGE IN 2010

Plants responded to *P. xylostella* damage with increased flower production or longer flowering duration (Fig. 7, Table S12). Plants subject to intermediate damage produced approximately 25% more flowers than plants in the least-damaged category (Fig. 7A, Table S12). In contrast, the most damaged plants reduced flower production by approximately 10% (Fig. 7A, Table S12), but flowered longer (Fig. 7B). These responses did not prevent progressive loss of fruit number or fruit mass with increasing damage

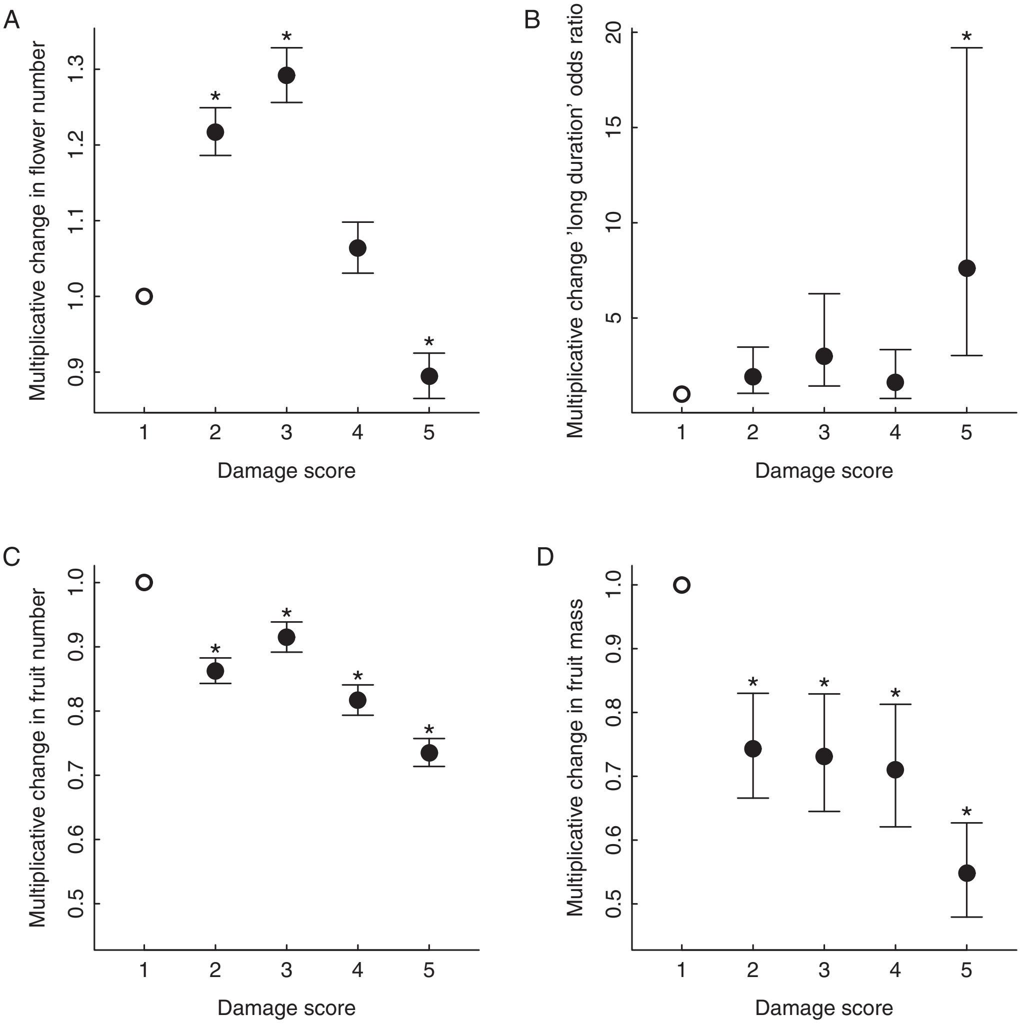

Figure 7. **Mean ± SE compensatory responses to damage by *Plutella xylostella* larvae (closed symbols), relative to plants in the least-damaged category (open symbols), including (A) multiplicative change in total number of flowers produced; (B) multiplicative change in odds of flowering >35 days; (C) multiplicative change in total fruit number; and (D) multiplicative change in total fruit mass. All effects are corrected for JDF and population. All models calculated using only populations that exhibited all five damage categories. (*) = difference from least-damaged category (damage score 1) is significantly different from zero ($P < 0.05$).**

(Fig. 7C,D). For example, the approximately 10% reduction in flower production by the most damaged plants was accompanied by an approximately 25 and 50% reductions in fruit number and total fruit mass, respectively.

SELECTION BY *C. obstrictus*

Comparison of selection via seed mass and selection via fruit mass did not confirm that *C. obstrictus* acted as an agent of selection during 2010. Selection gradients on AAF through seed mass were not statistically stronger than those through fruit mass during 2010 (paired one-tailed *t*-test, $t = 0.84$, df $= 8$, $P = 0.\ 21$) or during 2011 (paired one-tailed *t*-test, $t = -0.28$, df $= 5$, $P = 0.60$).

Discussion

SELECTION FAVORS EARLY ENVIRONMENT OVER YOUNG AGE

Many studies show that selection favors early flowering (Harder and Johnson 2009; Munguía-Rosas et al. 2011), and this experiment was no exception. The unique finding here is that the fitness advantage of early flowering occurs primarily because early plants occupy a superior slice of the temporally varying environment during reproduction (Fig. 1, path *df*). The direct effect of AAF on seed production (Fig. 1, path *a*) was very weak, and the indirect effect through its correlation to plant condition (Fig. 1, path *cb*) just slightly stronger.

In this study, selection gradients (Fig. 5) and differentials (Fig. 6) on AAF varied with their correlation with JDF such that flowering on an early date (as opposed to at a young age) increased seed production, especially in 2010. There are at least three, non-mutually exclusive, reasons why female fitness may have declined with later JDF. First are effects of seasonal change in the abiotic environment on seed maturation. Photoperiod, temperature, and water availability in the days between a fruit reaching its final size (but still being green) and the point of final maturation can all affect seed maturation (Gutterman 2000). There was no evident temporal trend in precipitation in this study (Fig. 3B), and the populations were regularly watered. Day length and temperature, however, both decreased with JD (Fig. 3A), meaning that plants with a later JDF matured seeds during cooler, shorter days (JD of first fruit harvest increased with JDF: $r = 0.68$, $N = 334$ plants from 2011 populations [excludes two plants that produced no fruit]). This environmental change may have affected the mass of seed they produced.

Second, decreasing total seed mass with later JDF may have resulted from a temporal decline in pollinator service if, for example, the composition of the pollinator community or pollinator visitation rates varied across the season (e.g., Forrest et al. 2011; Rafferty and Ives 2011, 2012). We did not conduct pollinator observations, but nor did we notice any remarkable change in the pollinator community during daily site visits. Further, the probability of precipitation did not vary linearly with JD during either study year (Fig. 3B, Table S5), eliminating one reason to suspect a decrease in pollinator service over the season (insect pollinators are generally less active on rainy days, e.g., Visscher and Seeley 1982). Pollinator observations and hand pollination experiments (e.g., O'Neil 1999; Sandring and Ågren 2009) are required to definitively test the role of pollinators as agents of selection on flowering time.

Finally, during 2010, herbivores and seed predators appeared to contribute to selection on AAF. Damage from *P. xylostella* increased with later JDF during 2010 (Fig. 4A), and plants were unable to fully compensate for this damage (Fig. 7). Damage by the seed predator *C. obstrictus* also increased with JDF (Fig. 4), but comparison of selection on AAF through seed mass to selection on AAF through fruit mass did not confirm that *C. obstrictus* acted as an agent of selection. However, *C. obstrictus* may have had a larger-than-anticipated effect on fruit mass: seed mass was not converted to frass mass at a 1:1 ratio. The larger difference in selection on AAF between treatments during 2010 than during 2011 is consistent with the hypothesis that *P. xylostella* and *C. obstrictus* acted as agents of selection, but a conclusive test of this hypothesis would require manipulation of herbivore and seed predator abundance across populations in a single study year.

Several other studies have identified herbivores and seed predators as agents of selection on flowering time (Pilson 2000; Nuismer and Ridenhour 2008; Parachnowitsch and Caruso 2008; reviewed in Kolb et al. 2007). These findings, and the results presented here, contrast with those of a meta-analysis finding no association between flowering time and seed predation (Munguía-Rosas et al. 2011). This discrepancy might be partly explained by methodology. In calculating the average correlation between flowering time and seed predation, Munguía-Rosas et al. (2011) included both the sign and value of correlations extracted from the literature, thereby assuming enemies consistently attack early (or late) plants. If enemies differ in their timing of attack (Kolb et al. 2007), the absolute value of the correlation would be better suited to testing the role of enemies as agents of selection. Of course, a consistent enemy preference for late-flowering plants would have been required to implicate enemies as the major cause of the selection for early flowering documented by Munguía-Rosas et al. (2011).

INDIRECT SELECTION THROUGH CORRELATION WITH CONDITION

In addition to selection on AAF through its correlation with environment (Fig. 1, path *df*), we also detected some selection through correlation with size (Fig. 1, path *cb*). Larger taproots were associated with greater seed production during both study years (Fig. 5), and AAF and taproot size were positively correlated (Table S4). During 2011, selection differentials on AAF (Fig. 5) were more positive than selection gradients on AAF (Fig. 6); this may have been due to indirect selection on AAF through its correlation with size.

Our results regarding condition address a question that Munguía-Rosas et al. (2011) were unable to resolve with data available at the time of their study. These authors reported that flowering time tends to correlate negatively with size, a finding since bolstered by Forrest (2014). They were unable, however, to assess the importance of indirect selection on flowering time because few studies reported both selection differentials and selection gradients on flowering time from models including size. The experiment here has provided a clear answer: selection on flowering time was not fully attributable to indirect selection through correlation with size, whether size was positively (the assumed direction of genetic correlation, Forrest 2014) or negatively (the typical direction of phenotypic correlation, Forrest 2014) correlated with JDF. This result was particularly strong for 2010 data.

One prior study that did examine both differentials and gradients found little evidence of direct selection on flowering time or rosette size (Sandring et al. 2007). The discrepancy between this result and those presented here may be due in part to differences in life history: Sandring et al. (2007) studied a perennial (*Arabidopsis lyrata*), and selection on flowering onset tends to be stronger in annuals such as *B. rapa* (Munguía-Rosas et al. 2011).

Moreover, while AAF and size were positively correlated in our study (Table S9), the time–size correlation varied among populations of *A. lyrata* (Sandring et al. 2007). Finally, vertebrate herbivores were the major agent of selection in the studied populations of *A. lyrata* (Sandring et al. 2007), whereas plants in our experiment experienced invertebrate enemies only. Any of these factors may contribute to differences in selection.

DIRECT SELECTION ON AGE

Some direct effects of AAF (Fig. 1, path *a*) may have also occurred in our experiment. During 2011, gradients on AAF were centered on a value less than zero (Fig. 5A). This could reflect indirect selection on AAF through phenotypic correlation with a trait not included in the selection model. Alternatively, negative selection gradients on AAF could be due to a decreasing reproductive capacity with later AAF. Such a decline could, for example, arise if the branching probability of axial meristems decreases with plant age (Kudoh et al. 1995; Duffy et al. 1999). Compensatory response to herbivory during 2010 (Fig. 7) may have overwhelmed this effect, if this compensation was achieved by activating quiescent meristems.

IMPLICATIONS FOR LIFE-HISTORY THEORY

Classic life-history theory for annual plants balances fitness gains of delaying flowering to achieve greater size (and thus greater reproductive investment) against the risks of flowering too near the end of the season (Cohen 1976; King and Roughgarden 1983; Kozłowski 1992). Within this framework, individual models vary in their assumptions about what determines the rate of photosynthesis (plant size as predictor: King and Roughgarden 1983; environmental conditions as predictor: Johansson et al. 2013), the constancy of return on reproductive investment (Ejsmond et al. 2010), and other factors. Empirical observation is required to determine which factors prevail under a given set of conditions.

A challenge in applying experimental results to the development of life-history theory is that the latter assumes plants vary in flowering time only. In real (or experimental) populations, associations between fitness and flowering time may reflect not only the balance of size, time, and environment as modeled in life-history theory, but also among-individual variation in growth rates and other traits. Encouragingly, a recent simulation found that such variation does not overwhelm the signal of size, time, and environment (Weis et al. 2014). Assuming that among-individual variation in traits, such as growth rate, similarly had little effect on the association between flowering time and fitness in our experiment, results presented here can help inform model development.

The environment in which an individual reproduced affected the mass of seed it produced. During both years, declining photoperiod and temperature over the season meant that plants with a later JDF matured seed in a less favorable environment. This cost outweighed benefits of flowering at a larger size. Additionally, during 2010, seed predator attack around JD 222 caused the loss of seeds maturing at that time, that is, the first seeds of the last plants to flower. Both factors lead to temporally varying return on investment in reproduction. A model styled after Ejsmond et al. (2010), incorporating seasonality, productivity as function of size, and temporally varying returns on reproductive investment, is probably best suited to describing trade-offs in our experiment.

FINAL CONSIDERATIONS

This study has demonstrated that manipulating the genotype–environment correlation between AAF and JDF can reveal causal mechanisms of selection on flowering time. It further points to several avenues for future investigation. First, analogous experiments in different systems, sites, and years are required to test the generality of our result that JDF dominates the association between flowering time and female fitness. Decades of similar experiments in birds have shown that while environment is often the major determinant of the association between breeding time and fitness, the role of individual quality at breeding is sometimes stronger (reviewed by Verhulst and Nilsson 2008; see also Grüebler and Naef-Daenzer 2010; Mark Hipfner et al. 2010; Garcia-Navas and Sanz 2011). The range of findings in birds underscores the need for additional manipulative experiments in plants.

Second, future experiments might use manipulative techniques to examine the role of frequency-dependent selection on flowering time. There is reason to expect such selection; for example, asynchronous plant populations are less able to attract pollinators and satiate seed predators than are highly synchronous populations (Augspurger 1981). Further support is again found in birds: optimal breeding time may depend more on when neighbors are breeding than on individual condition or environmental quality (Meijer and Langer 1995; Verhulst and Nilsson 2008). A plant's male fitness, which may depend on access to mates, could be particularly sensitive to how early (or late) it flowers relative to the rest of the population.

Finally, flowering time may affect not only offspring production, but also offspring survival, and the relative contributions of age, condition, and environment to total selection on flowering time could vary for different components of fitness. Such effects are particularly likely for plants, such as *Campanulastrum americanum* (Campanulaceae), in which maternal flowering time influences offspring life history (Galloway and Burgess 2009).

Flowering time is a multifaceted trait that may affect fitness in multiple ways. Its evolution is implicated in climate change response (Franks et al. 2007), plant species invasion (Montague et al. 2007), reproductive isolation (Lowry et al. 2008), gene flow between crops and wild plants (Burke et al. 2002), and several other processes of theoretical and practical concern. Understanding the nature of selection on this trait requires explicit recognition of its many possible fitness effects, and continued experimentation to tease these effects apart.

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DATA ARCHIVING

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Supporting Information

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

Figure S1: Midparent–offspring regression for bolting time following two generations of mating in the glasshouse.
Figure S2: Map depicting locations of experimental populations.
Table S1: Bolting groups in 2010 glasshouse generation.
Table S2: Transplant schedule during 2010.
Table S3: Transplant schedule during 2011.
Table S4: Variation in associations between traits and fitness among replicate populations.
Table S5: Logistic regression coefficients from analysis of probability of precipitation by JD.
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Table S12: Generalized linear model coefficients from analysis of compensatory responses to *P. xylostella* damage.