Int. J. Plant Sci. 167(1):000–000. 2006.

1058-5893/2006/16701-00XX$15.00

A STEEP CLINE IN FLOWERING TIME FOR *BRASSICA RAPA* IN SOUTHERN CALIFORNIA: POPULATION-LEVEL VARIATION IN THE FIELD AND THE GREENHOUSE

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We examined clinal variation in flowering time in a series of *Brassica rapa* populations occupying a 4-km-long urban greenbelt in southern California. Field studies on two populations showed that mean flowering date for populations of this winter annual differed by up to 3 wk during the 1998–1999 season, when an El Niño event elevated precipitation well beyond normal levels. The degree of population difference was compressed in 1999–2000, when drought delayed onset of the growing season. In both years, the population occupying the drier site flowered earlier. Fitness functions, estimated separately for the two sites, indicated directional selection for early flowering in the dry site and a stabilizing component to selection at the wet site. Greenhouse experiments using other populations along the cline and conducted under different photoperiod conditions showed that when grown in a common environment, dry-site plants flower earlier than wet-site plants. This indicates a genetic basis for between-population variation. The degree of among-population variation in flowering phenology is compressed by long photoperiods.

Keywords: cline, common garden experiment, flowering time, genetic variation, local adaptation

Introduction

Many plant species are divided into subpopulations that differ in reproductive phenology, often on a very short geographic scale (Clausen et al. 1940; Antonovics and Bradshaw 1970; Hamrick and Holden 1979; Schmitt 1983; Rajakaruna et al. 2003; see Mazur and LeBuhn 1999). Environmental gradients can have two effects on flowering time. First, direct impacts on physiology can accelerate or retard mean flowering time of local populations across a gradient. Second, the same environmental gradients can impose a gradient of selection (Endler 1980) that pushes resident populations to different phenological optima (Linhart 1974). Generally, gene flow genetically homogenizes closely spaced local populations and thus thwarts local adaptation. However, variation in flowering schedules leads inevitably to phenological assortative mating (Fox 2003; Weis and Kossler 2004; Weis 2006), which restricts gene flow between phenologically divergent populations and thereby facilitates local adaptation (Kirkpatrick 2000).

The "decision" on when to flower may be the most crucial of an annual plant's life history. Plants blooming too early or too late can suffer a fitness disadvantage for several reasons. Among these is the trade-off between size and age at first reproduction, which has long been posited by life-history theory (Stearns 1992; Roff 2002). An early switch from exponential vegetative growth to reproduction forces the plant to initiate and mature seeds while they still have limited capacity for resource gathering (few leaves and roots). A delayed switch increases resources for seed production but may not allow enough time for seed maturation. Models based on this reasoning predict stabilizing selection on flowering time (Cohen 1976; Kozlowski 1992), with earlier optima for short-season habitats than for long-season habitats.

Additional selective forces can act on top of this trade-off. Seasonal shifts in pollinator service (Schemske 1977; Gross and Werner 1983; Torres et al. 2002) or flower and seed predation (English-Loeb and Karban 1983; Gross and Werner 1983; Biere and Honders 1996; Bishop and Schemske 1998; Pilson 2000; Mahoro 2002) can delimit a safe time to flower. Mate limitation may select against very early or very late flowering (Augspurger 1981; Campbell 1989; Devlin and Ellstrand 1990; Ollerton and Diaz 1999). Although selection on phenology may be variable (Ollerton and Lack 1992), few studies (e.g., Maad 2000; Buide et al. 2002) have reported no selection on flowering time (although a possible publication bias against negative results should be kept in mind).

We found a series of subpopulations of *Brassica rapa* along a 4-km urban greenbelt stretching from coastal bluffs, continuing along a creek drainage, and terminating in commercially developed land. Casual observation indicated the subpopulations at the opposite ends of this series differed by as much as 4 wk in date of first flower. In addition, we observed that annual variation in precipitation caused variation in germination date, which in turn caused plants to develop under different photoperiods in different years. This article is the first of several that will explore the causes and consequences of this clinal variation. Here we document the phenological variation between populations in the field. We also report experimental results that point to a genetic basis for this variation and describe how interannual variation in

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Manuscript received December 2004; revised manuscript received August 2005.

precipitation interacts with photoperiod to amplify or diminish variation.

Material and Methods

Brassica rapa is a self-incompatible, naturalized winter annual found in disturbed and mesic areas in southern California. This species germinates with the arrival of winter rains, which occurs sometime between late October and late January. Plants grow as rosettes until flowering in mid- to late winter. The growing season is terminated by the onset of annual summer drought. Thus, the length of the growing season for the whole assemblage of subpopulations can vary between years because of variation in the beginning and end of the rainy season. The length of the growing season within any one locality may vary because of local edaphic and community factors that alter the seasonal rise and fall of water availability. Variation in germination date also causes among-year variation in photoperiod during development.

The most coastal of our clinal populations is located along the sandy bluffs above Upper Newport Bay, also known as Back Bay (BB), in Orange County, California. The Michelson/Carlson population (M/C) is at the opposite end of the cline, approximately 4 km inland. This inland site has a number of artificial ponds that have been managed for waterfowl conservation for over 80 yr. Three populations lie in between; the one along San Diego Creek in the San Joaquin Freshwater Marsh (SJM) and the one in the marsh adjacent to the University of California, Irvine, Arboretum (ARB) lie approximately 2.7 km inland from BB, while the one at the Irvine Ranch Water District's property (IRWD) is approximately 3.2 km inland. The latter two populations also occur along long-established artificial ponds. Among all five populations, plants nearest the ocean flowered earliest, with populations that are more inland flowering later. Soon after we identified these populations and collected seed from each, habitat restoration programs were begun to eliminate nonnative species at the M/C and IRWD populations. Although remnants of these populations survive, we necessarily focused field studies on the ARB and BB populations.

The ARB and BB habitats differed in several ways. Soils at ARB are darker, and the woody vegetation includes Arroyo willow (*Salix lasiolepis*) and mule fat (*Baccharis glutinosa*), both of which indicate mesic conditions. The BB site has little woody vegetation, with California sage (*Artemesia californica*) and bladderpod (*Isomeris arborea*), both indicative of xeric conditions, being common woody species. Annual Q1 species, such as *Brassica nigra* and *Raphanus raphanistrum*, senesce earlier at the more xeric BB site.

At these clinal sites, most leaf herbivory is caused by snails (*Helix aspera*) and slugs, with occasional light herbivory by the insects *Trichoplusia ni, Pieris rapae*, and *Phyllotreta cruciferae* (cabbage looper, diamondback moth, and flea beetle, Q2 respectively; Franke 2004). After bolting, *Brevicoryne brassicae* attacks the maturing infloresences (Franke 2004). Various birds eat seeds within the maturing fruits. Successful fruits eventually dehisce, scattering their contents over the surrounding ground, usually starting in late April. Seeds lie dormant over the dry summer and fall, and their germination is triggered by the next winter rainy season.

Field Studies

Soil moisture capacity for the BB and ARB sites was determined from five soil samples per site. These were saturated and then dried to a constant mass at 40°C. Samples were also sieved (53-μm mesh) and weighed to determine the mass percentage of the sand fraction. The sand was further sieved (125-μm mesh) to obtain a coarse/fine sand particle ratio. Mean moisture capacity, sand percentage, and the coarse/fine sand ratios were compared by *t*-test. We had brief access to a time domain reflectometry device to measure soil moisture in the field during the later half of the 2001–2002 growing season, and we measured moisture percentage at five sampling points centered in four dense *B. rapa* stands per site on a weekly basis until plants senesced. We obtained precipitation data collected by the National Weather Service between 1970 and 2003 at the Orange County Airport, located ca. 5 km from the sites, to correlate with between-year variation in flowering time.

We conducted field studies during the 1998–1999 and 1999–2000 growing seasons at the BB and ARB sites. At each site we established 10 plots (each 0.5×0.5 m) at the time of initial seedling emergence. Specific plot locations

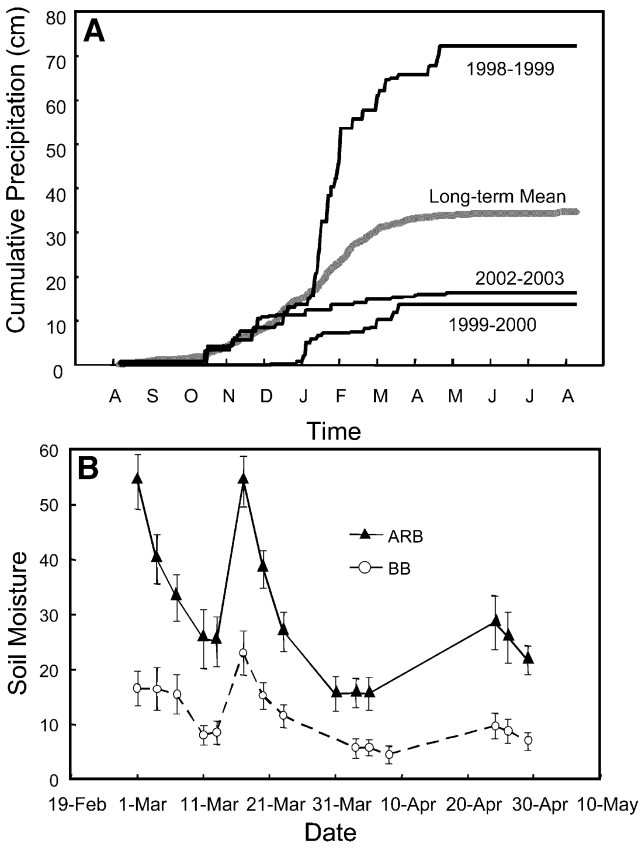

Fig. 1 Regional precipitation and study site soil moisture. *A*, Cumulative precipitation curves, recorded from August through July at Orange County Airport, which is located ca. 5 km from the field sites. The long-term mean is based on records from 1970 through 2003. *B*, Soil moisture percentage at the BB and ARB study sites in spring of 2003. Error bars indicate 95% confidence intervals.

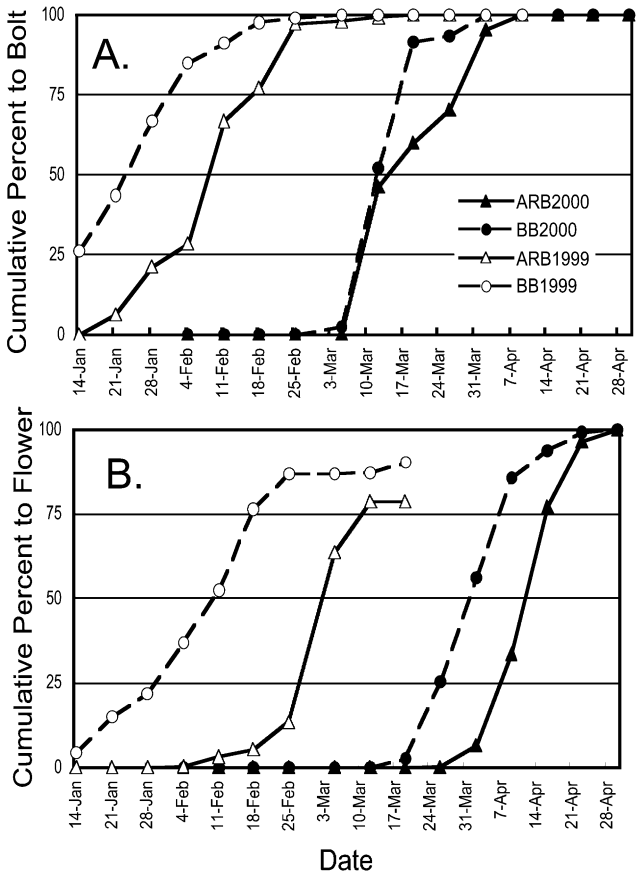

Fig. 2 Reproductive phenology at the ARB and BB sites in the 1998–1999 and 1999–2000 growing seasons. Points are the cumulative percentages of plants to bolt (*A*) and to flower (*B*) in the census plots.

were chosen haphazardly at the beginning of the season, with a minimum of 3 m between plots and at least one *Brassica* seedling visible per plot (Franke 2004). Starting in December, plots were censused weekly, and the number of plants bolting and the number coming into flower were recorded. Population differences in the dates of bolting and first flowering were tested by failure time analysis (Fox 2001) as implemented in the LIFEREG procedure in SAS (SAS Institute 2004). Population, year, and the population × year interactions were tested by Wald χ^2. The LIFEREG analysis is sensitive to assumptions on the underlying distribution of the dependent variable (Fox 2001), so we ran the analysis using the Weibul, lognormal, and gamma error distributions. Residuals fit the last distribution the best, so we assumed gamma-distributed errors for hypothesis testing.

We also examined between-population variation in leaf herbivory on the 20 plants, using a 0–4 scale: 0 = none, 1 =< 25%, 2 ≅ 25% – 49%, 3 ≅ 50% – 74%, 4 ≅ 75%–
Q3 100%. Stem diameter was measured at the base of the plant using calipers. In the second year (1999–2000), we also recorded the numbers of rosette and cauline leaves, leaf area (length × width summed over all leaves), stem height, and stem diameter at time of flowering. Once seed set was complete but before pods dehisced, seeds were separated from Q4 other plant material and weighed without drying to retain viability. The remaining material was dried and weighed. We recorded number of pods, number of pods damaged by birds, mass of seeds, and final aboveground biomass. Populations were compared by ANOVA, implemented in the GLM procedure in SAS.

In the 2001–2002 season we estimated the fitness function for flowering time in the BB and ARB populations. Sites were visited every 5 d. On each visit we haphazardly chose and marked 10 plants that had bolted but not yet flowered. The marked plants were widely distributed through representative *B. rapa* patches at each site. Plants were rechecked on all subsequent visits until they flowered. The flowering season extended over a longer period in the ARB population, so the total sample size there was over twice that at BB. As each plant senesced, fruits were collected and the aggregate mass of seeds recorded for each plant; assuming seed size varies negligibly between plants, aggregate mass estimates fitness through female function. Theory predicts stabilizing selection on flowering time (Cohen 1976; Kozlowski 1992), so we first fitted data to the Gaussian function

$$S = b \times \exp - \frac{(D-d)^2}{w},$$

where the dependent variable, S, is the aggregate mass of seeds produced per plant, and the independent variable, D, is the day of first flowering. Parameters are d, the optimal date of first flowering; b, mean seed production at the optimal date; and w, which describes the decline in production with increasing deviations from the optimum (small w indicates strong selection). We chose this function over the quadratic because theory indicated that fitness should asymptotically approach zero as flowering time goes to extreme values. This nonlinear regression was performed with the NLIN procedure in SAS. If this function did not fit, data were reanalyzed by standard linear regression.

Common-Environment Experiments

We conducted four experiments to determine if phenological differences among the BB, SJM, and M/C populations persist when plants are grown in a common environment. These were carried out in greenhouses under several photoperiods and with various pot sizes. Plants were started from wild-collected seed, except where noted, and grown in a 3 : 1 mixture of soilless potting medium and sand. Plants were grown singly per pot, and pots were always placed on

Table 1

Failure Time Analysis of the Bolting Time and Flowering Time Differences between the BB and ARB Populations in the 1998–1999 and 1999–2000 Growing Seasons

	Bolting time		Flowering time	
	Wald χ^2	*P*	Wald χ^2	*P*
Population	4.01	0.0456	10.17	0.0014
Year	710.35	<0.0001	342.70	<0.0001
Population × year	3.99	0.0457	10.15	0.0014

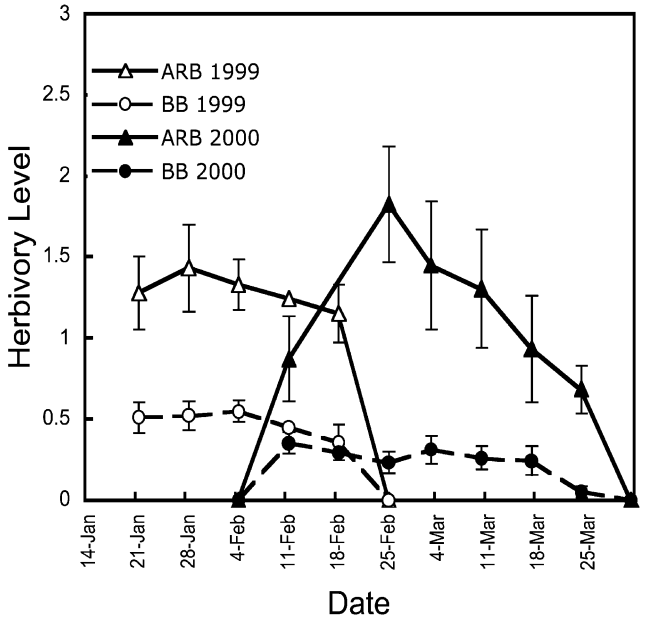

Fig. 3 Herbivory scores at the ARB and BB sites in the 1998–1999 and 1999–2000 growing seasons. Error bars are ±1 SE.

the bench in randomized order. Plants were watered five to six times a week and fertilized (10 : 10 : 10 liquid, according to manufacturer's directions) the first week after planting and again during the fourth week. Several of these experiments were performed for additional purposes, but here we report the results that pertain to the effects of growing conditions on the population differences in flowering time.

We bulk-collected wild seed in the spring of 1997. Haphazard transects were walked though each population and all seed collected from the plant at each ca. 0.5-m interval. A minimum of 400 plants contributed to the collection at each site.

Experiment 1 was done in the phytotron at the Centre for Terrestrial Ecology, Netherlands Institute for Ecology. Other aspects of this experiment are described by Weis et al. (forthcoming). Pots were 10 cm in diameter and 25 cm deep, with 16 pots per population. Seed was planted on December 5, 1997. Natural light was supplemented by sodium vapor lamps set to a 14-h photoperiod. Plants were examined every 2–3 d to determine time to flowering.

All other experiments were performed in the greenhouse at the University of California, Irvine. In these experiments, plants were examined daily to determine the time to flowering.

Experiment 2 was started on November 16, 1998, so that plants experienced a photoperiod that matched that often experienced in the field (ca. 10 h). We used 52 plants per population, drawn at random from the bulk collection. Pots were 12 cm wide and 35 cm deep. No supplemental lighting was used.

Experiment 3 was started on May 25, 1999. Plants experienced a longer photoperiod (ca. 14 h) than that of their normal growing season. The sample size was 64 plants per population. Pots were 10 cm wide and 12 cm deep. No supplemental lighting was provided. The length and width of the longest leaf and the diameter of the stem were measured early in development. Stem height, number of leaves, and number of flowering branches were measured several times over development. After flowering, plants were harvested, dried, and weighed.

Experiment 4 plants were grown from seed produced under greenhouse conditions, which muted potential environmental maternal effects on flowering time imposed by site differences in the natural environments. The plants in this final experiment were the progeny of randomly assigned matings between plants from experiment 2. In that experiment,

Table 2

Comparison of ARB and BB Populations in 1999–2000

	ARB		BB				
Variable	Mean	SE	Mean	SE	Type III *F*	df	*P*
Growth traits:							
Leaf area at flowering (cm^2)	71.96	15.34	12.04	3.31	20.02	1, 27	**0.0001**
Number of rosette leaves at flowering	5.16	0.83	3.05	0.35	6.77	1, 27	**0.0149**
Number of cauline leaves at flowering	8.08	1.49	4.47	0.57	6.50	1, 27	**0.0168**
Stem diameter at flowering (cm)	0.56	0.07	0.35	0.03	7.68	1, 30	**0.0095**
Height at flowering (cm)	48.13	4.47	24.06	2.35	24.26	1, 30	**0.0001**
Number of branches per plant	2.94	0.84	0.20	0.14	11.46	1, 36	**0.0017**
Aboveground vegetative biomass (g)	1.60	0.50	0.15	0.07	9.21	1, 36	**0.0044**
Herbivory levels:							
Number of aphids at flowering	2.53	2.53	1.35	1.25	0.20	1, 33	ns
Leaf herbivory damage score	0.87	0.19	0.18	0.14	2.9	1, 36	0.098
Percentage of pods damaged by birds	17.35	6.05	9.16	5.60	0.99	1, 36	ns
Reproductive traits:							
Number of pods	29.50	10.54	3.10	1.52	6.81	36	**0.0131**
Mass of seeds (g)	0.24	0.12	0.03	0.02	3.48	36	0.0705

Note. These data are based on individual plants marked and followed throughout the season and then harvested. Boldface indicates a significant difference between the populations.

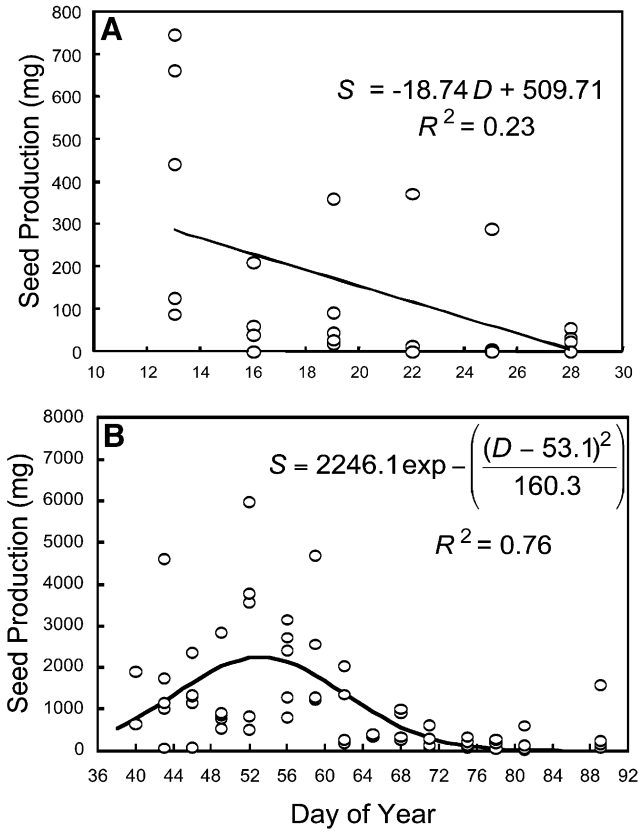

Fig. 4 Fitness functions for the 2002–2003 growing season. Time is measured as days since January 1, 2003. *A*, At BB, selection was directional, without a stabilizing component. *B*, At ARB, selection had a strong stabilizing component, with peak fitness for plants flowering on February 22.

plants within a mating pair were randomly designated to be pollen donor or recipient and the seed collected (18–23 successful parings per population). Each mating pair contributed 10–15 seeds to a bulk pool for their population. Seeds for experiment 4 were then randomly drawn from these three pools and sown in 46 pots per population. The experiment was started on December 18, 1999, without supplemental light, so that plants grew under a normal photoperiod (ca. 10 h). We used Super Cell–size Cone-tainer pots (Stuewe and Sons, Corvallis, OR), which were 3 cm wide and 18 cm deep.

A different set of populations was examined in a fifth experiment, started on October 5, 2000. We wanted to know if the BB population, which is the largest, showed substructuring. We also wanted to know if populations from drier sites that are also farther inland than M/C, like the inland marshy sites along the San Diego Creek drainage, flowered late, or, alternatively, if they flower early, as does the drier coastal BB Q5 population. We compared plants from the ARB and M/C populations, from two different areas of the BB site (one more coastal and one 0.3 km more inland), and, finally, from a population adjacent to Harvard Park (HP) in Irvine, California, which is 16 km inland. Like those at the BB site, the HP soils appear to dry early, as indicated from the resident vegetation. Sample size was 23 per population. Plants were grown in Super Cell Cone-tainers with supplemental light set to a 14-h photoperiod.

In all five common-environment experiments, we tested for population differences in the mean number of days to first flowering among populations by the failure time analysis (Fox 2001), as implemented in the LIFEREG procedure of SAS (SAS Institute 2004). Flowering times were best described by a gamma distribution. Bonferroni-adjusted multiple comparisons among populations were made by *Z*-test (Fox 2001).

Growth data from experiment 3 were examined to determine if populations differed in other respects. We performed

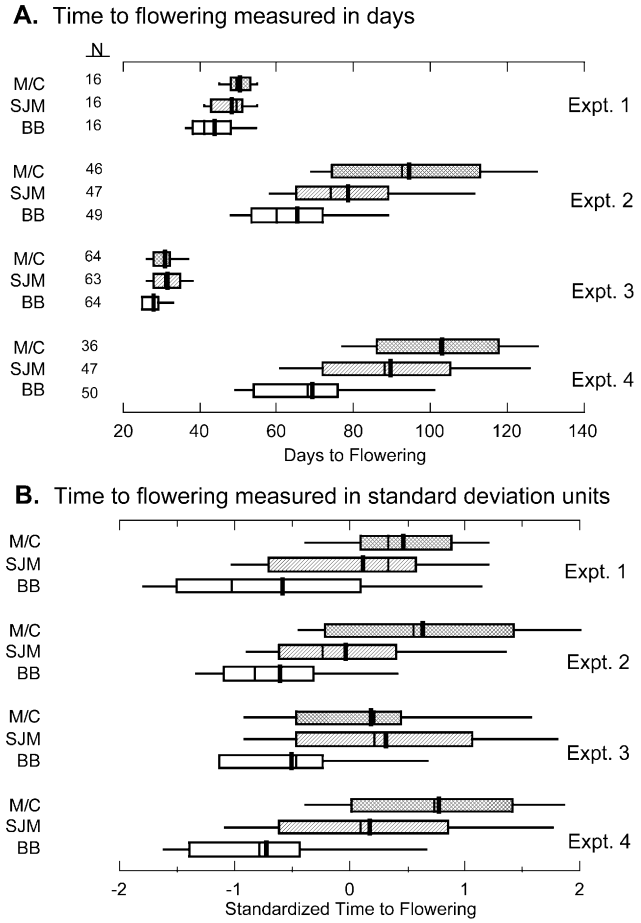

Fig. 5 Box plots of flowering time for three populations of *Brassica rapa* in four different common-environment experiments. Conditions for the experiments were as follows. Experiment 1: large pots, winter, long photoperiod; experiment 2: large pots, winter, short photoperiod; experiment 3: small pots, summer, long photoperiod; experiment 4: small pots, winter, short photoperiod. Thick vertical lines indicate mean days to flowering; thin vertical lines indicate the median flowering time. The boxes span from the twenty-fifth to the seventy-fifth percentiles, and the thin horizontal lines extend to the tenth and ninetieth percentiles. *A*, Absolute time to first flowering. Data plotted as number of days since germination. *B*, Relative time to first flowering. Days to first flowering for each individual was divided by the experiment-specific standard deviation in days to first flowering. BB = Back Bay; SJM = San Joaquin Marsh; M/C = Michelson/Carlson.

Table 3

Differences in Population Mean Days to Flowering in Common-Environment Experiments

	Conditions	Failure time analysis		Homogeneity test		
		Wald χ^2	P	Back Bay	San Joaquin Marsh	Michelson/Carlson
Experiment 1	Large pots, winter, long photoperiod	17.77	<0.0001	a	b	b
Experiment 2	Large pots, winter, short photoperiod	55.89	<0.0001	a	b	c
Experiment 3	Small pots, summer, long photoperiod	34.11	<0.0001	a	b	b
Experiment 4	Small pots, winter, short photoperiod	134.58	<0.0001	a	b	c

Note. Flowering time distributions illustrated in figure 4. Homogeneity of means determined by Fisher's least significant difference test.

MANOVA to determine if there are overall differences among populations using the GLM procedure. Then we performed one-way ANOVA for each trait. To see if the effects of maturation rate (transition from vegetative to reproductive mode) were distinct from the effects of growth per se (increase in size), we performed principal components analysis on growth data. Stem height, number of leaves, and number of branches, all of which were measured at least three times during development, plus final biomass and days to flowering, were analyzed by the PRINCOMP procedure of SAS. One-way ANOVA was performed on the principal component scores to see if populations differed for the underlying factors.

Results

Field Studies

At saturation, the mean moisture content of the ARB soil was 41.5% ± 8.14% (95% confidence interval) by mass, whereas BB mean soil moisture content was only 21.5% ± 6.84% ($t = 5.35$, df = 8, $P < 0.001$). Total sand content was approximately 80% of dry mass at both sites. However, the ratio of coarse to fine sand was significantly lower at ARB than at BB (0.60 ± 0.48 vs. 1.13 ± 0.57; $t = 2.02$, df = 8, $P = 0.02$).

In 1998–1999, the growing season began at the end of October, with near normal precipitation until January, when an El Niño event brought much greater than normal precipitation (fig. 1*A*). In 1999–2000, measurable precipitation was delayed until mid-January, causing a later than normal start to the growing season. The later start exposed the 1999–2000 plants to longer photoperiods during early development than plants in the previous generation. For instance, photoperiod on the day the first plant flowered ranged from 10 h, 3 min for the BB population in 1998–1999 (January 9) to 12 h, 14 min in the ARB population in 1999–2000 (March 25). The 2002–2003 season started normally, but the annual drought began in January, 3 mo earlier than usual (fig. 1*A*).

Table 4

Means and ANOVA Results for Experiment 3 (Small Pots, Summer, Long Photoperiod)

	Back Bay		San Joaquin Marsh		Michelson/Carlson			
Character	Mean	SD	Mean	SD	Mean	SD	F	P
Leaf length (cm):								
Day 10	5.49^{A}	0.77	5.54^{AB}	0.86	5.79^{B}	0.60	2.86	0.059
Leaf width (cm):								
Day 10	3.08^{A}	0.45	3.23^{AB}	0.53	3.36^{B}	0.46	5.64	0.004
Stem diameter (mm):								
Day 17	3.39^{A}	0.53	3.48^{A}	0.62	3.78^{B}	0.68	7.06	0.001
Number of leaves:								
Day 4	3.94^{A}	0.50	3.98^{A}	0.49	4.22^{B}	0.46	6.33	0.002
Day 22	9.29^{B}	1.89	8.50^{A}	2.22	8.65^{A}	1.65	2.94	0.055
Day 35	6.43	3.47	7.25	2.68	7.06	2.29	1.43	ns
Stem height (cm):								
Day 15	5.72^{B}	5.18	3.72^{A}	5.89	3.87^{AB}	4.73	2.79	0.063
Day 22	41.76^{B}	13.22	31.74^{A}	19.96	39.39^{B}	18.72	5.66	0.004
Day 35	79.23^{A}	10.74	83.49^{AB}	16.62	90.66^{B}	11.74	11.87	<0.001
Day 43	92.37^{A}	16.5	99.75^{B}	13.83	106.81^{C}	11.61	16.48	<0.001
Number of branches:								
Day 22	1.08^{B}	1.02	0.63^{A}	1.06	0.71^{A}	0.97	3.53	0.031
Day 35	4.67	2.41	4.05	2.50	3.90	1.78	2.04	n.s.
Day 43	12.24^{B}	4.56	10.49^{A}	4.03	10.46^{A}	3.31	4.07	0.019
Final biomass (g)	3.49^{A}	1.27	3.64^{A}	0.72	4.03^{B}	0.83	5.27	0.006
Final shoot-to-root ratio	10.57	5.58	9.87	3.84	10.36	2.66	0.21	ns

Note. All F-ratios had 2 over 188 degrees of freedom. Means with the same superscript letter are not different by Fisher's least significant difference test.

During that spring, soil moisture, by volume, at the BB site was significantly lower than at the ARB site (fig. 1*B*).

Plants in the BB population were phenologically advanced compared to those in the ARB population during both years (fig. 2). Furthermore, both populations were phenologically earlier in the wetter 1998–1999 season than the drier 1999–2000. Population differences were diminished in the late-starting dry year, when plants were exposed to longer photoperiods during development. In the earlier and wetter 1998–1999 season, the BB plants bolted approximately 2 wk earlier than ARB plants. However, in the later, drier 1999–2000 season, both populations started to bolt at the same time, although bolting extended over a longer period for the ARB populations (fig. 2*A*). Date of first flower showed similar patterns. The median flowering date in the BB population was ca. 3 wk earlier than that of ARB during the earlier, wetter 1998–1999 season. This difference was cut in half in the later, drier 1999–2000 season (fig. 2*B*). Failure time analysis showed significant effects of population, year, and their interaction (table 1).

Leaf herbivory levels at the two field sites differed from each other in both years (fig. 3). ARB plants suffered higher levels of herbivory than BB plants in both years. The standing level of damage was high early in the growing season when plants were in the rosette stage but declined as plants matured. Once plants bolt, they shed their rosette leaves, and so standing leaf herbivory decreases toward zero. In the 1999–2000 season we found no differences between populations for aphid infestation or bird attack levels (table 2).

Plant size also differed between sites in 1999–2000; leaf area at flowering, number of rosette and cauline leaves, stem diameter at flowering, height at flowering and end of season, and number of branches were all greater at ARB than at BB (table 2).

The fitness function for the ARB population during the 2002–2003 season showed an optimal flowering date in late February (fig. 4*A*). The optima occurred before the median flowering date, which suggests a combination of directional and stabilizing selection on flowering time. In the BB population, however, fitness declined in a linear fashion with flowering date (fig. 4*B*), indicating directional selection for earlier flowering. Winter rains ended in early January during this generation (fig. 1), so the differences in fitness function shape may result from the faster drying rate of the BB soil.

Common-Environment Experiments

In the greenhouse environment, plants derived from the BB population consistently flowered earlier than those from the SJM and M/C populations (fig. 5*A*; table 3). Interestingly, the SJM population flowered significantly earlier than the M/C population in the two short-day (natural photoperiod) experiments, but in the two long-day experiments the median flowering time was identical (fig. 5*A*). Inspection of the box plots in figure 5*A* showed a strong general effect of photoperiod on the number of days to flowering. Long days cut the median time from germination to first flower to about half that of the short-day experiments. The variation in flowering time was also constricted, with the interval between the tenth and ninetieth percentiles being approximately 85% narrower under long days than under short days. Although plants grew larger in larger pots, they did not appear to flower earlier. Although photoperiod had a strong effect on absolute time to first flowering, it did not have a strong effect on the relative time to flowering. We standardized flowering time for each experiment by dividing the days to flowering for each individual by the experiment-wide standard deviation. Figure 5*B* shows substantial similarity among the four experiments when time is set in relative units.

The growth measurements taken on experiment 3 (small pots, summer, long photoperiod) revealed overall population differences in plant size (MANOVA: Wilkes $\lambda = 0.48$, $F_{44,328} = 3.22$, $P < 0.001$). M/C plants tended to be larger than BB plants (table 4). The exceptions occurred around day 22, which was about 10 d before the median flowering day for BB; the increase in stem height and leaf number at this time reflects earlier bolting by this population. Not only did the M/C population bolt later, but it retained its rosette leaves longer (table 4).

When we subjected the growth data to principal components analysis, we found three components with eigenvalues greater than one (table 5). The first of these, which explains 35% of the variance among plants, shows a negative relationship between early size and flowering time; i.e., plants that bolted early also flowered early. The number of flowering branches was also associated with this factor. Thus, this principal component (PC) primarily describes plant phenology. The second PC explains 21% of the among-plant variance and describes final size, as indicated by high loadings for late stem height and final biomass. The third PC associates delays in bolting and leaf senescence with increases in branching and final biomass. In one-way ANOVAs followed by Fisher's least significant difference (LSD) test, we found that BB differed from the other two populations for the first

Table 5

Principal Component (PC) Analysis of Growth in Experiment 3 (Small Pots, Summer, Long Photoperiod)

Character	PC 1	PC 2	PC 3
Number of leaves:			
Day 4	0.15	**0.35**	−0.02
Day 22	**0.38**	0.03	0.18
Day 35	−0.11	0.00	**0.64**
Stem height (cm):			
Day 15	**0.35**	−0.07	**−0.30**
Day 22	**0.40**	0.16	−0.15
Day 35	0.11	**0.55**	−0.08
Day 43	−0.03	**0.56**	−0.12
Number of branches:			
Day 22	**0.39**	−0.13	−0.06
Day 35	**0.37**	−0.8	**0.36**
Day 43	**0.32**	−0.01	**0.40**
Final biomass (g)	0.05	**0.41**	**0.31**
Days to flowering	**−0.35**	0.14	0.12
Eigenvalue (variance explained)	4.22	2.46	1.64
Proportion of variance explained	0.35	0.21	0.14

Note. PC 1 indicates that early flowering is associated with early stem elongation and increased branching. PC 2 indicates that final size is unrelated to time of flowering. PC 3 associates delays in stem elongation and leaf senescence with increased branching and final biomass. Boldface indicates component loadings that exceed 0.20.

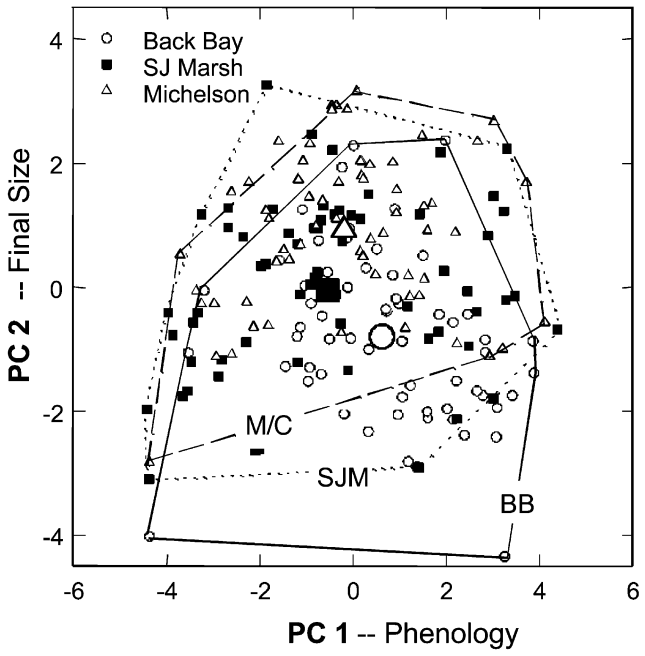

Fig. 6 Bivariate plot of the first two principal components (PCs). BB differs from the other two populations along the phenology PC. All three populations differ from one another along the second PC. Small symbols indicate individual plants; large symbols represent corresponding population centroids.

PC; this parallels results for flowering time alone. (Note that the LSD test is considered liberal, and so a lack of significant difference suggests a true lack of phenological difference between the SJM and M/C populations under long days.) All three populations were significantly different from one another for the second PC, which parallels the result for final stem height. No population differences were found for the third PC. Thus, although the phenology PC explained most of the among-individual variance, it did not explain most of the among-population variance (fig. 6).

In the final common-environment experiment we found no substructuring of the BB populations for flowering time (fig. 7). The relative flowering times of the other San Diego Creek drainage populations were consistent with previous experiments. Plants derived from the HP population, which is 16 km inland, did not differ from those of the BB sites but were significantly earlier than those of the ARB and M/C sites. This pattern indicates that the flowering time cline seen along the San Diego Creek drainage is unlikely to reflect selection caused by a simple coastal-inland climate gradient and is more likely a result of differences in the length of the growing season caused by higher soil moisture in the creek drainage sites.

Discussion

Hypothetically, several environmental factors that vary across the San Diego Creek drainage could directly cause the observed differences in flowering time (fig. 2) and in other growth traits (table 2). A number of environmental stresses are known to either directly accelerate or retard flowering in the related species *Sinapis arvensis* (Stanton et al. 2000). The very open BB habitat allows *Brassica rapa* to grow in full sun through the entire day for the whole season, whereas light competition with willows and other tall shrubs could delay flowering in more inland habitats. Drought typically causes delayed flowering (Fox 1990); however, lower water-holding capacity could limit nutrient uptake at BB and thus cause a stress-induced acceleration in flowering (Stanton et al. 2000). Alternatively, increased herbivory at ARB and the other inland sites (fig. 3) could cause a stress-induced delay in flowering (Winterer and Weis 2004). Although we did not measure temperature at the two sites, sunnier conditions at BB could increase temperatures and thus advance flowering. However, these populations continued to express clinal variation when grown in a common greenhouse environment (figs. 5, 7), and this strongly indicates a genetic basis for the phenological differences. The limited data provided by the 2002–2003 fitness functions at BB and ARB (fig. 4) indicate that one or more of these environmental factors has caused an evolutionary divergence among these populations.

In a future article we will present results from quantitative genetic experiments that show the observed flowering time differences are caused by variation at multiple unidentified segregating loci. Results in this article give some clues for speculation on which developmental pathways may have differentiated among these populations.

Recently, developmental geneticists have made great strides in uncovering the genetic controls for the shift from vegetative growth to reproduction, especially in *Arabidopsis*. A recent review listed no fewer than 30 loci that can influence flowering date in this species, and these loci are distributed among four developmental pathways (Puterill et al. 2004). *Brassica* and *Arabidopsis*, both crucifers, have largely collinear genomes (Lagercrantz et al. 1996), and so one would expect homology in the genetic controls for flowering time.

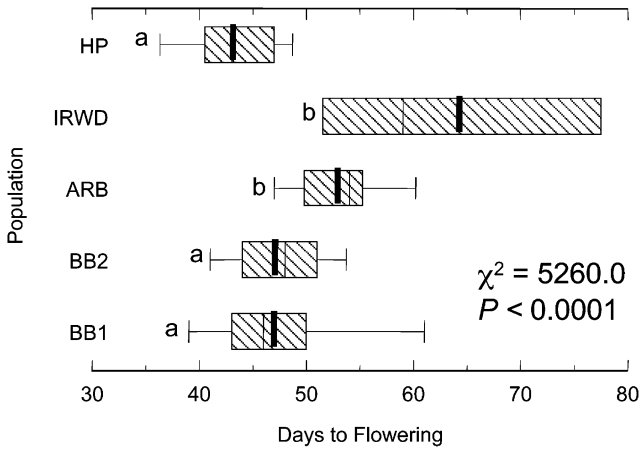

Fig. 7 Box plots of days to first flowering in five *Brassica rapa* populations. Populations are ordered from top to bottom by their location relative to the Pacific coast. Symbols are as in fig. 5. The significant Wald χ^2 indicates that failure time analysis revealed overall population differences in flowering time. Populations marked with the same letter do not significantly differ from one another. Sample size is 29 for all populations except M/C, where poor germination limited sample size to 6.

Variation along this cline is unlikely to be caused by genetic differences in the vernalization pathway, which triggers flowering after young plants are chilled. This pathway is regulated by the *FRIGIDA* locus and is involved in latitudinal clines in *Arabidopsis* flowering time (Stinchcombe et al. 2004). However, all plants readily and rapidly flowered under summer greenhouse conditions (figs. 5, 7).

Our data indicate a possible role for the photoperiod response pathway in this cline. This pathway triggers flowering through elevated expression of the *CO* locus when stimuli from the external photoperiod coincide with a sensitive period set by the internal diurnal clock (Puterill et al. 2004). All three tested populations flowered faster under long days (fig. 5). The absolute difference between BB and the more inland populations decreases with increasing photoperiods, both in the field (fig. 2) and in the greenhouse (fig. 5). Furthermore, a difference between the SJM and M/C populations was detected in short-day greenhouse experiments but not under long days (experiments 1 and 3 in fig. 5), and the ARB and M/C populations do not differ under long days (fig. 7). Diminished statistical differences between the two inland populations under long days could reflect low statistical power, but it could also indicate that these more inland populations differ through the photoperiodic response, which is more strongly expressed under the shorter winter photoperiods (experiments 2 and 4 in fig. 5). Under very long summer days, however, all plants in these populations may be past their critical photoperiod from the start, causing genetic differences in critical time not to be expressed. Persistence of the BB versus inland differences under long days indicates that this difference could result in part from genetic differentiation at the endogenous or gibberellic acid pathways (Puterill et al. 2004). Schranz et al. (2002) found that some biennial *B. rapa* strains differ from annual strains by having more replicates of the *BrFLC* gene. This flowering inhibitor is regulated by the endogenous pathway and is a candidate for flowering time variation in the clinal populations.

In summary, studies presented in this article strongly indicate that the flowering time cline in *B. rapa* along the San Diego Creek drainage is genetically based and caused by selection imposed by differences in soil moisture or other environmental factors correlated with soil moisture. Further studies are required to determine which loci are responsible for the clinal variation and to confirm the role of season length mediated by soil moisture as the agent of selection on flowering phenology.

Acknowledgments

We thank G. LeBuhn, D. R. Campbell, and L. D. Mueller for advice and assistance in several aspects of this study. The manuscript was greatly improved through suggestions from G. A. Fox, A. G. Stephenson, and an anonymous reviewer. S. Honders, M. Cuevas, A. Calusen, and M. Vega helped in the greenhouse. Grants from the Mildred Matthias Fund (to D. M. Franke and A. G. Ellis) and the National Science Foundation (DEB98-15873 and DEB03-45030 to A. E. Weis) provided financial support.

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Queries

Q1 "Brassica nigra" correct expansion of B. nigra? (IJPS style is to spell out genera on first reference to each species.)
Q2 Franke 2004 is not included in Lit. Cited. Please provide a reference for this article.
Q3 This sentence ("We also examined...") seemed to have some extra words in it. I've provided a possible rephrasing, but please check carefully that it accurately reflects your intended meaning.
Q4 "Once seed set was complete but before pods dehisced, seeds were separated..." OK to repair sentence fragment? Or did you mean "...at time of flowering, once seed set was complete but before pods dehisced."?
Q5 Sentence beginning "We also wanted to know..." slightly rephrased for clarity; does it accurately reflect your intended meaning?
Q6 "later, wetter 1999–2000 season" changed to "later, drier..."; is this correct?
Q7 Added note to table 2, "Boldface indicates a significant difference between the populations." Is this correct? If not, what does boldface indicate?
Q8 Change of "indication directional selection" to "indicating directional selection" correct?
Q9 Changed citation of table 4 here to table 3; is that correct? If not, please cite table 3 somewhere between table 2 and 4 citations, since table 3 is not currently cited in the paper.
Q10 Change of capital B to lowercase b in upper-right cell of table 3 OK?
Q11 "Principal component" correct expansion of "PC"?
Q12 Can you provide a page range for the Mazur & LeBuhn chapter?
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