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Parental environments and interactions with conspecifics alter salinity tolerance of offspring in the annual *Medicago truncatula*

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Summary

1. Based on expectations of the stress-gradient hypothesis for conspecific interactions, stress-sensitive genotypes may be able to persist in stressful environments when positive interactions between individuals occur under stressful environments. Additionally, we test how parental environmental effects alter responses to stress and outcomes of conspecific interactions in stress. While the stress-gradient hypothesis focuses on plant growth, earlier flowering may provide stress avoidance in short-lived organisms.

2. We studied responses to soil salinity and conspecific neighbour using genotypes of *Medicago truncatula* (Fabaceae) originating from saline or non-saline environments, utilizing seeds from parental plants grown in saline or non-saline environments. During the early stages of reproduction, we quantified leaf number, as a measure of vegetative growth, and number of flowers, as a measure of early reproduction potential.

3. Based on leaf counts, non-saline genotypes were better competitors than saline-origin genotypes and benefited from neighbouring plants in saline environments. This positive interaction was detected only when seeds were matured on parental plants grown in non-saline environments. Saline-origin genotypes displayed greater salinity tolerance in early flowering than non-saline genotypes. Plants with neighbours had greater early flowering, regardless of origin, consistent with facilitative interactions in stressful environments.

4. Transgenerational plastic responses influenced neighbouring plant interactions on plant growth, and results suggest that facilitative interactions may be transient only persisting for one generation. However, earlier flowering of non-saline genotypes when grown with a neighbouring plant is consistent with facilitative interactions resulting in reproductive benefits in saline environments, if earlier flowering is favoured in saline environments.

5. Synthesis. Adaptation to stressful environments allows tolerant genotypes to persist in these environments. Less appreciated is that stress-sensitive genotypes lacking such adaptations may persist in stressful environments via positive interactions with other individuals. Thus, positive interactions between individuals may explain the persistence of stress-sensitive genotypes within a population adapted to stressful environments.

Key-words: competition, facilitation, flowering time, interactions, intraspecific, plant population and community dynamics, salinity, stress tolerance

Introduction

Environmental stress and competition between individuals are two major axes shaping species distribution and abundance patterns (e.g. Grime 1977, 1979; Chapin, Autumn &

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Pugnaire 1993; Tilman 1997; Stowe *et al.* 2000; Pennings, Grant & Bertness 2005; Greenwood & MacFarlane 2008). Environmental stress reduces productivity and performance (e.g. Chapin, Autumn & Pugnaire 1993; Callaway 2007), but has also been associated with facilitative interactions that ameliorate stress among con- and interspecific individuals (Bertness & Callaway 1994; Liancourt, Callaway & Michalet 2005; Maestre *et al.* 2009; He, Bertness & Altieri 2013). Support for the stress-gradient hypothesis, in which interactions between individuals move from competitive to facilitative across a benign to stressful environmental gradient, appears to hold among species for a wide range of environmental stressors from water, nutrients, salinity and temperature (reviewed by Brooker *et al.* 2008; Maestre *et al.* 2009; Dohn *et al.* 2013).

Species in benign environments generally have greater competitive ability than species from stressful environments, while those in stressful environments are more stress tolerant than species from benign environments (e.g. Grime 1977). Tolerance is defined as the ability to maintain performance when exposed to different levels of environmental stress (Strauss & Agrawal 1999; Stowe *et al.* 2000) and is associated with traits that increase resource use efficiency (Lambers & Poorter 1992). Traits that confer greater vegetative growth rates have a positive association with competitive ability (e.g. Grime & Hunt 1975; Wilson & Tilman 1993; Schwinning & Weiner 1998; Strauss & Agrawal 1999; Keddy *et al.* 2002). While most of the empirical tests of the stress-gradient hypothesis and trait association along the stress gradient are from interspecific comparisons, here we test these hypotheses using the genotypes from a single species.

The differences in interactions and traits associated with species along stress gradients (Maestre *et al.* 2009; Malkinson & Tielbörger 2010; Bornhofen, Barot & Lattaud 2011; Dmitriev 2011) may also apply to patterns of genotypic variation within species. For species that can live across a variety of environments, we predict that genotypes that are favoured in benign habitats might be better competitors than those favoured in stressful environments. We also expect that these genotypes could be more or less facilitative across environments. More specifically, extending the stress-gradient hypothesis to genotypic expectations predicts that non-tolerant genotypes will benefit from interactions between individuals more than tolerant genotypes in stressful environments (Maestre *et al.* 2009). While direct tests of the stress-gradient hypothesis at the intraspecific level exist, they are limiting; results from studies suggest the importance of interactions between density, environmental stress and genotype (e.g. Stanton, Thiede & Roy 2004; McNutt *et al.* 2012). For example, McNutt *et al.* (2012) demonstrated facilitative interactions of *Solanum carolinense* when exposed to herbivory and genetic variation for herbivory tolerance.

For short-lived annuals, earlier reproduction in the season is a commonly observed strategy of temporal avoidance of seasonally stressful environments (e.g. Stanton, Roy & Thiede 2000; Griffith & Watson 2005; Verhoeven *et al.* 2008; von Wettberg, Remington & Schmitt 2008; Brachi *et al.* 2012). In

populations where the environmental stress builds up during the growth phase (e.g. drought, soil salinity), earlier reproduction may allow plants to reproduce prior to reaching lethal levels. Studies have demonstrated that earlier reproduction response to stress that evolved in response to one type of stress may also be favoured under a different type of stress (Stanton, Roy & Thiede 2000; Stanton, Thiede & Roy 2004), suggesting that phenological patterns are also consistent along the stress gradient.

Transgenerational parental environmental (PE) effects can also influence within-species variation in stress tolerance (Mousseau & Fox 1998; Sänen, Laurila & Merilä 2003; Galloway 2005; Galloway & Etterson 2007; Räsänen & Kruuk 2007). Transgenerational PE effects are broadly defined as parental influences on offspring phenotype beyond the influence of Mendelian inheritance (Lynch & Walsh 1998). Adaptive transgenerational plastic responses have been documented in a number of systems and environmental stressors (Galloway 2005; Gustafsson, Rengefors & Hansson 2005; Räsänen & Kruuk 2007; Herman *et al.* 2012; but see Weiner *et al.* 1997), including soil salinity (Van Zandt & Mopper 2004). For example, offspring size may increase when parental plants are grown under stressful conditions (e.g. Hereford & Moriuchi 2005; Räsänen, Laurila & Merilä 2005; reviewed by Roach & Wulff 1986) because larger individuals may increase the ability to establish and give a competitive advantage. If parental exposure to stressful environments increases offspring tolerance through changes in growth rates or efficiency, we expect that this will also influence within-species interactions of the offspring.

Soil salinity is a major environmental stress, currently estimated to decrease crop yield in 800 million hectares of agricultural land throughout the world (FAO 2008). Populations have evolved different mechanisms to deal with salinity including sequestration of ions, avoidance of salinity and tolerance (reviewed by Cheeseman 1988; Munns & Tester 2008). Traits that confer salinity tolerance are often associated with respiration, ion transport and storage costs (Cheeseman 1988; Munns & Tester 2008), resulting in slower growth. Because of these costs, saline-tolerant genotypes are expected to have lower performance and be worse competitors when grown in non-saline soils relative to saline-non-tolerant genotypes, but have greater survival to reproduction in saline environments.

Using genotypes of *Medicago truncatula* from populations located in saline and non-saline habitats, we predict that in non-saline environments, non-saline-origin genotypes will have faster individual growth rates and will be better competitors compared to saline-origin genotypes. In saline environments, saline-origin genotypes are predicted to have greater salinity tolerance and greater earlier reproduction compared to non-saline-origin genotypes. Competition in saline environments is expected to have less influence on growth and reproduction than differences due to stress-tolerant and stress-non-tolerant genotypes. When grown with another individual, we predict that plants – particularly non-saline-origin genotypes – may experience facilitation in saline environments if these interactions ameliorate abiotic stress.

Materials and methods

Medicago truncatula Gaertn (Fabaceae) is a self-compatible annual plant from the Mediterranean region found in a range of environments including saline and non-saline soils in Tunisia (Lazrek *et al.* 2009; Friesen *et al.* 2010). The genotypes used in this study are part of a larger collection of 39 genotypes from two populations of saline origin [i.e. Enfidha (TN1) and Soliman (TN8)] and from two populations of non-saline origin [i.e. El Kef (TN7) and Bulla Regia (TN9); (Lazrek *et al.* 2009)]. Previous research has demonstrated that three of the four populations (i.e. TN 1, 8 and 9) used in this study are genetically differentiated and linked by moderate levels of gene flow, but are locally adapted to soil salinity levels (Friesen *et al.* 2010). Saline populations' environments have 14 times greater soil sodium and 4.6 times greater magnesium levels than non-saline populations' environments (Table S1 in Supporting Information). Non-saline populations' environments have 3.6 times greater soil nitrogen levels and greater intraspecific density (von Wettberg, Friesen & Strauss, pers. obs.) compared to saline populations' environments (Table S1).

Seeds of four genotypes from non-saline soil origins and eight genotypes from saline soil origins were used in this study (Table S2). Genotypes used were part of a larger study on PE effects on salinity adaptation and were chosen based on available seeds at the time of the study. Seeds were from a glasshouse experiment where plants were grown under 0 mM NaCl or 100 mM NaCl treatments throughout their life span in 2009, hereafter referred to as PE. Salinity treatments during the parental generation were applied biweekly throughout the plants' life span by mixing NaCl with 1× Fahraeus solution. In addition to twice-weekly application of the salinity treatments, plants were watered twice a day throughout the extent of the experiment with deionized water until soil was saturated but not dripping out of the pot. A subset of viable seeds from each genotype were counted and weighed to quantify PE effects on seed size (see Data S1).

Seeds were scarified and subsequently germinated under non-saline conditions in petri dishes filled with moistened sand and dark conditions during the first week of February 2010. As seeds germinated, roots were allowed to develop for an additional day, measured (mm) and transplanted into 164-mL cone pots filled with 2 : 1 University of California, Davis soil/sand mixture. Five replicates per soil origin, PE and offspring salinity treatment [i.e. offspring environment (OE)] were transplanted with a competitor. Plants were transplanted with a seedling that germinated at the same time and having the same initial root length, while the genotype and soil origin of the neighbour were assigned haphazardly. Plants that died within 1 week from the time of transplant were excluded from the analyses, and neighbour classification for the surviving plant was reassigned to no neighbour. The resulting pairs of plants in the neighbour treatment resulted in 22 saline-origin, saline-origin pairs; 12 saline-origin, non-saline-origin pairs; and two non-saline, non-saline genotype pairs. To be able to compare how plants respond to growing alone vs. growing with a competitor, we incorporated data from an additional 129 seedlings of the same genotypes, parental and OE and growing conditions, and of the same germination age, but had seedlings transplanted individually in pots. The final sample size was 175 plants. Once seedlings were sown, plants were moved to an open field on campus and salinity treatments and additional water were applied as in the parental generation except NaCl was mixed in with 0.1× Fahraeus solution. After 6 weeks from the time of transplant and at the start of reproduction, we quantified leaf number and early reproduction (number of flowers) of plants grown alone and with a neighbouring plant.

DATA ANALYSIS

All analyses were performed in SAS v. 9.2 (SAS Institute 2010). To test the effects of PE, OE, soil origin, neighbour treatment and all interactions on two traits measured during the experiment (i.e. number of leaves and early flowering numbers), we performed fixed-effects ANOVA (PROC GLM). Identity of the neighbouring plant (i.e. saline or non-saline origin) did not approach significance for any of the traits ($F < 0.70$; $P > 0.40$); thus, neighbour identity was excluded from all analyses. Due to low survival of transplants of non-saline-origin plants from the 0 mM NaCl parental generation into the 100 mM NaCl OE with neighbour treatment, the four-way interaction of PE by OE by tolerance by neighbour treatment was excluded from the model. Because of unequal sample sizes, ANOVAS on each of the four traits were performed using type IV sums of squares. To meet ANOVA assumptions of normality and homoscedasticity of the ANOVA, number of leaves was square-root-transformed.

Because of the large number of zeros for number of flowers on the plant measured after 6 weeks from transplant, these data were analysed using a generalized linear model (PROC GENMOD LINK=LOG DIST=NEGBIN) fitting number of flowers as a negative binomial distribution using the same factors as the ANOVA described above. Because very few non-saline-origin plants flowered in the 100 mM NaCl OE and there was no neighbour treatment, the three-way interaction of OE by neighbour treatment by origin was excluded in the model. *Post hoc* comparisons of means were tested using least-square means comparisons (LSMEANS).

Results

LEAF NUMBER

Parental exposure to saline environment compared to non-saline environment resulted in 34.8% fewer leaves on offspring (Table 1). Soil origin and neighbour treatment

Table 1. *F*-values and significance from ANOVA for leaf number counts and χ^2 -values and significance from generalized linear model for number of flowers measured on 6-week-old seedlings of *Medicago truncatula* originating (Origin) from saline and non-saline environments

Trait	Leaf number SQRT <i>F</i>	Flower number χ^2
Parental environment (PE)	8.52**	0.25
Offspring environment (OE)	1.76	11.78***
Neighbour treatment	0.06	9.84**
Origin	0.73	4.33*
PE × OE	4.72*	0.07
PE × neighbour treatment	3.48 [†]	0.33
OE × neighbour treatment	2.67	3.75 [†]
PE × origin	3.17 [†]	0.54
OE × origin	2.33	7.01**
Neighbour treatment × origin	9.42**	2.00
PE × OE × neighbour treatment	4.50*	0.12
PE × OE × origin	4.03*	0.00
PE × neighbour treatment × origin	0.95	0.80
OE × neighbour treatment × origin	0.82	NA

[†]0.05 < P < 0.10, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

interaction had the largest effect on the number of leaves (Table 1), with the pattern supporting the hypothesis that non-saline-origin genotypes are better competitors than saline-origin genotypes (Fig. S1a). In the no-neighbour treatment, saline-origin plants had 49% more leaves than non-saline-origin plants ($P = 0.002$), while in the neighbour treatment, non-saline-origin plants had 46% greater number of leaves than saline-origin plants ($P = 0.038$, Fig. S1a). For offspring whose mothers grew in non-saline environments, plants growing in the saline environment with a neighbour had 54% more leaves than when grown alone (Fig. 1a), while plants growing in the non-saline environment did not differ in the number of leaves with or without a neighbour (Fig. 1a). However, when offspring were from mothers that grew in saline environments, no difference in number of leaves was detected for neighbour treatment or offspring salinity environment (Fig. 1b). When mothers were from non-saline environments and offspring were grown in saline environments, non-saline-origin plants had 57% more leaves compared to saline-origin

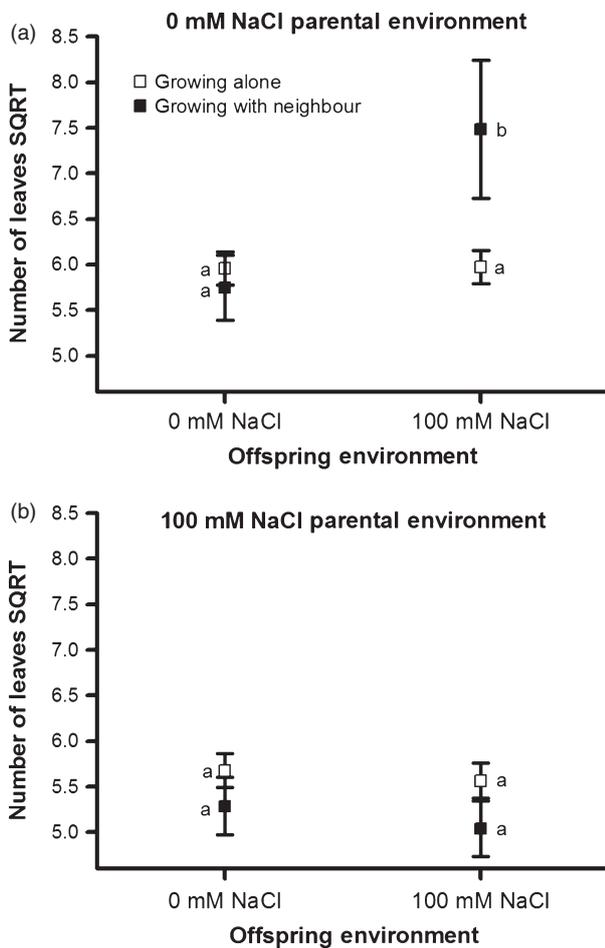


Fig. 1. Means and \pm one standard error bars for neighbour treatment by offspring salinity environment for (a) offspring from parental plants grown in non-saline environment and (b) offspring from parental plants grown in saline environments, along with significant differences between means for total number of leaves measured after 6 weeks; significant differences ($P \leq 0.05$) in means are indicated by different letters.

plants, but did not differ when offspring were grown in non-saline environments (Fig. S1b). When mothers were grown in saline environments, offspring did not differ in number of leaves for either OE or between saline- and non-saline-origin plants (Fig. S1b). This evidence for facilitative interactions was supported by three-way interactions that included parental and OE with either neighbour treatment or origin (Table 1 and Fig. 1a,b). Unfortunately because of death of transplants in certain treatment combinations, we are unable to tease apart which of these interactions has a greater influence on leaf number (see Materials and methods).

NUMBER OF FLOWERS EARLY IN REPRODUCTION

As expected, saline-origin genotypes produced 49% more flowers than non-saline-origin genotypes (Table 1). Non-saline OE supported 2.5 times greater flower production than saline environments. Plants with neighbours had produced 59% more flowers than plants without neighbours (Table 1).

Flower production of saline-origin genotypes was less sensitive to saline OE relative to non-saline-origin genotypes (Table 1 and Fig. 2a). Saline-origin plants maintained flower

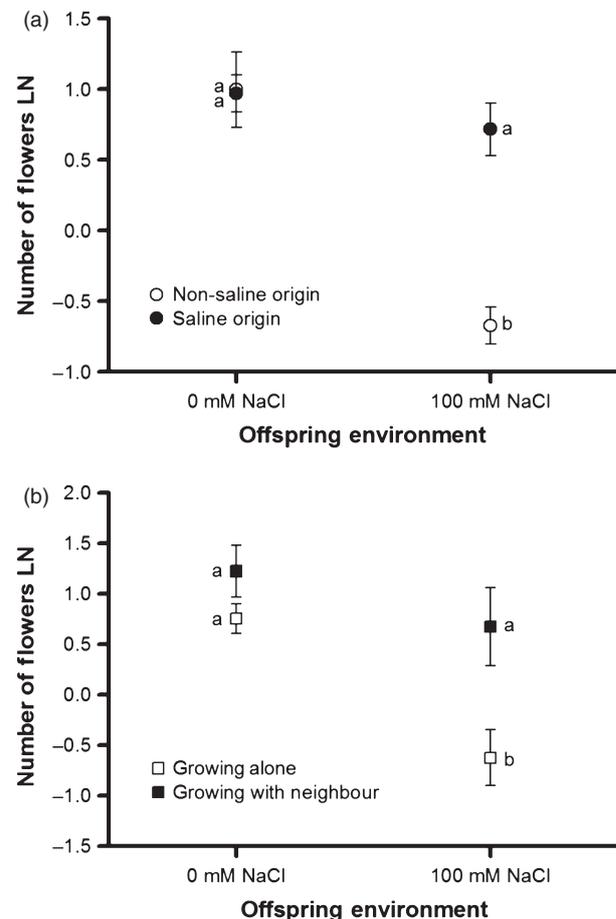


Fig. 2. Mean and \pm one standard error bars for significant effects on number of flowers on plant during the start of flowering: (a) soil origin by offspring environment (OE) interaction, (b) neighbour treatment by OE interaction; significant differences ($P \leq 0.05$) in means are indicated by different letters.

numbers between OEs ($P = 0.283$), while non-saline-origin genotypes showed a fivefold decrease in flower number in saline compared to non-saline OE ($P = 0.002$, Fig. 2a). Although the interaction between OE and neighbour treatment was marginally significant (Table 1), *post hoc* comparisons of the means indicated that plants in the neighbour treatment had equal numbers of flowers in both OEs ($P = 0.178$), but plants in the no-neighbour treatment showed a decline in flower number in the saline OE relative to the non-saline OE ($P < 0.0001$, Fig. 2b). Thus, flowering data are consistent with the hypotheses that salinity stress results in greater early reproduction and saline-origin genotypes have evolved earlier reproduction (Fig. 2a), consistent with 'escaping' unfavourable environmental conditions. Furthermore, there is some support that producing greater flower production earlier in the presence of a neighbour in saline compared to non-saline OEs may be a mechanism that can result in facilitative interactions under stressful environments (Fig. 2b).

Discussion

In this study, we report results from a soil salinity and neighbouring plant experiment that used seedlings of *M. truncatula* genotypes originating from populations occurring in saline and non-saline environments. Consistent with expectations on neighbouring plant outcomes on vegetative growth, non-saline-origin genotypes were better competitors than saline-origin genotypes, as measured by number of leaves (Fig. S1a). Furthermore, facilitative interactions for leaf number were dependent upon parental and offspring salinity levels (Fig. 1a,b) and suggest that positive interactions between individuals may be transient. Estimates of early flower numbers are consistent with the hypothesis that saline-origin genotypes evolved avoidance strategies to stressful environments by increasing their investment in early reproduction (Fig. 2a). Additionally, flowering number data suggest that facilitative interactions may result in fitness increases via greater earlier reproduction when growing with a neighbouring plant in stressful than in benign environments (Fig. 2b). Overall, responses to soil salinity were dependent upon previous exposure to salinity, both short and long term, as well as interactions with neighbouring plants.

We find support for the stress-gradient hypothesis that positive interactions between individuals should be more common between non-tolerant than between tolerant genotypes, and as the environment becomes more stressful when we use leaf number as an estimate of vegetative growth (Brooker *et al.* 2008; Maestre *et al.* 2009; He, Bertness & Altieri 2013). While the general applicability of the stress-gradient hypothesis at the within-species level will require more studies, the predictions can provide insights into the different mechanisms and constraints on patterns of adaptations across populations. First, there is growing evidence for selection favouring traits associated with greater plant growth in benign environments and greater tolerance in stressful environments (e.g. McGraw & Chapin 1989; La Peyre *et al.* 2001; Stanton, Thiede & Roy 2004). Thus, our data are consistent with general patterns

detected at the species level that benign environments tend to favour greater competitive ability, while tolerance is favourable in more stressful environments.

The vegetative growth patterns we observed are consistent with other studies comparing species interactions along a salinity gradient (e.g. Callaway & Pennings 2000; Liancourt, Callaway & Michalet 2005; but see Pennings *et al.* 2003), as well as other abiotic stress gradients (reviewed by Brooker *et al.* 2008; Maestre *et al.* 2009; He, Bertness & Altieri 2013). Overall, there is well-documented support for the stress-gradient hypothesis from among-species data sets. Our study shows that the stress-gradient hypothesis also holds within a single species. Thus, non-tolerant genotypes within a population occupying a stressful habitat may perform well if densities are high, which could be important in the early stages of establishment with high dispersal. However, our data suggest that the benefits of facilitation may only persist for one generation (Fig. 1a,b), suggesting that these beneficial interactions may be transient and influenced by PE effects. Interestingly, in this study, PE effects do not appear to be mediated via differential allocation to seed weight (Data S1). Following initial colonization, genetic changes providing adaptations to salinity could occur or maladaptive PE effects may build up over generations (Miao, Bazzaz & Primack 1991; Räsänen, Laurila & Merilä 2005). The facilitation we observe could be a component by which salinity adaptations evolve, and a potentially important factor maintaining genetic variation within populations.

Earlier reproduction within a season as a means of avoiding stressful environments has been observed across a range of stressful environments (e.g. Stanton, Roy & Thiede 2000; Griffith & Watson 2005; Verhoeven *et al.* 2008; von Wettberg, Remington & Schmitt 2008; Brachi *et al.* 2012) including soil salinity (Munns & Tester 2008). Soil salinity levels in saline populations of *M. truncatula* increase during the vegetative reproductive season before it peaks during the Saharan summer (Noy-Meir 1973). Earlier flowering allows short-lived self-compatible annuals, such as *M. truncatula*, to escape environmental conditions that would otherwise increase mortality prior to reproduction. For example, Stanton, Roy & Thiede (2000) and Stanton, Thiede & Roy (2004) demonstrated that for the annual *Sinapsis arvensis*, selection favoured earlier reproduction when grown under stressful than under benign environments. An emerging pattern from multiple studies indicates that flowering time can rapidly respond to natural selection (Elzinga *et al.* 2007; Franks, Sim & Weis 2007; Galloway & Burgess 2012), suggesting either ample standing genetic variation for flowering time within populations or ample gene flow between populations with differing phenological set points. The generality of earlier flowering in response to multiple sources of environmental stress suggests that phenology may be an important trait responding to differences in environmental stress, along with growth and competitive ability.

Predictions of the stress-gradient hypothesis are typically tested using biomass (e.g. Grime 1977; Maestre *et al.* 2009). Within a species that varies in salinity tolerance, we found

that plants growing with a neighbour had greater earlier reproduction than plants growing alone (Fig. 2b). Unlike neighbouring effects on vegetative growth, PE effects did not influence interactions between individuals (Table 1), suggesting that positive interactions between individuals may persist. Because of the greater overlap in flowering time between soil origins in the presence of a neighbour in stressful environments, we would expect greater potential for gene flow when individuals are grown in high-density than in low-density populations.

How plants respond to environmental stress and how interactions among individuals can alter such responses remain an overarching challenge in plant biology. This challenge is further complicated by environment-specific transgenerational plasticity. Though growing single plants in pots may be adequate in tests for mechanisms of stress tolerance, manipulating both stress and density may provide more robust tests on explaining genetic diversity observed in natural populations. Furthermore, studies manipulating the types and magnitude of stress across parent and offspring generations are warranted to elucidate these patterns more generally and to identify how patterns of natural selection change across the stress gradient. The general patterns of competitive ability or stress tolerance also suggest a fundamental genetic trade-off which may be useful in crop breeding programmes and testing assumptions of constraints on adaptive evolution. Identifying the genetic basis of adaptation to stressful environments and mechanism of adaptive transgenerational plasticity will yield insight into how plants evolve in natural environments with multiple sources of stress.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Environmental characteristics of the four populations where genotypes originated from Northern Tunisia.

Table S2. List of genotypes of the Tunisian *Medicago truncatula* used in this study.

Figure S1. Means and \pm one standard error bars for significant effects found for total number of leaves measured after 6 weeks.

Data S1. Soil origin and parental environmental influences on seed size.