

USING EXPERIMENTAL EVOLUTION TO INVESTIGATE GEOGRAPHIC RANGE LIMITS IN MONKEYFLOWERS

Amy L. Angert,^{1,2,3} H. D. Bradshaw Jr.,^{4,5} and Douglas W. Schemske^{6,7}

¹Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824

²E-mail: angert@mail.colostate.edu

⁴Department of Biology, University of Washington, Seattle, Washington 98195

⁵E-mail: toby@u.washington.edu

⁶W. K. Kellogg Biological Station, 3700 East Gull Lake Drive, Hickory Corners, MI 49060

⁷E-mail: schem@msu.edu

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Every species occupies a restricted geographic distribution, but it is unclear why natural selection at the range margin fails to increase tolerance to limiting environmental variables and thereby allow continual range expansion. Models indicate that the interplay of demographic asymmetries, dispersal, divergent natural selection, and adaptive trade-offs across spatially varying environments can give rise to stable range limits. Here we examine sister species of the monkeyflowers *Mimulus cardinalis* and *M. lewisii* to identify traits that might contribute to the evolution of the species' ranges and to ask whether adaptive trade-offs between environments can limit their geographic distribution. In the Sierra Nevada Mountains of California, *M. cardinalis* is found from low to mid elevation and *M. lewisii* is found from mid to high elevation. We transplanted segregating populations of interspecific hybrids to low and high elevation and cross-pollinated those that survived to flowering to create selected populations that evolved at low or high elevation. When grown in a common environment, the progeny of hybrids selected at high elevation flowered earlier compared to a greenhouse control population, whereas hybrids selected at low elevation displayed increased warm-temperature photosynthetic capacity. If adaptation to one environment entails a cost to adaptation in other environments, then selected hybrid populations should display reduced fitness, relative to an unselected control population, when grown in an environment in which they were not selected. Two such trade-offs were observed in this study, where hybrids selected at high elevation displayed reduced biomass when grown in temperatures characteristic of low elevation and hybrids selected at low elevation showed reduced resistance to freezing. These results identify traits under selection for range expansion and suggest that adaptive trade-offs can contribute to limiting the geographic distribution of species.

KEY WORDS: Flowering time, geographic distribution, natural selection, physiological adaptation, trade-off.

Species' distribution boundaries have long fascinated ecologists and biogeographers seeking explanations for why species fail to occur beyond their present limits (Griggs 1914; Grinnell 1917; Good 1931; Dahl 1951). Most studies of distribution limits have

focused on identifying the proximate ecological factors that give rise to a distribution boundary. Such studies may determine whether local species abundance decreases toward the range margin (Brown et al. 1996; Sagarin and Gaines 2002) or whether marginal populations are demographic sinks or more prone to extinction than central populations (Carter and Prince 1981; Lennon et al. 1997; Mehlman 1997; Guo et al. 2005; Angert 2006a).

³Present address: Department of Biology, Colorado State University, Fort Collins, Colorado 80523.

Many other investigations of distribution limits focus on individuals, asking whether survival and reproduction decrease toward the range margin (Marshall 1968; Pigott and Huntley 1981; McKee and Richards 1996; Garcia et al. 2000; Hennenberg and Bruelheide 2003; Angert and Schemske 2005), and, if so, which environmental variables are responsible for variation in components of fitness (McNab 1973; Root 1988; Cumming 2002; Angert 2006b). However, even when ecological and demographic factors that limit the range are identified, it remains unclear why natural selection does not continually improve adaptation to limiting environmental variables and overcome current distribution limits. To solve this conundrum, we must know which traits are under selection at and beyond the range boundary, and why they do not evolve to allow range expansion.

Many mechanisms have been proposed to limit the potential for adaptive evolution at range boundaries. Some hypotheses propose that marginal populations lack genetic variation in traits necessary for range expansion, perhaps due to genetic drift in small populations, increased environmental variation in stressful environments that masks additive genetic variation, or depletion by persistent strong natural selection (Parsons 1991; Hoffman and Blows 1994; Eckert et al. 2008). A related class of hypotheses posits that marginal populations lack appropriate multivariate genetic variation, although additive genetic variation in any given trait may not be low, due to negative genetic correlations among traits or fitness components (Antonovics 1976; Bradshaw 1991; Blows and Hoffman 2005; McGuigan and Blows 2007). A third class of hypotheses focuses on the maladaptive effects of gene flow from centrally adapted populations (Haldane 1956; Garcia-Ramos and Kirkpatrick 1997; Kirkpatrick and Barton 1997). Although these hypotheses do make some distinct predictions, they are not necessarily mutually exclusive and may act synergistically to constrain range expansion.

Theoretical studies have focused on the latter class of hypotheses, and these studies illustrate how a complex interplay of demographic asymmetries, dispersal, natural selection, and adaptive trade-offs can give rise to stable range limits across environmental gradients (Garcia-Ramos and Kirkpatrick 1997; Kirkpatrick and Barton 1997) or between central and marginal habitat patches (Holt and Gaines 1992; Holt and Gomulkiewicz 1997; Gomulkiewicz et al. 1999; Kawecki and Holt 2002; Holt 2003). A common premise of these models is that environments within and beyond the range favor different phenotypes, and species are best adapted to the environment of the range center. Even when dispersal is random with respect to direction, greater population density at the range center yields a net flux of migrants from the center to the edge. In this scenario, gene flow from central populations swamps marginal populations with maladapted alleles and hinders adaptation to marginal environments. Alleles that could increase fitness in the marginal habitat incur a fitness

cost at the range center where population densities are greatest (Holt and Gomulkiewicz 1997; Kawecki 2000). Thus, due to the combined effects of demographic asymmetries, dispersal, and trade-offs, adaptive evolution is biased toward habitats in which the population already resides, and results in the establishment of stable range limits.

Adaptive trade-offs such as those underlying models of range limit evolution are fundamental to evolutionary ecology theory and presumed to be a general outcome of divergent natural selection (Levins 1968; MacArthur 1972; Gupta and Lewontin 1982; Futuyma and Moreno 1988). When trade-offs exist due to antagonistic pleiotropy, selection acts in different directions on a quantitative trait and favors alternate alleles at loci underlying the trait. Although negative correlations between fitness in different environments are often observed in interspecific or interpopulation comparisons, they do not provide definitive evidence of genetic trade-offs (Futuyma and Moreno 1988; Fry 2003). This is because it is possible that populations may exhibit divergent adaptation and apparent adaptive trade-offs due to the accumulation of mutations that are favored in the native environment but neutral in alternate environments. In this scenario, selection has acted on independent loci in each environment and recombination following gene flow could yield broadly adapted genotypes, quite the opposite of range-limited genotypes. Recent studies have demonstrated many loci underlying quantitative traits contribute to fitness in one environment only or are globally favored, rather than having opposing effects as is generally assumed (Gardner and Latta 2006).

The evolution of range limits is a multi-faceted problem that ultimately requires knowledge of population density and dynamics across the range, the relative strengths of selection and gene flow across environmental gradients, and genetic variation and genetic architecture of the traits under selection. To tackle such a complex problem requires the development of systems in which these diverse pieces of information can be gathered. Toward that broader goal, this study had two specific aims: (1) to estimate phenotypic selection across the environmental gradient to identify traits that are required, or closely linked to required traits, for the species to expand its range and (2) to assess whether adaptive trade-offs have the potential to bias evolution toward the range center and thus contribute to stable range limits.

There are several possible approaches to estimating phenotypic selection and testing for adaptive trade-offs. Standing genetic variation could be used for estimating phenotypic selection gradients in marginal environments and for split-family designs (Fry 1996) to quantify genetic trade-offs between environments. This approach has the benefit of utilizing natural phenotypic and genetic variation within a population, but would potentially suffer from low power because variation within populations is likely to

be reduced by stabilizing selection (Endler 1986; Kingsolver et al. 2001). Split-family designs may also fail to reveal genetic trade-offs even when they exist (Fry 1996). Alternative approaches would use experimental evolution beyond the species range to identify traits that evolve under “quasinalural” selection (Kassen 2002) and to assess the fitness costs of adaptation to the marginal environment. The base population for such an experimental evolution approach could again be a sample from natural marginal populations. This would best mimic the processes under study, but the generation time of most organisms would preclude this approach.

To increase the tractability of experimental evolution approaches for a broader range of organisms, many researchers opt for increasing genetic variation, and hence selection response, within the base population, for example by experimental hybridizations of divergent populations or closely related species (Conner 2003; Fry 2003). Here we have adopted the latter approach by creating advanced-generation hybrids between *Mimulus cardinalis* and *M. lewisii*, sister species of monkeyflower with parapatric elevation distributions. Previous work in this system has revealed no local adaptation to elevation or temperature among populations within each species (Angert and Schemske 2005; Angert 2006b). In addition to the expanded range of phenotypic and genetic variation, advanced-generation hybrids also have the advantage of decoupling trait combinations that covary within species. This provides a means to identify traits that are targets of natural selection (or closely linked to true targets) and to assess the fitness costs of adaptation to a novel environment without the confounding effect of linkage disequilibrium that arises from population history. This approach assumes that the genetic architecture of differences between closely related species can inform us about adaptive trade-offs that also would be present within each species (Bradshaw and Schemske 2003; Hall et al. 2006; Albert et al. 2008). The source populations used here were from the species’ shared range limit, so we were effectively asking what traits are required for a given species to expand its range, and if this involves trade-offs.

Previous experiments have demonstrated that each species is most fit at its elevation range center (low elevation for *M. cardinalis*, high elevation for *M. lewisii*), less fit at the shared mid-elevation range boundary, and unable to survive or reproduce when transplanted to elevations beyond its current range (Hiesey et al. 1971; Angert and Schemske 2005). For *M. lewisii*, reduced fitness at low elevation results primarily from high mortality within the first growing season. For *M. cardinalis*, reduced fitness at high elevation is due primarily to limited growth and reproduction (Angert and Schemske 2005). Many features of the environment that affect plant survival, growth, and reproduction change with elevation, most prominently temperature and length of growing season. In growth chamber experiments, *M. cardinalis*

and *M. lewisii* display differences in survival, growth, leaf photosynthetic physiology, and freezing resistance when grown in temperature regimes that mimic their natural distributions (Angert 2006b). The species also differ in phenological traits that may contribute to differences in fitness across an elevation gradient. When grown in a common environment, *M. lewisii* flowers earlier than *M. cardinalis* (Hiesey et al. 1971), suggesting that the ability to flower and mature fruits quickly may be favored in short growing seasons at high elevation. In this study, we measure natural selection on leaf photosynthetic rates, freezing resistance, and flowering phenology. We hypothesized that genetic variation in these traits affects the ability to survive and reproduce at different elevations.

We created advanced-generation hybrids between *M. cardinalis* and *M. lewisii* and transplanted them to low and high elevation (Fig. 1A, B). We then cross-pollinated surviving plants that had reached the flowering stage within each environment (Fig. 1C) and grew the progeny in common environments to determine which physiological and phenological traits evolved at low and high elevation, relative to a control hybrid population maintained in a permissive greenhouse environment (Fig. 1D). We apply two criteria to assess trait evolution. First, if a particular trait is itself a target of natural selection or is genetically correlated with a trait that is the target of natural selection, then its mean value should differ significantly from the unselected control population. Second, if parental trait values are adaptive, then trait means of selected hybrids should evolve toward the phenotypic value of the parent native to that environment. Based on these criteria, we hypothesized that hybrids selected at high elevation will flower more rapidly, exhibit less tissue damage following freezes, and display greater leaf photosynthetic capacity in cool temperatures characteristic of high elevation than the greenhouse control population. Likewise, we hypothesized that hybrids selected at low elevation will flower later, incur greater freezing damage, and display greater leaf photosynthetic capacity in warm temperatures characteristic of low elevation than the greenhouse control population.

To determine whether adaptation to low elevation entails a cost to adaptation at high elevation, and vice versa, we measured phenotypes of hybrids grown in two temperature regimes: one characteristic of low elevation and one characteristic of high elevation. If adaptation to one environment entails a cost in another environment, then selected hybrid populations should display reduced fitness, relative to the control, when grown in the environment in which they were not selected. Alternatively, if reduced fitness in the unselected environment is not evident as a pleiotropic or correlated byproduct of evolution in the selected environment, then we can conclude that fitness across the environmental gradient is not constrained by between-environment fitness trade-offs.

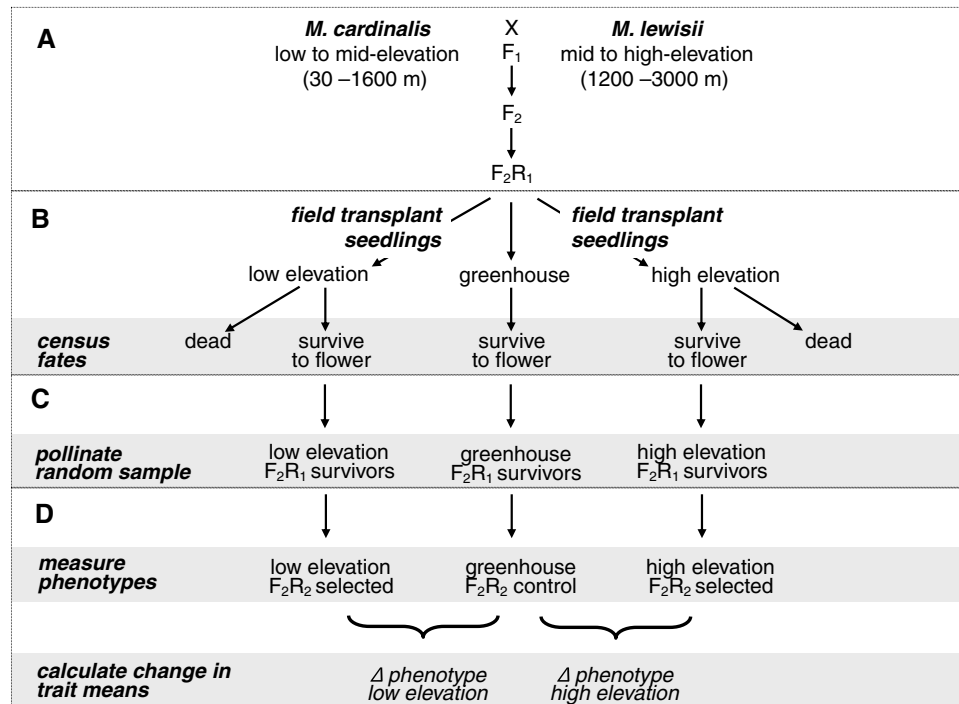


Figure 1. Schematic of experimental design. (A) Pollinations to generate segregating hybrid population. (B) Field transplants to low and high elevation, with greenhouse control population. (C) Field pollinations to generate selected hybrid populations. (D) Measurement of change in phenotypic trait means due to selection.

Materials and Methods

The genus *Mimulus* has become a model system for evolutionary ecology because of its tremendous ecological and phenotypic diversity and ease of experimental propagation (Wu et al. 2007). In addition, the availability of a species-level molecular phylogeny provides an excellent platform for comparative studies (Beardsley et al. 2004). *Mimulus cardinalis* and *M. lewisii* (Phrymaceae) are closely related perennial herbs of riparian habitats in western North America. *Mimulus cardinalis* occurs from southern Oregon to northern Baja California, Mexico and from the coast of California inland to Arizona and Nevada. *Mimulus lewisii* is composed of two partially incompatible races (due to at least two reciprocal chromosomal translocations; Hiesey et al. 1971), one occurring in the Pacific Northwest and the Rocky Mountains and one occurring primarily in the Sierra Nevada Mountains of California (Hiesey et al. 1971; Hickman 1993; Beardsley et al. 2003). Artificial hybrids between the Sierra Nevada populations of *M. lewisii* and *M. cardinalis* have regular meioses, with no evidence of chromosomal inversions, translocations, or rearrangements (Hiesey et al. 1971). In California, *M. cardinalis* and *M. lewisii* occupy different elevation ranges, with *M. cardinalis* occurring from sea level to 2400 m and *M. lewisii* occurring from 1200 m to 3100 m (Hickman 1993). In the Yosemite National Park region in which our research was conducted, *M. cardinalis* is not commonly found above 1500 m, *M. lewisii* is not commonly

found above 2800 m, and the species sometimes co-occur on the banks of larger watercourses between 1200 and 1500 m elevation (Angert 2005).

GENERATION OF SEGREGATING INTERSPECIFIC HYBRID POPULATIONS AND PARENTAL SPECIES CONTROLS

Seeds of *M. cardinalis* and *M. lewisii* were collected from a naturally occurring sympatric population along the South Fork of the Tuolumne River (Carlon Day Use Area, Tuolumne County, California, 37.8152°N, 119.8657°W, 1320 m asl) in September 1999. Two individuals of each species from distinct maternal plants were grown to flowering in the University of Washington greenhouse under standard greenhouse conditions and crossed to generate two independent F₁ hybrid lines, using *M. lewisii* as the maternal parent in each cross. Two F₁ individuals, one from each line, were grown to flowering and crossed to generate a segregating F₂ population. One thousand F₂ individuals were grown to flowering and crossed to one another so that each plant served as pollen donor and recipient once (with no self- or reciprocal pollinations), generating 1000 hybrid seed lots with an additional round of recombination (“F₂R₁”; Fig. 1A). These advanced-generation hybrids are similar to F₃, except that they were produced by sib-mating rather than selfing the F₂ population. Pure *M. lewisii* and *M. cardinalis* seedlings, with the same alleles

and inbreeding coefficient of the F_2R_1 interspecific hybrids, were produced by sib-mating intraspecific F_1 offspring (" F_1R_1 ") of the same two *M. lewisii* and two *M. cardinalis* used as parents of the segregating interspecific F_2R_1 hybrids.

TRANSPLANT GARDENS

Experimental gardens were established near Jamestown, California (37.9173°N, 120.4212°W, 415 m asl) and at White Wolf Ranger Station in Yosemite National Park (37.8718°N, 119.6507°W, 2395 m asl). These locations were chosen to represent elevations that are central within the elevation range for one species (415 m for *M. cardinalis*, 2395 m for *M. lewisii*) and beyond the range boundary for the other (2395 m for *M. cardinalis*, 415 m for *M. lewisii*). Seeds from 500 F_2R_1 hybrid seed lots were sown in flats in the University of Washington greenhouse five weeks prior to transport to garden sites. The average age of transplanted seedlings was approximately three weeks after germination, when the seedlings were approximately 6 cm tall. In July 2001, 8110 F_2R_1 seedlings (16–17 individuals from each of 500 seed lots) were transplanted in random order at White Wolf (*M. lewisii* range center, 2395 m; Fig. 1B). To assess the strength of selection in each environment, 319 F_1R_1 control seedlings of each parental species were randomly interspersed among the hybrid individuals. In April 2003, 6000 F_2R_1 seedlings were transplanted to Jamestown (*M. cardinalis* range center, 415 m) following identical methods (11–12 from each of the same 500 F_2R_1 hybrid seed lots plus 156 of each F_1R_1 pure species control; Fig. 1B).

Garden plots were covered in landscape fabric to suppress weed growth and irrigated daily to approximate conditions in the species' native riparian habitat and to standardize water treatments across environments. Due to irrigation system failure in one area of the Jamestown garden, 27 *M. cardinalis*, 24 *M. lewisii*, and 933 hybrids were excluded from analysis. At Jamestown (415 m), most *M. lewisii* were dead after one growing season, and the majority of surviving plants had reached the flowering stage, thus censuses at this site were restricted to 2003. Observations were conducted over a longer time period at White Wolf (2395 m) because of the longer time necessary for plants to reach reproductive maturity at high elevation. Survival and day of first flowering were recorded at approximately two-week intervals from 2001 to 2003 at White Wolf and in 2003 at Jamestown. Records from the closest regional weather stations (Sonora, National Climate Data Center station #8353 and Bridgeport, NCDC station #1072) indicate that the 2001 and 2002 growing seasons were close to long-term average temperatures but 2003 was 1.5 – 2°C warmer than average.

GENERATION OF SELECTED AND CONTROL HYBRID POPULATIONS FROM THE EXPERIMENTAL GARDENS

To compare phenotypes in a common environment, we generated a seed population for each elevation from plants that had

experienced selection ("selected" populations) and grew these progeny (" F_2R_2 ") in common environments for trait measurement. Selected seed populations were made by crossing subsets of hybrid individuals that were able to survive and flower within the transplant gardens (Fig. 1C). We enclosed all floral buds in fine mesh bags to prevent pollinator visitation and conducted hand pollinations because we were taking precautions not to introduce interspecific hybrid pollen to natural populations in Yosemite National Park. Due to low survival to flowering of parental species grown outside their native range, we did not pollinate parental species controls. At White Wolf, pollinations of hybrids were conducted at two-week intervals in 2003, beginning two weeks after flowering commenced and proceeding throughout the flowering period. Up to 80 individuals were crossed to one another within each pollination cohort, using only those individuals that began flowering within the interval. Each plant served as pollen donor and recipient only once. When more than 80 individuals began flowering within the two-week period, individuals were haphazardly selected from throughout the garden. Because this method of crossing potentially flattened the flowering time distribution of the offspring, for subsequent experiments we included fruits from each pollination cohort in proportion to the total number of individuals that began flowering within the interval.

At Jamestown, we were unable to conduct pollinations on hybrids during the growing season of 2003, so dormant rhizomes of individuals that survived and flowered in 2003 were transported to the Michigan State University greenhouse in February 2004, where plants were regrown to flowering. Pollinations of Jamestown plants grown in the greenhouse were conducted following identical methods to those used at White Wolf, defining pollination cohorts by the flowering times previously recorded within the transplant garden. An additional population of hybrids from 250 of the original 500 F_2R_1 hybrid seed lots was grown under favorable conditions in the greenhouse, where selection was assumed to be minimal (survival 100%, only 6 out of the initial 250 lines not included in crosses due to pollen inviability), and crossed following identical methods to generate an unselected F_2R_2 control population of hybrid seeds.

MEASUREMENT OF PHENOTYPIC TRAITS

We investigated phenotypic selection both within and between hybrid populations. Phenotypic selection on flowering time within populations were measured within the F_2R_1 population in the field at White Wolf (2395 m) by examining the relationship between flowering time and seed set. Seed set per fruit was quantified for hand pollinations conducted at two-week intervals (see previous section). In the laboratory, samples of approximately 150–200 seeds per fruit were counted under a dissecting microscope and weighed to determine the relationship between seed mass and seed number. To examine the relationship between date of first flower

and total seed set, total seed number per plant was estimated for each pollination cohort by multiplying the number of flowers each plant produced by the expected seed set per flower based on hand-pollinations. Phenotypic selection on leaf photosynthetic traits, tissue resistance to freezing damage and flowering phenology between populations was measured as changes in trait means between selected and unselected control F_2R_2 populations grown in common environments in the experiments described below (Fig. 1D).

Growth chamber experiment

Selected and unselected control hybrids (F_2R_2 , Fig. 1D) were grown in two growth chambers (Model GC-20BDAF-REFR404, Econair, Winnipeg, Canada), one simulating a temperature regime characteristic of low elevation (35/15°C day/night, with 42°C daytime maximums on days 50 and 64) and the other grown in a temperature regime characteristic of high elevation (23/4°C day/night, with -2°C freezes on nights 50 and 64). These regimes were based on July temperatures measured within the Jamestown and White Wolf transplant gardens, which reproduce the patterns of differential growth and survival observed in reciprocal transplants at these sites (Angert 2006b). Data loggers (Hobo Pro Temp/External Temp; Onset Computer, Bourne, MA) placed at plant height within the transplant gardens recorded 34 nights when air temperatures dropped below freezing during the 2003 growing season at White Wolf, with freezing nights increasing in frequency toward the end of the season. At Jamestown during the same period, temperatures recorded by data loggers never dropped below zero. During simulated freezing events in the growth chamber, plants were transferred for a period of 24 h to a chamber capable of holding subzero temperatures for the 4-h freeze treatment (Model GC-20BDAF-REFR-22, Econair, Winnipeg, ON, Canada). Chambers were programmed for 14/10 h day/night cycles (approximating the natural photoperiod in July), with daily maximum and minimum temperatures held for 4 h each with gradual ramps between the maximum and minimum. Light averaged 350 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at plant height.

In October 2004, seeds of selected and control F_2R_2 hybrid populations were sown in either the low or the high elevation temperature regime in 6 cm rose pots (Anderson Die and Manufacturing Company, Portland, OR) filled with Baccto High Porosity Soil-less mix (Michigan Peat Company, Houston, TX). Pots were placed in random order within wire frames, and the frames were placed in trays for subirrigation within the growth chamber. Frames were rotated several times per week to minimize position effects. Approximately 10 seeds were sown per 10 cm pot and seedlings were randomly thinned to one seedling per pot three weeks after sowing so that each temperature regime

contained 35 individuals from each hybrid population plus 15 individuals of each parent species. After thinning, the cotyledon diameter of each remaining seedling was measured to account for potential differences in performance between selected populations due to maternal growth environment (greenhouse or 2395 m garden). However, cotyledon diameter did not differ between selected populations (one-way analysis of variance, low elevation temperature regime: $F_{2,163} = 0.12$, $P = 0.89$; high elevation temperature regime: $F_{2,155} = 1.01$, $P = 0.37$), indicating that seed quality did not significantly influence early seedling growth.

Simultaneous gas exchange and chlorophyll fluorescence measurements were performed with a portable open-flow gas exchange system equipped with leaf chamber fluorimeter and CO_2 mixer (LI6400, Li-Cor, Inc., Lincoln, NE) following the last extreme temperature event for each treatment to characterize leaf photosynthetic function in low and high elevation temperature environments. The youngest fully expanded leaf (second or third node) was enclosed within the leaf chamber and measured under the light intensity in which leaves developed (350 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$), a reference CO_2 concentration of 400 $\mu\text{mol mol}^{-1}$, a flow rate of 500 $\mu\text{mol s}^{-1}$, and block temperatures of 35°C (hot chamber) or 23°C (cold chamber). We measured the following parameters: (1) instantaneous net photosynthetic rate ($\mu\text{mol CO}_2\text{ m}^{-2}\text{s}^{-1}$); (2) effective quantum yield of photosystem II [$(F_m' - F_s)/F_m'$], which is the fraction of absorbed photons that a light-adapted leaf uses for photochemical reactions, determined by chlorophyll fluorescence readings; (3) stomatal conductance ($\text{mol H}_2\text{O m}^{-2}\text{s}^{-1}$), an indicator of the degree of stomatal openness, which determines leaf loss of water and gain of carbon dioxide; and (4) the ratio of intercellular to ambient CO_2 , which can indicate the degree to which stomatal closure limits the availability of CO_2 for photosynthesis. Because these response variables are statistically intercorrelated ($r = 0.54 - 0.87$, all $P < 0.001$), we present results for instantaneous net photosynthetic rate only, although all variables yielded similar patterns.

We quantified post-freeze tissue damage within the high elevation temperature regime by estimating the percentage of total leaf tissue damaged on each plant on the day following the first freeze event. After 87 days (low elevation temperature regime) and 127 days (high elevation temperature regime), plants were harvested for measurement of aboveground biomass. The difference in time period preceding harvest reflects large differences in growth rates between temperature regimes. Because very few plants (1 *M. lewisii*, 7 hybrids) flowered in the high elevation temperature regime, we conducted a second experiment in the greenhouse (see below) to examine the experimental evolution of flowering phenology.

Greenhouse common garden experiment

In May 2006, seeds of selected and unselected control F_2R_2 hybrid populations were sown on moist filter paper (Whatman #1) and then placed in an incubator (I-36LL, Percival Scientific, Perry, IA) for a one-week stratification treatment (10°C, no lights). One to four seeds were then transferred in a random order from the filter paper to 98-cell plug trays filled with Fafard Super Fine Germination Mix (Conrad Fafard, Agawam, MA, USA). Plug trays were placed under an automatic mist bench under greenhouse conditions and seed germination was recorded every three to four days. After the majority of seedlings had germinated (~ three weeks), the plug tray was removed and watered with an aqueous fertilizer. When seedlings were approximately five-week old, they were randomly thinned to one plant per cell, transplanted into rose pots as described above, placed in a random order, and sub-irrigated for the remainder of the experiment. Sample sizes were as follows: 157 Jamestown selected hybrids, 124 White Wolf selected hybrids, 150 greenhouse unselected control hybrids, 69 *M. lewisii* parents, 82 *M. cardinalis* parents. The date of first flower was recorded every three to four days for 70 days, at which point 92% of all plants had flowered; plants that did not flower were stunted and were unlikely ever to flower. We calculated days to flowering as the date of first flower minus germination date.

DATA ANALYSIS

All statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC). To examine differences among F_1R_1 parents and F_2R_1 hybrids in the probability of surviving and flowering at each elevation, we performed logistic regressions (PROC LOGISTIC), using the “contrast” statement to test for pairwise differences between parents and hybrids and the sequential Bonferroni procedure to control type I error rates. To examine differences among F_1R_1 parents and F_2R_1 hybrids in the day of first flower at each elevation, we performed analysis of variance (ANOVA) on log-transformed data (PROC GLM). Pairwise differences between parents and hybrids were evaluated with Tukey–Kramer adjusted comparisons of least square means. We used linear regression to examine the relationship between

pollination date and seed set per fruit for F_2R_1 hybrids at high elevation (PROC REG). We examined the relationship between date of first flower and total seed set per plant within the F_2R_1 hybrid population with cubic spline transformations of relative seed set as a function of mean-standardized flowering dates (Schluter 1988; PROC TPSPLINE). Following Schluter (2000), we used the value of the smoothing parameter lambda that minimized the generalized cross-validation function ($\log_{10}(n \times \lambda) = 0.6$).

We used univariate ANOVA to examine evolved differences among F_2R_2 hybrid populations after natural selection in the field, (Jamestown selected, unselected greenhouse control, and White Wolf selected) for each continuous response variable in each environment. Differences between each selected hybrid population and the greenhouse control were evaluated with Dunnett’s test for post hoc comparisons with a control (Dunnett 1955). Because space constraints in the growth chambers necessitated small sample sizes, we use data from parental species primarily to verify the direction of interspecific differences and the effect of each temperature regime. To test for differences in survival among hybrid populations, we used logistic regression as described above. Because survival of *M. cardinalis* was 100%, models of parental survival did not converge. We used one-way designs because different variables were measured in each temperature environment (e.g., freezing damage only in the high elevation temperature regime), precluding two-way designs that included the effect of temperature.

Results

PERFORMANCE OF *M. LEWISII*, *M. CARDINALIS*, AND F_2R_1 HYBRIDS IN RECIPROCAL TRANSPLANT GARDENS

At low elevation, annual survival of *M. cardinalis* was high (81%) and nearly every surviving plant flowered in the first growing season (Table 1). In contrast, survival of *M. lewisii* at low elevation was very low (17%), and fewer than half of all surviving plants flowered. At high elevation after three growing seasons, *M. cardinalis* survival was much lower (7%) than that of *M. lewisii*

Table 1. Survival and flowering of parental species and advanced-generation F_2R_1 interspecific hybrids at low elevation (Jamestown, 415 m) and high elevation (White Wolf, 2395 m) sites in the central Sierra Nevada Mountains, California. Data recorded at Jamestown after one growing season and at White Wolf after three growing seasons.

Location	Genotype	N Planted	N Alive (flowering)	N Alive (vegetative)	% survival	% flowering
Jamestown (1 year)	<i>M. cardinalis</i>	129	102	3	81	79
	<i>M. lewisii</i>	132	6	8	17	5
	Hybrid	4755	2978	83	64	63
White Wolf (3 years)	<i>M. cardinalis</i>	319	2	21	7	1
	<i>M. lewisii</i>	319	88	42	41	28
	Hybrid	8001	814	1206	25	10

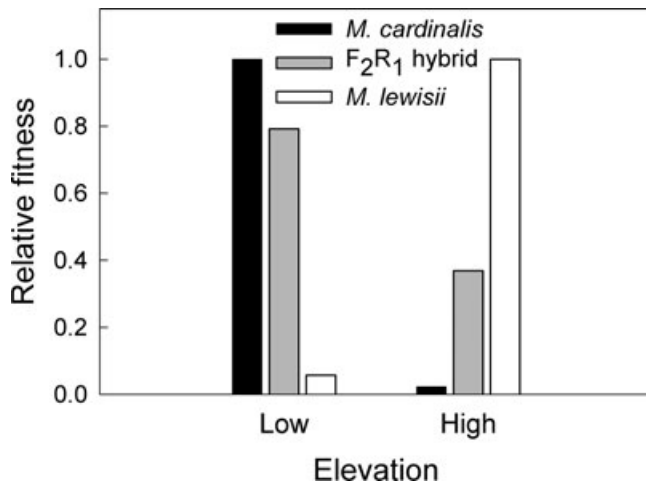


Figure 2. Relative fitness of parental species and advanced-generation F₂R₁ hybrids transplanted to a low elevation site characteristic of the range center for *M. cardinalis* (Jamestown, 415 m) and a high elevation site at the range center for *M. lewisii* (White Wolf, 2395 m).

(41%). Only two *M. cardinalis* plants flowered at high elevation, whereas approximately two-thirds of surviving *M. lewisii* flowered in the third growing season. Within each garden, survival and flowering of hybrids was intermediate to the parents (Table 1, Fig. 2). Logistic regressions of the probability of survival and flowering confirm that, within each garden, the species native to that elevation was more likely to survive and flower than either the nonnative species or hybrids (Table 2). Relative fitness of parents and hybrids within each garden was calculated by dividing the proportion of plants surviving to flower by the proportion observed for the species native to that elevation (Fig. 2). At low elevation, hybrid relative fitness was approximately 0.8, whereas at high elevation, hybrid relative fitness was approximately 0.4, suggesting stronger selection, on average, against hybrids at high elevation than at low (Fig. 2).

REPRODUCTIVE PHENOLOGY IN TRANSPLANT GARDENS

The date of first flower differed significantly among parents and hybrids at both low elevation (ANOVA, $F_{2,3083} = 6.54$, $P < 0.01$) and high elevation (ANOVA, $F_{2,901} = 41.63$, $P < 0.0001$) in 2003. At low elevation, *M. cardinalis* flowered on average four days later than hybrids ($t_{3083} = 3.60$, Tukey-Kramer adjusted $P < 0.001$). At high elevation, *M. cardinalis* and hybrids flowered significantly later than the native parent, *M. lewisii*. On average at this site, hybrids flowered approximately 13 days after *M. lewisii* ($t_{901} = 2.87$, $P < 0.05$; Fig. 3A), and the two *M. cardinalis* to flower did so approximately 35 days after *M. lewisii* ($t_{901} = 8.98$, $P < 0.0001$). Although *M. lewisii* flowered earlier on average, several late-flowering *M. lewisii* were also observed in the tail of the flowering time distribution. All plants at high elevation flowered approximately one week later in 2003 than in 2002, but the relative differences among hybrids and parents were similar in both years (data not shown). At high elevation, seed number per fruit declined with pollination date for F₂R₁ hybrids ($b = -16.75$, $N = 149$, $t = 3.41$, $P < 0.001$; Fig. 3B). We estimated the total seed set per plant by multiplying the expected seed set per flower based on hand pollinations (Fig. 3B) times the number of flowers each plant produced per pollination cohort (Fig. 3C). Total seed set per plant decreased toward zero as the date of first flower increased, indicating that selection favored early flowering at high elevation (Fig. 3D).

PHENOTYPIC DIFFERENCES WITHIN GROWTH CHAMBERS

High elevation temperature regime

Within the cold, high-elevation temperature regime characteristic of the *M. lewisii* range center, the parental species and hybrid populations did not differ in leaf photosynthetic rate at cool temperatures (parents: $F_{1,19} = 0.14$, $P > 0.05$; hybrids: $F_{2,83} = 0.67$, $P > 0.05$; Fig. 4A, B). Following exposure to freezing

Table 2. Chi-squares values from logistic regressions of the probability of survival and probability of flowering for *M. cardinalis*, *M. lewisii*, and interspecific hybrids grown at low (Jamestown, 415 m) and high (White Wolf, 2395 m) elevation. *** $P < 0.001$; **** $P < 0.0001$. All differences remain significant after sequential Bonferroni adjustment.

Location	Effect (Contrasts)	df	Survival	Flowering
Jamestown	Genotype	2	107.7833****	86.7512****
	(<i>cardinalis</i> vs. hybrid)	1	15.0678****	13.8389****
	(<i>cardinalis</i> vs. <i>lewisii</i>)	1	99.2659****	86.4138****
	(<i>lewisii</i> vs. hybrid)	1	91.6439****	72.2826****
White Wolf	Genotype	2	84.7034****	103.9157****
	(<i>cardinalis</i> vs. hybrid)	1	45.4358****	16.5353****
	(<i>cardinalis</i> vs. <i>lewisii</i>)	1	79.4660****	32.4326****
	(<i>lewisii</i> vs. hybrid)	1	37.0755****	86.2436****

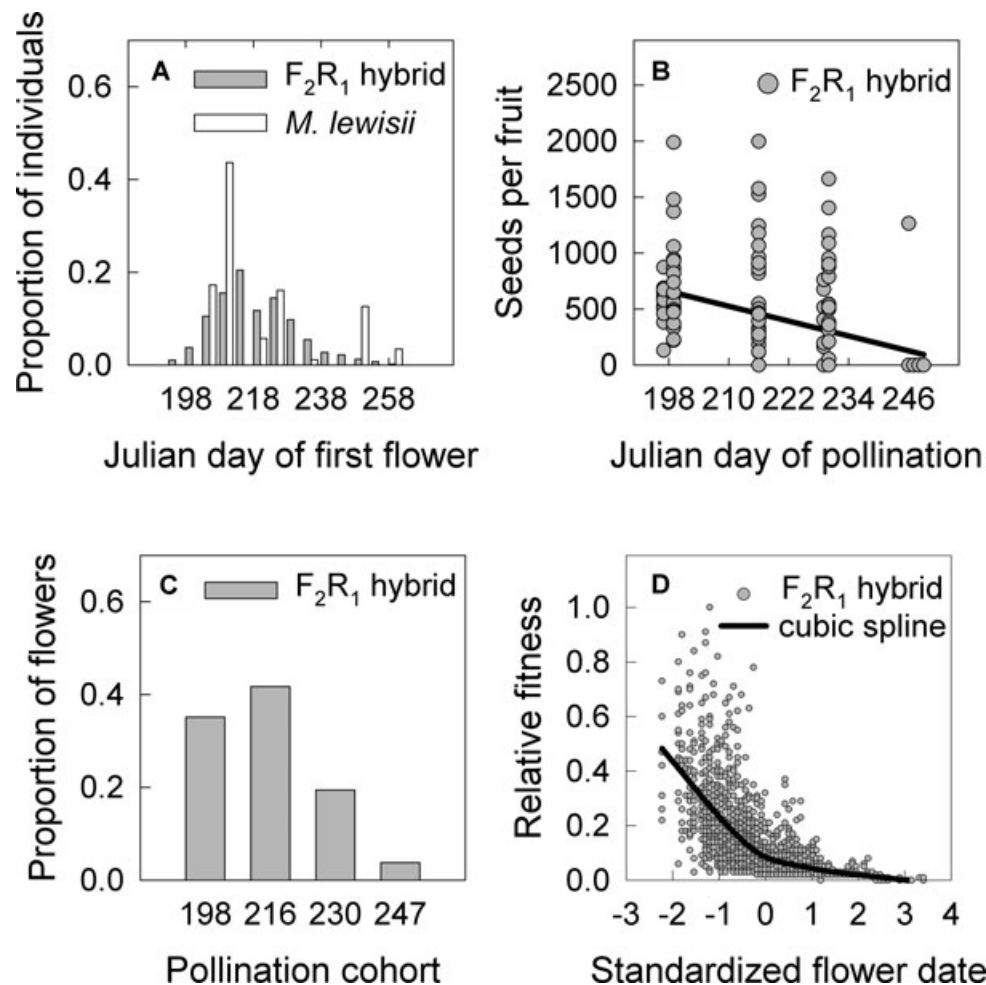


Figure 3. The relationship between reproductive fitness and flowering phenology at high elevation (White Wolf, 2395 m). (A) Distribution of the day of first flower for *M. lewisii* and F_2R_1 hybrids. (B) Linear regression of seed number per fruit versus pollination date for F_2R_1 hybrids. (C) Distribution of flowers among pollination cohorts for F_2R_1 hybrids. (D) Relative fitness (seed number per plant) versus the day of first flower (standardized to mean of zero and standard deviation of one) for F_2R_1 hybrids. Line depicts cubic spline fit.

temperatures, *M. lewisii* had lower tissue necrosis than *M. cardinalis*, although this difference was not significant ($F_{1,25} = 0.82$, $P > 0.05$; Fig. 4C). Hybrid populations differed significantly in the proportion of necrotic leaf tissue following exposure to freezing temperatures ($F_{2,95} = 3.93$, $P < 0.05$; Fig. 4D). The hybrid population selected at low elevation (Jamestown) showed marginally greater tissue damage after exposure to freezing temperatures than the greenhouse control population ($P < 0.05$; $P < 0.10$ with adjustment for multiple comparisons), suggesting that evolution at low elevation may have incurred a cost to performance in a temperature regime characteristic of high elevation. Although *M. cardinalis* had greater aboveground biomass than *M. lewisii* ($F_{1,25} = 72.15$, $P < 0.0001$; Fig. 4E), hybrid populations did not differ significantly in biomass ($F_{2,93} = 0.81$, $P > 0.05$; Fig. 4F). Survival of all genotype classes was high (*M. cardinalis*—100%; *M. lewisii*—92%; high elevation hybrids—97%;

greenhouse hybrids—94%; low elevation hybrids—91%, $\chi^2 = 0.89$, $P > 0.05$).

Low elevation temperature regime

When grown in a warm temperature regime characteristic of the *M. cardinalis* parental species range, *M. cardinalis* exhibited greater leaf photosynthetic rates in warm temperatures than *M. lewisii* ($F_{1,19} = 50.10$, $P < 0.0001$; Fig. 5A). Hybrid populations also showed significant differences in photosynthetic rates ($F_{2,92} = 6.71$, $P < 0.01$). Hybrids selected at low elevation (Jamestown) displayed greater photosynthetic rates than the greenhouse control populations of hybrids ($P < 0.01$), demonstrating evolution of photosynthetic capacity in warm temperatures at low elevation (Fig. 5B). Photosynthetic rates of high elevation hybrids did not differ from those of the greenhouse control ($P > 0.05$; Fig. 5B). In its native temperature regime,

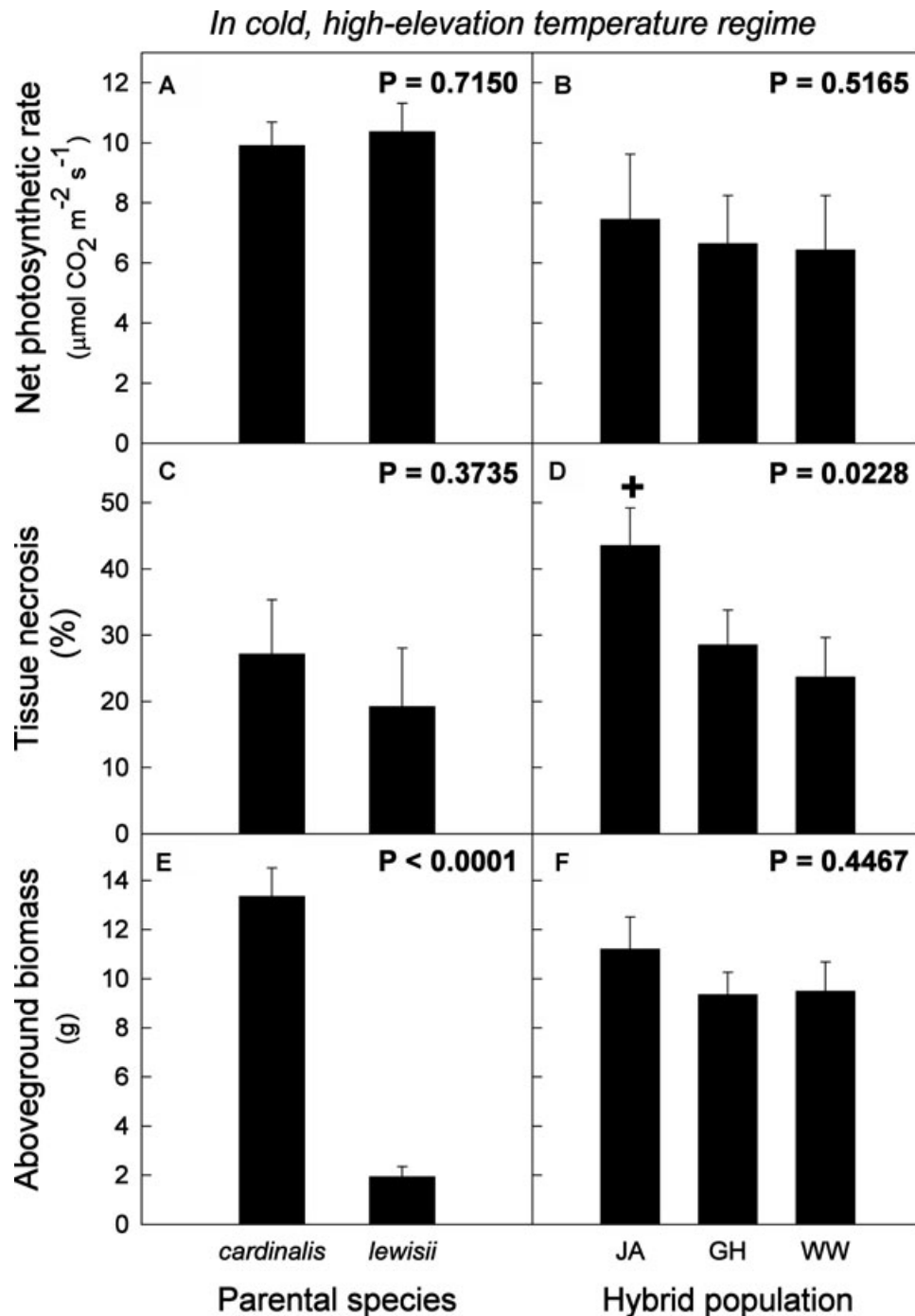


Figure 4. Comparison of instantaneous net photosynthetic rates (upper panels), tissue necrosis following freezing (middle panels), and aboveground biomass (lower panels) among parental species (left panels) and F₂R₂ hybrid populations (right panels) when grown in a cold temperature regime characteristic of the high elevation. Hybrid populations abbreviated as follows: JA, Jamestown selected low elevation population; GH, greenhouse control population; WW, White Wolf selected high elevation population. Plus sign (+) denotes selected hybrid population that differed marginally from the greenhouse control.

M. cardinalis attained much greater aboveground biomass than *M. lewisii* ($F_{1,21} = 68.86$, $P < 0.0001$; Fig. 5C). Hybrid populations also differed in aboveground biomass ($F_{2,98} = 6.03$, $P < 0.01$). The hybrid population selected at low elevation did not differ in biomass from the greenhouse control ($P > 0.05$).

However, the high elevation hybrid population had significantly less aboveground biomass than the greenhouse control, indicating that evolution at high elevation incurred a cost to performance in a temperature regime characteristic of low elevation ($P < 0.05$; Fig. 5D). *Mimulus cardinalis* survival was 100%, whereas

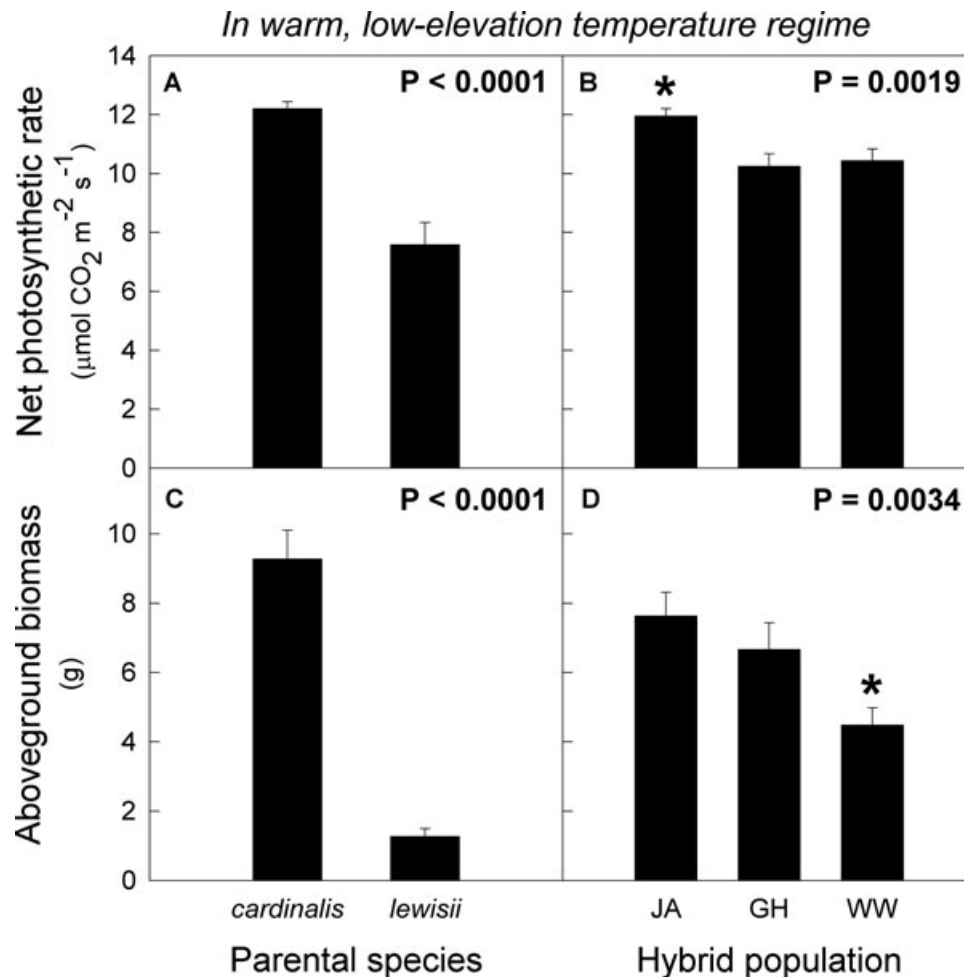


Figure 5. Comparison of instantaneous net photosynthetic rates (upper panels) and aboveground biomass (lower panels) among parental species (left panels) and hybrid populations (right panels) when grown in a warm temperature regime characteristic of the low elevation. Hybrid populations abbreviated as in Figure 4. Asterisks (*) denote selected hybrid populations that differ significantly from the greenhouse control.

M. lewisii survival was only 36%. Hybrid populations selected at low elevation displayed 97% survival, whereas hybrids selected at high elevation and in the greenhouse displayed a nonsignificant trend of lower survival (86% and 89%, respectively; $\chi^2 = 2.31$, $P > 0.05$).

DAYS TO FLOWER IN THE GREENHOUSE

When grown in a greenhouse common environment, *M. lewisii*, the parent native to high elevation, flowered approximately one week earlier than the low elevation parent, *M. cardinalis* ($F_{1,122} = 12.52$, $P < 0.001$; Fig. 6A). Hybrid populations also differed in the number of days from germination to first flower ($F_{2,376} = 5.75$, $P < 0.01$; Fig. 6B). The hybrid population selected at high elevation (White Wolf) flowered approximately five days earlier than the greenhouse control population ($P < 0.01$; Fig. 6B), indicating the evolution of rapid flowering at high elevation. Selection on the onset of flowering was not detected at low elevation, as indicated by the lack of difference in flowering time between

the low elevation and greenhouse control populations ($P > 0.05$; Fig. 6B).

Discussion

PHENOTYPIC SELECTION WITHIN AND BEYOND THE GEOGRAPHIC RANGE

In this study, we found that each parental species displayed greatest survival and reproduction at its range center and greatly reduced survival and reproduction beyond its range limit. On average, advanced-generation hybrids exhibited intermediate survival and reproduction in both environments. These hybrids also exhibited considerable variation in phenotypic traits and fitness components. This enabled us to quantify phenotypic selection on segregating traits and to identify traits that are required, or closely linked to required traits, for the species to expand its range. We found patterns consistent with natural selection favoring increased leaf photosynthetic rates in warm temperatures at low elevation

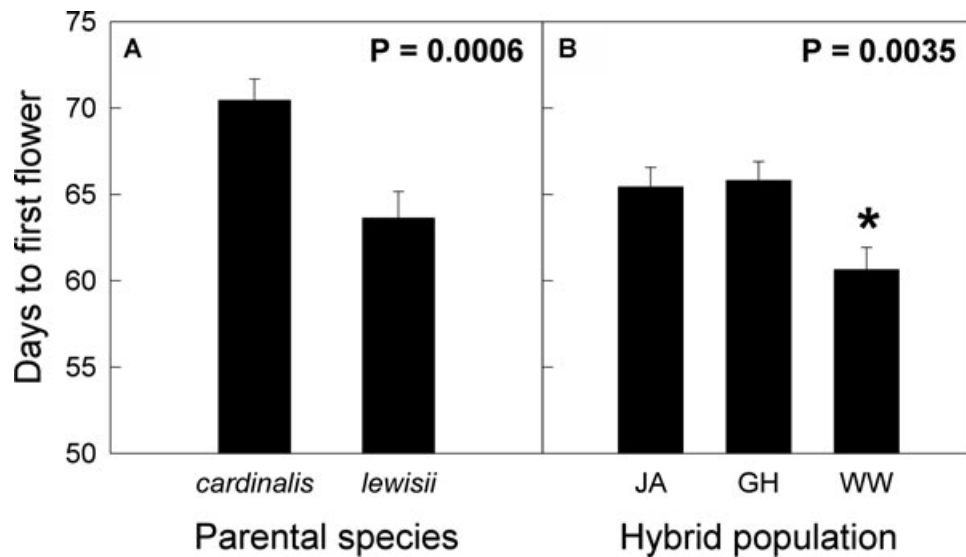


Figure 6. Comparison of flowering phenology (number of days from germination to first flower) among parental species and hybrid populations grown in a greenhouse environment. Abbreviations and symbols as in Figure 4.

and early flowering at high elevation. When significant patterns of phenotypic selection were observed, populations always evolved in the direction of the native parental species' trait value, supporting the hypothesis that *M. cardinalis* photosynthetic traits and *M. lewisii* flowering phenology are adaptive at their respective low and high elevation range centers.

The direction of natural selection on flowering phenology at high elevation was as we predicted. However, our assessment of the relationship between flowering time and reproductive fitness based on hand pollinations was independent of pollinator service, floral herbivory, or other biotic interactions. Thus, these results indicate that earlier flowering is favored by the abiotic environment, but it remains possible that pollinator service may not be reliable early in the growing season and early flowers would be pollen limited (Fleming 2006). If plants were open-pollinated rather than hand-pollinated, it is possible that the net effect of pollen limitation early in the season and physiological limitation late in the growing season would have resulted in stabilizing selection for an intermediate flowering time.

As expected, we observed the evolution of warm-temperature photosynthetic capacity in the hybrid population at low elevation. However, we did not detect selection on cool-temperature leaf photosynthetic rates at high elevation, nor did the warm-adapted low elevation population demonstrate a physiological cost (in terms of lower photosynthetic rates) when grown in cool temperatures characteristic of high elevation. Specialist/generalist trade-offs, such that an increase in performance at one temperature results in a decrease in performance at another temperature, are expected to be a pervasive feature of thermal reaction norm evolution (Levins 1968; Mongold et al. 1996; Angilletta et al. 2003). Changes in resource acquisition and allocation may mask

trade-offs even when they exist, or trade-offs may be manifest at different levels of physiological integration (Huey and Hertz 1984; Angilletta et al. 2003). On the other hand, dissection of the genetic architecture of specialization to contrasting environments can reveal surprising neutrality of adaptive alleles in an alternative environment, suggesting that adaptive trade-offs are not always the rule (Gardner and Latta 2006).

Other studies have used segregating hybrid populations to measure natural selection on leaf physiology (Heschel et al. 2002; Lexer et al. 2003; Heschel et al. 2004; Ludwig et al. 2004; Donovan et al. 2007) and have usually found selection operating in the direction of mean trait values in native populations or species as we found here. These studies used multivariate regression analysis to quantify within-generation phenotypic selection differentials and gradients on traits. In the present study, we evaluated selection as the difference in trait mean value between control and selected populations after one generation of evolution in each environment. This approach provides an assessment of the response to selection that could arise from direct phenotypic selection as well as underlying genetic correlations. Future studies combining within-generation multivariate selection analysis with measurement of between-generation selection responses would yield valuable information about the strength and direction of phenotypic selection, relationships among measured traits, and the trajectory of trait evolution.

Another consideration for the results presented here is the possibility of nongenetic effects introduced by variation in parental environment. High elevation progeny were created by pollinations in the field, whereas low elevation and greenhouse control progeny were created by pollinations in the greenhouse. To assess possible maternal effects due to differences in seed

provisioning, we measured cotyledon diameter and found no differences among populations despite differences in maternal environments. This is not surprising, as seeds of these species are very small (ca. 14 $\mu\text{g}/\text{seed}$) and not highly provisioned. Another nongenetic effect could arise from differences in seed age because seeds created in situ at high elevation were slightly older than seeds created in the greenhouse. Yet progeny from high elevation did not display reduced germinability, as might be expected for older seeds. High elevation progeny also did not display reduced growth or flowering across all environments, and in fact flowered earlier than progeny produced in the greenhouse environment. Although other nongenetic effects may yet be present, they are unlikely to explain a large percentage of phenotypic variation or to introduce bias to the results presented here. In support of this, the two populations of progeny produced in the greenhouse environment (low elevation selected and unselected control) displayed the same number of significant phenotypic differences as the comparison of populations produced in different environments (high elevation selected and unselected control). Had the parental environment introduced substantial bias, we would expect that the two populations produced in the same environment would display fewer phenotypic differences.

BETWEEN-ENVIRONMENT FITNESS TRADE-OFFS

One strength of the experimental evolution approach used here is the ability to examine not only patterns of trait evolution but also the fitness consequences of trait changes. Models for the evolution of species' ranges posit that adaptive trade-offs between environments interact with dispersal to create range limits. If adaptive trade-offs exist, then adaptation to one environment will entail a cost to adaptation in another environment, and selected populations should display reduced fitness relative to an unselected control population when grown in an environment in which they were not selected. Two such trade-offs were observed in this study, suggesting that between-environment fitness trade-offs may contribute to range limits along environmental gradients. First, hybrids selected at high elevation displayed reduced biomass (a fitness component) when grown in temperatures characteristic of low elevation, and second, hybrids selected at low elevation displayed reduced tissue resistance to freezing damage. Although high elevation hybrids did not differ from the unselected controls in their freezing resistance, we hypothesize that the reduced resistance observed in low elevation hybrids would incur a fitness cost at high elevation, given the frequency of freezing events observed during the growing season. The pattern of high photosynthetic rates but low total biomass of high elevation hybrids grown in warm temperatures suggests that increased respiration rates evolved at high elevation. High respiration rates in alpine plants are thought to increase growth capacity in the short, cold growing season of high elevations but

have detrimental effects on performance in warmer environments (Körner 1999).

This work suggests that adaptive trade-offs across the species range may contribute to the evolutionary stability of range limits, but there are two caveats to this conclusion. First, our approach assumes that genetic differences between closely related species are sufficiently similar to genetic differences among populations to be informative about genetic trade-offs within a species range. Significant relationships between the patterns of divergence across species and the genetic or phenotypic correlations within species have been observed in other systems (Schluter 1996; Baker and Wilkinson 2003; Begin and Roff 2004; Hunt 2007), suggesting that this is a reasonable assumption for the closely related species studied here (but see Pigliucci 2007; Doroszuk et al. 2008). Second, for adaptive trade-offs between habitats to play a role in range limits, dispersal from central to marginal populations must occur because an adaptive trade-off is only relevant if genotypes have the potential to be exposed to the environment in which the negative fitness consequences are manifested. At first glance, the lack of adaptive differentiation between central and marginal populations of these species (Angert and Schemske 2005; Angert 2006b) seems to suggest that dispersal from central to marginal populations would have no fitness consequences, and therefore that gene flow/trade-offs hypothesis may not be relevant to this system. However, a lack of local adaptation could be indicative of high levels of swamping gene flow across the environmental gradient (Slatkin 1978; Lenormand 2002). The potential for recurrent, long-distance dispersal exists for these riparian species (Lindsay 1964; Waser et al. 1982), but empirical estimates of gene flow within and between river drainages are necessary to determine whether this mechanism is operating in this system.

Some additional differences in fitness components between selected and control hybrid populations were suggestive of evolution of greater fitness within the selected environment at a cost to fitness within the unselected environment. For example, survival of both selected populations was numerically higher than the control in the selected environment and lower than the control in the unselected environment, but these differences were not significant. Low ability to detect differences in fitness among hybrid populations may be due to several factors. First, populations experienced less than one generation of selection in each environment, perhaps leaving considerable segregating variation within each population. Second, selected and unselected environments were simulated in growth chambers. The measurement of fitness components within growth chambers is not ideal for several reasons, including reduced flowering, small sample size, and the inability to simulate overwinter conditions. The latter limitations apply to the high elevation temperature regime in particular, in which the expected differences in trait means between the parental species were not always detected. Greater biomass of *M. cardinalis* in

the high elevation temperature regime was not surprising because *M. cardinalis* is a more robust plant than *M. lewisii*. Although *M. cardinalis* tends to be larger across all environments, for intraspecific comparisons in which plants are measured after the same amount of time in each environment, it grows relatively less in cold than in hot temperatures, whereas *M. lewisii* grows relatively more. Thus, there is a main effect of species on growth, but there is also an important species \times time interaction (Angert 2006b). Although the lack of significant interspecific differences for some traits may be due to low power (e.g., post-freeze tissue damage), other traits displayed very small differences that cannot be attributed to lack of power alone (e.g., low-temperature photosynthetic rates), suggesting that measurement conditions were not sufficiently favorable for *M. lewisii* and high elevation selected hybrids.

More definitive tests of the costs of adaptation to each environment will come from continued generations of experimental evolution and the reciprocal transplantation of selected populations to low and high elevation for a more thorough assessment of fitness. Such studies would also be improved by replication of selected populations and transplant sites. Replication of transplant sites would help prevent site-level differences in soil, canopy cover, and other microenvironmental factors from being confounded with differences due to elevation. However, we have previously demonstrated that soil type does not affect the relative performance of these species (Angert and Schemske 2005), and it is unlikely that other microenvironmental differences overwhelmed the gross effects of macroclimatic variables such as ambient temperature and length of the growing season.

A related approach to identify the causes and consequences of adaptation to alternate environments is to combine the study of quantitative trait loci (QTL) underlying traits of interest with field studies of their ecological effects. Segregating hybrid populations transplanted to low and high elevation can be used to identify QTL for fitness in each environment. The effects of major QTL can then be assessed with near-isogenic lines (NIL), containing single QTL regions from one species introgressed by repeated backcrossing into the genetic background of another, as has been done for a floral trait affecting pollinator preference between *M. cardinalis* and *M. lewisii* (Bradshaw and Schemske 2003). In this manner the phenotypic effects and fitness consequences of changes in single genomic regions can be characterized in environments within and beyond the species' range. These investigations are currently underway, and will help further our understanding of the mechanisms underlying genotype-by-environment interactions that contribute to evolutionary constraints on range expansion.

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