doi:10.1111/evo.12833

Increased susceptibility to fungal disease accompanies adaptation to drought in *Brassica rapa*

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Received November 20, 2014
Accepted November 23, 2015

Recent studies have demonstrated adaptive evolutionary responses to climate change, but little is known about how these responses may influence ecological interactions with other organisms, including natural enemies. We used a resurrection experiment in the greenhouse to examine the effect of evolutionary responses to drought on the susceptibility of *Brassica rapa* plants to a fungal pathogen, *Alternaria brassicae*. In agreement with previous studies in this population, we found an evolutionary shift to earlier flowering postdrought, which was previously shown to be adaptive. Here, we report the novel finding that postdrought descendant plants were also more susceptible to disease, indicating a rapid evolutionary shift to increased susceptibility. This was accompanied by an evolutionary shift to increased specific leaf area (thinner leaves) following drought. We found that flowering time and disease susceptibility displayed plastic responses to experimental drought treatments, but that this plasticity did not match the direction of evolution, indicating that plastic and evolutionary responses to changes in climate can be opposed. The observed evolutionary shift to increased disease susceptibility accompanying adaptation to drought provides evidence that even if populations can rapidly adapt in response to climate change, evolution in other traits may have ecological effects that could make species more vulnerable.

KEY WORDS: ***Alternaria brassicae*, drought, flowering time, rapid evolution, resurrection approach.**

Ongoing changes in climate, including warming and altered precipitation, have been increasing drought severity and frequency over the past 50 years (IPCC 2014). These changes in climate are having widespread effects in many populations, including rapid evolutionary shifts in traits such as phenology (Parmesan and Yohe 2003). Studies in the emerging field of eco-evolutionary dynamics suggest that such rapid evolutionary responses may potentially influence ecological interactions (Pelletier et al. 2009; Dargent et al. 2013), but our understanding of the ecological effects of evolutionary responses to climate change remains limited. Although adaptive evolutionary responses could potentially help populations cope with climate change (Penuelas and Filella 2001; Hoffmann and Sgro 2011; Franks et al. 2014), these benefits could be lost if the evolutionary shifts also result in increased susceptibility to predation or disease. This would be particularly likely if there are trade-offs between responses to increased abiotic stress and the ability to defend against natural enemies. Assessing the ecological effects and potential costs of climate change adaptation is critical for making predictions about the full effects of climate change.

Here, we examine the evolution and plasticity of multiple plant traits and the potential ecological effects of adaptation in a unique system in which a rapid evolutionary response to a change in climate has been demonstrated. Prior research found a rapid adaptive evolutionary shift to earlier flowering in response to a change in climate (drought) in southern California populations of the annual plant *Brassica rapa* (Franks et al. 2007; Franks and Weis 2008). In the current study, we investigate whether

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this adaptive evolutionary change may have ecological effects by exploring plant susceptibility to a fungal pathogen. Altered susceptibility is particularly likely because the early flowering plants, which are able to escape drought, allocate resources to rapid growth and development (Franks 2011), potentially leaving fewer resources available for defense. We hypothesized that post-drought descendant plants would show greater disease susceptibility than predrought ancestral plants, with the drought causing the evolution of increased susceptibility as a byproduct of selection for earlier flowering. We focused on defense against a fungal pathogen, *Alternaria brassicae*, which was commonly observed in our field sites with 21.9% (±21.1) to 35.2% (±32.7) of *B. rapa* tissue in quadrats sampled displaying symptoms (O'Hara et al. 2016). This fungus is also known to have important effects on both wild populations and agricultural varieties of crucifers (Tewari and Conn 1993). We used a resurrection approach (Franks et al. 2008), measuring pathogen response in ancestral predrought (seeds field-collected in 1997) and descendant postdrought (seeds field-collected in 2004) *B. rapa* populations grown under the same conditions in the greenhouse. We conducted a full-factorial experiment with the two plant populations (ancestral and descendant), two levels of drought treatment (well watered and drought stressed), and two levels of fungal inoculation (inoculated and noninoculated control). We assessed phenotypes including flowering time, disease susceptibility, and specific leaf area (SLA) to determine how adaptive evolution in response to drought in *B. rapa* affected disease susceptibility.

Methods

STUDY SYSTEM

The plant-pathogen system we used is the foliar fungal pathogen *A. brassicae*, which causes Alternaria black spot in its host *B. rapa* L. (Brassicaceae, field mustard) (Conn et al. 1990). *A. brassicae* is a necrotrophic fungus that causes damping off, leaf spots, defoliation, and reduced seed yield in *B. rapa* (Tewari 1991; Koike et al. 2006). Brassicas have multiple lines of defense against Alternaria fungi, including a waxy cuticle that forms a barrier to invasion (Tewari and Skoropad 1976) and induced defenses upon successful invasion, governed by multiple genes, including phytoalexins, which may impart partial, but not total resistance to the disease in *B. rapa* (Nowicki et al. 2012). Because *B. rapa* is an important crop species (bok choi, napa cabbage, oilseed, turnip, polish canola), its response to this costly and destructive pathogen has been extensively studied in agriculture (Rotem 1994; Meena et al. 2010).

B. rapa PROPAGATION

Previous to this study, a large number of seeds (>10,000) were collected from ripened seedpods (siliques) along a transect in a natural population of *B. rapa* located on the University of California Irvine campus in May of 1997 (ancestors) and June of 2004 (descendants). The temporally distinct ancestral and descendant populations are hereafter referred to as our populations. Plants were grown for a generation (about 90 days) and crossed within-population under greenhouse conditions to reduce maternal effects (Franks et al. 2007). These F1 plants were crossed within-population prior to this study to reduce storage effects and the F2 (refreshed) generation was used in the current study. For all crosses, at least 500 plants per population were crossed at random once they started flowering, using a feather to transfer pollen, and visiting each plant at least two times every 3 days.

A. brassicae CULTIVATION

B. rapa tissue infected with *A. brassicae* was collected from Bodega Bay, California. This collection site is distant (702 km) from our natural *Brassica* populations to avoid the potential issue of coevolution affecting differential disease susceptibility in ancestral versus descendant plants. *A. brassicae* fungal spores were isolated from the plant tissue and identified by the Oregon State University Plant Clinic. Spore plugs were grown on carrot dextrose agar plates for one week followed by a week on carrot agar plates under 12 hours of light and 12 of dark to encourage sporulation. Fresh spores were collected the day of inoculation, strained through gauze to remove hyphae, and adjusted to a concentration of 1×10^6 spores/ml in 0.05% Tween. All fungal work was conducted in sterile conditions, and was permitted under APHIS license #P526P-11-00130.

EXPERIMENTAL DESIGN

Using *B. rapa* F2 seeds, we conducted a greenhouse experiment (from February 18th to June 17th, 2012) growing 288 ancestral and 288 descendant plants from seed. Both populations were subjected to a full-factorial combination of a pathogen treatment (mock inoculated or inoculated with spores) and a drought treatment (well watered or drought stressed).

For cultivation, seeds were planted individually in separate 8 × 8 × 13 cm pots filled with Sunshine Mix #1 growth media (Sun Gro Horticulture, Vancouver, BC, Canada), with 1.4 g of slow release 14-14-14 Osmocote fertilizer and supplemented with Miracle Gro All Purpose 20-20-20 fertilizer weekly during watering (3.0 g/l) (Scotts, Marysville, OH, USA). To avoid room position effects, plants were moved in blocks among randomized coordinates in the greenhouse every 5 days. Blocks were small (about eight plants) and included both populations. Inoculated plants were kept separate from control plants to avoid cross-infection. Light hours were gradually lengthened from 12 to 14 hours to mimic the growing season. Because *B. rapa* is self-incompatible, plants were hand pollinated between randomized pairs of plants every three days, once they started flowering. All open flowers

were pollinated. All plants were watered daily to saturation for two weeks to allow establishment. After two weeks, we began the drought and inoculation treatments.

Plants that received a drought treatment were watered to saturation every 4 days. Plants that did not receive the drought treatment continued to be watered to saturation daily. Soil moisture was monitored using a Field Scout TDR 100 Soil Moisture Meter (Spectrum Technologies). The moisture level in the soil of drought treated plants was significantly lower than well-watered plants (wet = 28.57% soil moisture (±0.30), dry = 21.55% soil moisture (±0.22), F = 351.7, $p < 0.001$). This drought treatment was designed to mimic field conditions based on field observations and precipitation records of the study population, which was characterized by a wet period pre- and immediately postgermination followed by limited precipitation, rather than a sudden stop in rain (Franks et al. 2007). Using this study design, plants were kept alive but also experienced a drought treatment by infrequent watering.

Plants were inoculated with *A. brassicae* by wounding 2-week-old leaves (one leaf per plant and two wounds per leaf) with a sterile pipette tip and placing 10 μl of a fresh spore solution on each wound. Control plants were wounded and treated with 10 μl of 0.05% Tween. We wounded the leaves to inoculate our plants because *A. brassicae* enters leaves through wounds as well as through stomata and by enzymatically degrading the cuticle and cell wall and forming specialized penetration structures (Tsuneda and Skoropad 1978). Immediately following inoculation, plants were kept at 90% humidity for 3 days and then placed at ambient humidity and either well watered or drought treated (as described above). High humidity following inoculation is standard protocol in plant pathology studies because it is known to encourage spore germination. These conditions also mimic field conditions for our study population that experience more moisture early in the growing season and a high incidence of *A. brassicae* infection (O'Hara et al. 2016).

TRAIT MEASUREMENTS

Host susceptibility was assessed in terms of disease severity, with plants showing greater damage scored as more susceptible. The disease severities of the leaves for a subset of 277 randomly selected plants, including both noninoculated control and inoculated plants, were scored 21 days postinoculation, using a visual index (Fig. S1) that ranged from 1 to 10 based on the amount of chlorosis and necrosis (Buchwald and Green 1992). Generally, disease severity scores were independently verified by two researchers who were blinded to whether they were assessing ancestral or descendant plants. Infected leaves displayed a highly significant increase in disease severity (one-way ANOVA comparing inoculated vs. control plants: inoculated mean = 4.62 (±0.20), noninoculated mean = 3.64 (±0.16), $F_{1,117} = 41.34$, $p < 0.001$), demonstrating the efficacy of this treatment.

We quantitatively validated our visual index and the efficacy of the inoculation with a detached leaf assay of 50 leaves. Prior to inoculation, fully expanded leaves were detached from plants and placed in petri dishes on filter paper premoistened with distilled water and inoculated following the same procedure previously described. Four days postinoculation, leaves were cleared, stained, and visualized through a microscope. Leaves were cleared using a 1:3 acetic acid to ethanol solution and shaken overnight at a low speed, followed by a 1:5:1 acetic acid, ethanol, and glycerol solution. After rinsing in water, leaves were boiled for 3 minutes in a solution of 5% Parker black ink and distilled white vinegar, and then destained using water that was acidified with a few drops of vinegar, followed by a 5% vinegar wash (Vierheilig et al. 1998). The number of spores invading leaf tissue was counted at 100× magnification. Infected, stained leaves had an average of 9.5 (±8.7) spores per wound, while uninfected plants were free of symptoms and spores. We also found that spore counts were correlated with the disease severity scores (Pearson correlation: $r = 0.784$, $p = < 0.001$).

Plants were monitored daily and the date of flowering was recorded for all 527 plants that germinated. The experiment was conducted until all plants senesced. Specific leaf area (SLA), the ratio of the light capturing surface area of a leaf per unit of dry leaf mass (Milla and Reich 2007), was also measured. SLA is often altered in response to stress and is informative about resource allocation (Cornelissen et al. 2003). To calculate SLA, the newest fully expanded leaf was collected from a randomly selected subset of 280 plants 58 days postplanting, scanned, desiccated with silica beads, and then weighed. Leaf area in scanned images was measured using ImageJ (Schneider et al. 2012). SLA was calculated by dividing the area of each leaf by its dry weight.

DATA ANALYSIS

To determine if evolutionary shifts in plant traits (flowering time, disease susceptibility, and SLA) occurred, we compared ancestral and descendant plants, following the resurrection protocol (Franks et al. 2008). We tested for differences in trait means under all treatments using a three-way ANOVA, with population (ancestor or descendant), pathogen treatment (fungal or mock inoculated), drought treatment (well watered or drought stressed) and their interactions as fixed effects. A two-way ANOVA was used to test for an effect of population and drought treatment on disease susceptibility since only inoculated plants display disease susceptibility. We then tested two hypotheses using one-way ANOVAs: (1) traits (flowering time, disease susceptibility, and SLA) evolved in this population, which we tested by comparing trait means of ancestors to descendants for each trait within inoculation and drought treatments, and (2) traits displayed plasticity in response to a drought

treatment in the greenhouse, which we tested by comparing trait means of wet to dry treated plants within inoculation treatment and temporal populations. One-way ANOVAs were used because they are direct tests of these a priori hypotheses. Trait values (flowering time, disease susceptibility, and SLA) were dependent variables, with each treatment tested (population, inoculation, and drought treatment) modeled as fixed effects in specific analyses. For all models of disease susceptibility, only data collected for inoculated plants were used. All analyses were conducted on transformed data (Table S1) using R 3.0.1 stats package (R Core Team 2013).

Results

EVOLUTION OF FLOWERING TIME AND DISEASE SUSCEPTIBILITY

We found evidence for the rapid evolution of earlier flowering, with population significantly affecting flowering time (three-way ANOVA; Table 1). We also found the same pattern of descendants flowering earlier than ancestors in each treatment, but this shift was only significant under the well-watered/noninoculated condition (ANOVA: $F_{1, 131} = 5.278$, $p = 0.023$; Fig. 1A).

We found evidence for an evolutionary shift to greater pathogen susceptibility (two-way ANOVA; Table 1). When we analyzed each treatment separately, we also found that descendants were more susceptible to the pathogen than the ancestors under both well watered/inoculated (ANOVA: $F_{1, 67} = 16.25$, $p < 0.001$; Fig. 1B) and drought treated/inoculated (ANOVA: $F_{1, 65} = 5.302$, $p = 0.025$; Fig. 1B) conditions. Thus the evolutionary shift to earlier flowering was accompanied by an evolutionary increase in disease susceptibility.

We found evidence for the rapid evolution of increased SLA (three-way ANOVA; Table 1). We also found the same pattern of descendants having greater SLA than ancestors in each treatment, although this was only significant under the well watered/noninoculated condition (ANOVA: $F_{1, 69} = 4.160$, $p = 0.045$; Fig. 1C).

PLASTIC RESPONSES TO DROUGHT

We found a plastic response in flowering time to water availability, based on a significant effect of the drought treatment (three-way ANOVA; Table 1). However, the pattern among the different treatment groups was not consistent. Flowering time was significantly earlier for well watered versus drought stressed ancestral/inoculated plants (ANOVA: $F_{1, 130} = 5.360$, $p = 0.022$) and descendant/inoculated plants (ANOVA: $F_{1, 133} = 6.761$, $p = 0.010$), but later, although nonsignificantly, for well-watered versus drought stressed ancestral/noninoculated plants, while descendant/noninoculated plants were unaltered by watering (Fig. 1A).

Disease susceptibility showed a plastic response to the drought treatment (two-way ANOVA; Table 1). We analyzed each treatment separately and found that disease susceptibility was not affected by watering for ancestral/inoculated plants, but that descendant/inoculated plants were significantly more susceptible to disease under well-watered conditions (ANOVA: $F_{1, 64} = 8.60$, $p = 0.005$; Fig. 1B).

There was evidence for plasticity of SLA in response to water availability based on a significant watering treatment effect (three-way ANOVA; Table 1). However, one-way ANOVAs within each treatment group showed no significant plastic response to watering. Instead we saw a consistent but nonsignificant pattern of lower SLA in the wet treatment for all groups (Fig. 1C).

Discussion

In this study, we found that a natural population of *B. rapa* that evolved to flower earlier also evolved increased susceptibility to disease and greater SLA (thinner leaves) within seven generations during the course of a natural drought (Fig. 1; Table 1). This work adds to a growing body of evidence that rapid adaptive evolution can occur in natural populations and contribute to climate change responses (Penuelas and Filella 2001; Hoffmann and Sgro 2011; Franks et al. 2014). These findings also support the idea that contemporary evolution can play an important role in shaping ecological interactions, which is the central premise of the recent field of eco-evolutionary dynamics (Pelletier et al. 2009). As with prior work in this system (Franks et al. 2007) and other recent studies (Nevo et al. 2012; Sultan et al. 2013; Bustos-Segura et al. 2014), the resurrection approach (Franks et al. 2008) allowed direct assessment of evolutionary change.

The evolutionary shift to earlier flowering we found is consistent with previous studies using these predrought ancestral and postdrought descendant populations (Franks et al. 2007; Franks and Weis 2008; Franks 2011). While the shift to earlier flowering was seen previously, the evolution of greater pathogen susceptibility shown in this study is a novel finding. Variation in disease resistance in natural plant populations has been mapped at a spatial scale looking at local adaptation, but to our knowledge, previously had not been followed over time (Thrall and Burdon 2003; Laine et al. 2011; Pautasso et al. 2012). In contrast to the evolution of early flowering following drought, which is adaptive and allows plants to increase fitness by escaping drought conditions (Franks et al. 2007), the reason for the evolution of increased susceptibility to pathogenic disease following drought is less clear. Although not tested directly in this study, one possibility is that selection for earlier flowering resulted in plants with thinner leaves and lower defenses that were more susceptible to disease (due to a trade-off between growth and defense). Our finding of an evolutionary increase in SLA resulting in thinner leaves following

Table 1. Evolution and plastic responses to treatments.

	Df	Mean square	*F* value	*p* value
Response variable—flowering time				
Population	**1**	**0.080**	**11.907**	**<0.001**
Inoculation	**1**	**0.026**	**3.892**	**0.049**
Watering	**1**	**0.030**	**4.490**	**0.035**
Pop × Inoc	1	0.001	0.106	0.745
Pop × Watering	1	0.001	0.156	0.693
Inoc × Watering	**1**	**0.059**	**8.823**	**0.003**
Pop × Inoc × Watering	1	0.002	0.299	0.585
Error	519	0.007		
Response variable—disease susceptibility				
Population	**1**	**0.772**	**21.778**	**<0.001**
Watering	**1**	**0.425**	**11.993**	**0.001**
Pop × Watering	1	0.056	1.575	0.212
Error	132	0.036		
Response variable-SLA				
Population	**1**	**37.52**	**5.994**	**0.015**
Inoculation	**1**	**192.94**	**30.824**	**<0.001**
Watering	**1**	**62.68**	**10.013**	**0.002**
Pop × Inoc	1	4.36	0.696	0.405
Pop × Watering	1	0.02	0.004	0.952
Inoc × Watering	1	0.24	0.039	0.844
Pop × Inoc × Watering	1	0.28	0.044	0.834
Error	272	6.26		

Shown are the results of three-way and two-way ANOVAs with population (ancestors or descendants), inoculation (inoculated or noninoculated, but dropped when modeling disease susceptibility since only inoculated plants are included), watering (well watered or drought stressed) and their interactions as fixed-effects in analyses conducted on transformed response variables: flowering time, disease susceptibility, and SLA. Significant effects of population indicate evolution, while significant effects of the inoculation and watering treatments indicate plasticity. Parameters significantly different from zero ($p < 0.05$) are bolded.

drought is consistent with this scenario. However, it is also possible that selection did act directly to increase susceptibility, or that selection acted on other correlated traits. It is also possible that the increase in susceptibility was due to bottleneck and drift events, although the population size remained qualitatively large during the drought, suggesting that this was not a major factor. Future studies that examine the genetic basis of these traits, or that directly manipulate the traits, would be useful in testing among these alternatives and determining the mechanism of the evolutionary shift to increased susceptibility and its relationship to change in flowering time.

Our experimental design allowed us to investigate both evolution in response to a natural drought and plasticity in response to an experimentally induced drought. Plasticity and evolution in many systems are predicted to be in the same direction (Pigliucci et al. 2006; Wund et al. 2008), but studies have also found that this relationship varies between highly adaptive, moderately adaptive and maladaptive plasticity (Ghalambor et al. 2007; Ghalambor et al. 2015). We found that some traits were plastic and some were not, and we also found that plastic responses in general (for flowering time and disease susceptibility but not SLA) did not match evolutionary responses. Flowering time evolved over seven generations of drought to become earlier, but plants flowered later as a plastic response to experimentally induced drought (Fig. 1; Table 1). Similarly, experimentally induced drought resulted in a plastic reduction in disease susceptibility, while the evolutionary response over seven generations was increased susceptibility (Fig. 1; Table 1). The contrast in these two patterns suggests that plasticity is not likely to have played a role in the evolutionary increase in susceptibility observed in descendant plants and that, in fact, the rapid evolutionary changes we observed occurred despite plasticity generally acting in opposition to the direction of evolution. For disease susceptibility, it is also possible that the well-watered treatment may directly favor the success of the pathogen (Agrios 2005). In contrast, evolution and plasticity were in the same direction for SLA (Fig. 1; Table 1). Although the reasons for this were not directly tested in this study, we hypothesize that drought causes thinner leaves due to resource limitation, resulting in a plastic increase in SLA under drought conditions, and earlier flowering results in thinner leaved due to

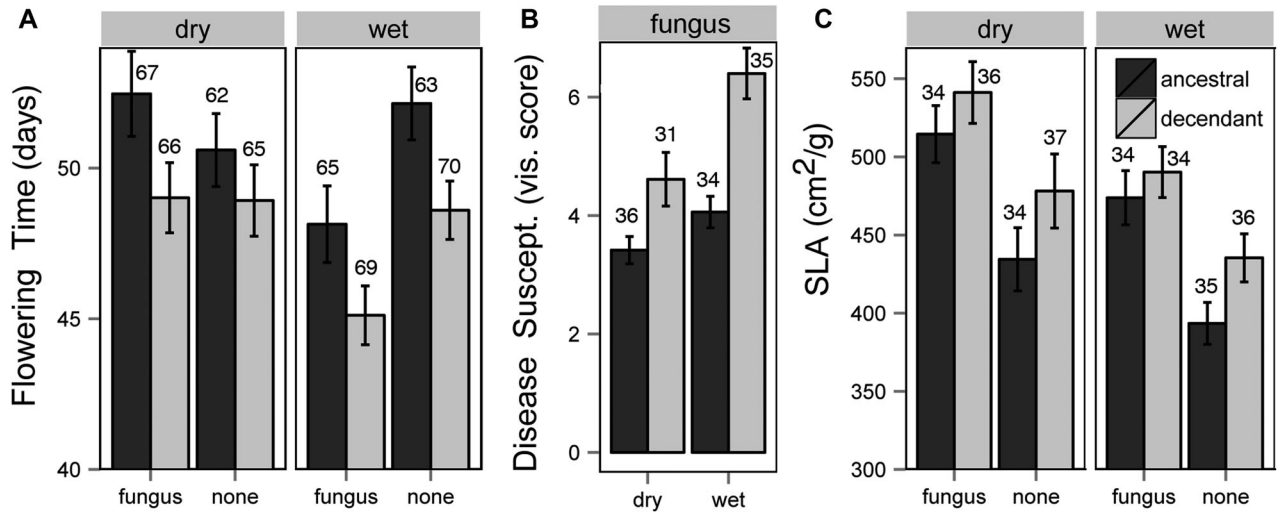

Figure 1. . **Evolutionary changes in a natural *B. rapa* population between predrought ancestors (dark bars) and postdrought descendants (light bars). We found a significant evolutionary shift to (A) earlier flowering, (B) greater disease susceptibility, and (C) increased specific leaf area (SLA). There was also evidence for plasticity in these traits, with the direction of the response opposite to the evolutionary response (A–B) except for SLA (C). The plants were grown under well-watered (wet) or drought stressed (dry) treatments and were inoculated (fungus) or mock inoculated (none) with fungal spores. A three-way ANOVA (two-way for disease susceptibility) was conducted on transformed data (Table S1) with population, inoculation, and watering treatments as fixed-effects, followed by specific pairwise comparisons within treatments conducted using one-way ANOVAs. Untransformed means are shown with standard errors. Statistics are provided in Table 1 (two- and three-way ANOVAs) and the text (one-way ANOVAs). Sample sizes are shown above each bar.**

a trade-off between flowering time and growth, with selection for earlier flowering causing an evolutionary increase in SLA. In any case, the results of this study indicate that plastic and evolutionary responses are not necessarily congruent, and that rapid evolution can occur despite plasticity acting in the opposite direction.

An important caveat here, as with any greenhouse study, is that the effects on all traits of our experimental drought in the greenhouse could be different from natural drought in the field, and field-based estimates of these traits may produce different results. We attempted to approximate field conditions in the greenhouse as much as possible by watering early in the season to mimic the wet spring and then drought treating by infrequent watering, as experienced in the field in the dry summers.

While plants can show some rapid evolutionary and plastic responses to climate change, there are also constraints that may substantially limit the ability of many plant populations to sufficiently cope with the ongoing rapid rate of changes in climate (Franks et al. 2014). One type of constraint to evolution that could hinder responses to climate change is negative genetic correlation, which can be the result of trade-offs (Conner and Hartl 2004, Etterson and Shaw 2001). One important trade-off in plants involves defense against natural enemies such as herbivores and pathogens, and growth or reproduction (Simms and Rausher 1987). This trade-off between growth and defense has been well studied and underlies much of plant defense theory (Mole 1994). Plants are under selection for increased growth and competitive ability at the expense of defense allocation in introduced ranges where specialist natural enemies are absent (Blossey and Notzold 1995). In this case, the adaptation conferring greater competitive ability results in an ecological cost of decreased defense, which is only paid if natural enemies are present. Though rarely considered, ecological costs to contemporary adaptive evolution could also hinder the ability of populations to respond to climate change. The evolutionary shifts to earlier flowering, increased SLA, and greater susceptibility to disease we found in our study are consistent with such a trade-off, particularly if the shift to earlier flowering caused plants to produce thinner leaves that left them more vulnerable to disease. Although our study was not designed to test for such a trade-off directly, the fact that such trade-offs in growth and defense appear to be common indicate that climate-induced selection on one trait could cause correlated evolutionary responses in other ecologically important traits.

Earlier flowering is a ubiquitous response to both drought and the earlier start to growing seasons seen globally as the climate warms (Penuelas and Filella 2001; Parmesan and Yohe 2003; Miller-Rushing and Primack 2008; Munguia-Rosas et al. 2011). Although this general shift to earlier flowering could be taken as evidence that plant populations can adapt to climate change, there could be negative consequences and costs to such adaptations. Specifically, our study shows that a shift to earlier flowering in response to a climatic change in a natural plant population is accompanied by increased susceptibility to fungal disease. Such

ecological consequences of adaptation could impose constraints on the long-term ability of populations to persist as climatic conditions continue to change.

ACKNOWLEDGMENTS

We thank A.E. Weis for seed collection, J. Gurevitch for help with experimental design, D. Futuyma, J. J. Weber, A.L. Mila, J.R. True, and J. Gurevitch for feedback on this manuscript, E. Lowry and M. O'Neal for counsel and supplies, the Stony Brook greenhouse for support, E.P. Lauture, J.J. Lee, F. Chowdhury, H. Tahir, W. Amidi-Abraham, D.T. Cassaro, A. Gill, and M. Flenniken for greenhouse help. Financial support included an American Fellowship from AAUW to N. O'Hara, a grant (DEB-1142784) from the National Science Foundation to S. Franks, and a grant (R01GM108904) from NIGMS-NIH to J.S.R. The authors have no conflict of interest to declare.

DATA ARCHIVING

Dryad: http://dx.doi.org/10.5061/dryad.h3p05.

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Associate Editor: A . Angert
Handling Editor: J . Conner

Supporting Information

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

Figure S1: The visual index that was used to measure disease severity of B. rapa plants infected with A. brassicae 21 days post inoculation.
Table S1: Fixed factors and response variables with transformations conducted.