Phenotypic plasticity and specialization along an altitudinal gradient in *Trifolium repens*

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Abstract: Phenotypic plasticity and specialization represent seemingly contrasting strategies to heterogeneous environments. Specialization is associated with the loss of phenotypic plasticity, particularly in functional traits. However, it is equivocal if this loss of plasticity is observed only in the specific habitat and stress type where the specialization occurs or a general loss of plasticity is seen across habitats. We examined populations of *Trifolium repens* L. following an expansion during the colonization of Australia from relatively good low altitude habitats to more stressful high altitude habitats in the Blue Mountains region, New South Wales, Australia. We examined if specialization to abiotic stress causes a loss of adaptive plasticity in functional traits under competition treatments (a different type of stress rather than abiotic stress). We found that both low and high altitude populations experienced a loss in performance in competition treatments and did not express a shade avoidance response under competition. Specialization to higher abiotic stress was associated with the loss of adaptive plasticity in functional traits. Our results suggest that specialization may limit the responses of plants to future environmental changes.

Key words: Phenotypic plasticity, specialization, altitude, competition, abiotic stress, shade avoidance, *Trifolium repens*

1. Introduction

Phenotypic plasticity (particularly adaptive plasticity) is an important strategy for plants to survive and flourish in heterogeneous environments (Sultan, 2001; Griffith and Sultan, 2012; Turcotte and Levine, 2016) and often facilitates the expansion of species into new and stressful environments (Pfennig et al., 2010; Barros et al., 2011). Adaptive plasticity can increase environmental tolerance and fitness across habitats and also promote the persistence of species in a range of environmental conditions (Griffith and Sultan, 2012; Gratani, 2014). The degree of plasticity can change across environments (Huber et al., 2012; Gibbin et al., 2017; Ozbucak et al., 2017), and the expression of phenotypic plasticity is frequently associated with the evolution of adaptive strategies (Grime, 1979). Phenotypic plasticity is predicted to be costly (Weinig et al., 2006; Murren et al., 2015).

Following specialization to a given environment (that is, the evolution of an ecological strategy maximizing performance potentially at a cost of reduced performance in other environments), individuals may lose plasticity to mitigate the costs of plasticity. For example, the evolution of a stress tolerance strategy is often associated with

the loss of plasticity (e.g., Grime, 1979). More broadly, the evolution of specialization (not limited to stressful environments) has been argued to be associated with the loss of plasticity (Pigliucci et al., 2006; Richards et al., 2006; Fazlioglu et al., 2017).

The specialization hypothesis is a framework for predicting how changes in performance plasticity (i.e. plasticity in size and fitness-related traits) are associated with specialization to different types of environment and it is a null hypothesis for adaptive plasticity (Taylor and Aarssen, 1988; Lortie and Aarssen, 1996). Under the specialization hypothesis, specialization to an environment (underpinned by selection driving genetic differences between populations) will result in loss of the ability to perform well in other environments (i.e. a loss in adaptive plasticity). Thus, a good environment specialist (an organism/genotype specialized/adapted to good environments) will probably suffer a dramatic loss in fitness in bad environments. Similarly, a bad environment specialist (an organism/genotype specialized/adapted to bad environments) will probably not increase its performance in a good environment (Lortie and Aarssen, 1996).

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Although there are a few studies that tested the specialization hypothesis in shrubs (Valladares et al., 2000) and tree species (Balaguer et al., 2001), a direct testing of the hypothesis has rarely been carried out in herbaceous plants (Fazlioglu and Bonser, 2016; Fazlioglu et al., 2017). It is also unknown if the loss of plasticity after specialization (Lortie and Aarssen, 1996) is specific to traits related to the environmental gradient to which the population has specialized or if populations experience a more general loss in plasticity to environmental variability. If the latter is the case, we assume that specialization may limit the capacity of these populations to respond to future environmental variability.

We tested whether a plant population shift from low to high abiotic stress (as the species moved from coastal and lowland plain areas to higher elevation and higher stress areas during the colonization of Australia) precipitated a change in adaptive plasticity to the presence of competitors (i.e. plasticity not related to the change in abiotic stress). We suggest that the evolution of stress tolerance may follow 3 contrasting scenarios, depending on whether the shift from low stress to high stress environments involves:

a) a simple shift from good to bad environment specialists. Then low stress and high stress populations should express low adaptive plasticity generally in functional traits, and plants from both populations should experience a steep drop in performance (i.e. fitness) under high competition.

b) a loss of plasticity in functional traits with increasing stress. Then high stress populations will express a higher loss in fitness under competition compared to low stress populations.

c) an increase in functional trait plasticity with increasing stress. Then high stress populations will express high adaptive plasticity in functional traits and a lower loss in fitness under competition compared to low stress populations.

As a model system, we examined the shade avoidance plasticity response (see Weijschedé et al., 2006; Vermeulen et al., 2008, Botto, 2015) to test for adaptive plasticity under competition in the short-lived perennial clonal plant *Trifolium repens* L. The growth form of *T. repens* is primarily horizontal. Therefore, shade avoidance response (foraging for light) usually occurs through plastic responses in petiole length (Huber et al., 2008; Weijschedé et al., 2008), and plasticity in petiole length is highly adaptive in *T. repens*.

We analyzed shade avoidance responses and plant performance in low and high stress environment populations (low versus high altitudes representing low and high abiotic stresses, respectively) across competition treatments. We examined the patterns of adaptive plasticity in functional traits (i.e. petiole length and specific leaf area - SLA) and in performance traits (i.e. total biomass and reproductive mass) across competition treatments to test if specialization to good and/or bad environments (as plants expanded into higher elevation, they experienced new abiotic stresses such as freezing) is associated with a general loss of plasticity (Lortie and Aarssen, 1996).

T. repens is susceptible to frost and populations experiencing freezing temperatures have to adapt (specialize) quickly (Junttila et al., 1990; Frankow-Lindberg, 2001), which is an important aspect of specialization to abiotic stress that is clear across the altitudinal gradient. We tested the following hypotheses:

1) Plant populations from different altitudes will express plasticity in performance traits under competition (testing the specialization hypothesis) and there can be a general loss of plasticity in plant populations following the specialization to abiotic stress.

2) The evolution of stress tolerance for *T. repens* during the colonization of Australia can be explained by one of our scenarios (a, b, or c).

2. Materials and methods

2.1. Study species and populations
Trifolium repens L. (white clover) is a short-lived clonal perennial plant capable of both sexual reproduction through the production of seeds and vegetative reproduction by the production of stolons. *T. repens* has prostrate growth form (laterally growing), and petiole length determines leaf height and light harvesting in shaded environments. We sampled populations from the Blue Mountains region, NSW, Australia, in spring 2012. Seeds from three low altitude populations were gathered around Emu Plains, NSW (33°44′44″S, 150°40′29″E; 33°45′16″S, 150°39′52″E; 33°44′33″S, 150°39′17″E), 30 m above sea level. Low altitude populations were collected from grassy fields near farmlands and parks. Four high altitude populations were sampled from Lawson to Katoomba, NSW (33°43′30″S, 150°25′22″E; 33°41′57″S, 150°22′19″E; 33°43′05″S, 150°17′40″E; 33°43′30″S, 150°18′33″E), at an elevation of 900–1000 m above sea level. High altitude populations were collected from herbaceous plant communities growing at the edge of forests.

The climate data from the last 30 years indicated that the mean number of clear days in high altitudes was 10 days fewer and the average maximum temperature was 7.7 °C lower than in low altitudes. Moreover, the average number of days below 0 °C (freezing/frost days) are 1 and 12 for low and high altitudes, respectively (Australian Bureau of Meteorology, http://www.bom.gov.au/climate/data). High and low altitude populations were approximately 32–45 km apart. Seeds were collected from 5 to 6 individuals haphazardly selected from each population. Populations within low or high altitude were at least 400 m apart. An

isolation distance of 100 m can be adequate to minimize gene flow for *T. repens* populations (Woodfield et al., 1995).

2.2. Experimental design

In August 2012, grass seeds of a natural competitor (*Polypogon monspeliensis*) were sown in flats to be used in low and high competition treatments (see below). Pots (175 mm in diameter, 2.8 L in volume) were filled with potting mixture by using peat, river sand, nutrients (N, P, and K), trace elements (Ca, Fe, Zn, and Mn), and slow-release fertilizer (Osmocote - 1.66 g/L). We used the same soil resources for all plants. In September, *T. repens* seedlings were individually transferred to prepared pots.

Seedlings were randomly assigned to three competition treatments. For the low and high competition treatments, one clover seedling (at the emergence of the first true leaves) was planted in the pot center and surrounded by three grass seedlings planted 6 cm away from the pot center at 120° intervals. Under low competition treatment, grass individuals were cut weekly to maintain their height at 3 cm throughout the experiment. Thus, clover individuals competed with grass only for soil resources but not for light. The clipped grass was probably also less dense belowground and a poorer underground competitor. Therefore, the low competition treatment represents low competition belowground and no competition aboveground.

Under high competition treatment, the grass was not clipped during the experiment, and clover (*Trifolium repens* L.) competed with the grass for both nutrients and light (high competition below- and aboveground). These two levels of competition treatment were used to establish competition conditions where shade avoidance plasticity would be adaptive (under high competition due to competition with tall grass for the light) or would not be adaptive (under low competition due to the presence of short grass). Therefore, separating the effects of competition and shade avoidance should be possible.

Numerous other studies examining shade avoidance plasticity also include the full suite of competitive interactions (Schmitt, 1997; Weijschedé et al., 2006; Huber et al., 2008; Vermeulen et al., 2008) and therefore shade avoidance in the presence of full competition should be an appropriate test of this competitive response. In the control treatment, there was no competition with grass. We replaced dead individuals with healthy ones for the first 3 weeks of the experiment to maximize the establishment of seedlings.

Each competition treatment was replicated eight times per population. In total, 168 pots were established (8 replicates × 7 populations × 3 treatments). Replicates were randomly arranged in 8 blocks on glasshouse benches and watered as required. The experiment ran for 9 weeks from October to December 2012 (austral spring and early summer) under a natural photoperiod in a glasshouse on the UNSW Australia campus (temperature range: 20–24 °C) until *Trifolium* individuals started to grow laterally on the benches and increase their ramet number.

It is possible that stoloniferous clonal plants can forage for light by growing laterally rather than increasing the petiole length. However, in this experiment, the lateral expansion of plants was limited and increasing ramet density away from our competition treatment was not possible due to the length of the experiment. We also acknowledge that shade from neighboring plants might have occurred, but we kept pots far from each other (pots were separated by about 12 cm) during the experiment, and within each block, we changed the positions of pots biweekly.

2.3. Data collection and analysis

Petiole length of 10 mature leaves per individual (selected haphazardly each week) and the total number of leaves and inflorescences were recorded weekly during the experiment. At the end of the experiment, only the aboveground parts of clover (*Trifolium repens* L.) were harvested due to very intense grass root density tangled with clover roots. Aboveground biomass was separated into leaf, stems, and inflorescences and placed separately on drying papers to remove moisture. All plant material was dried at 60 °C for 48 h to a constant mass. Biomass of leaves, inflorescences, and stolons was recorded separately for each individual. Three mature leaves per individual were scanned, and lamina area was calculated by Leaf Area Measurement software (version 1.3). Another ten mature leaves from each individual were chosen to measure leaf lamina mass/petiole mass ratio.

The lamina mass and petiole mass are probably linked, and we examined this relationship to test for possible costs of producing large petioles at the expense of lamina area. For example, if the relative allocation to lamina diminishes with increased petiole size, then advantages of long petioles under competition or shade should be reduced due to decreased light harvesting. Therefore, we examined the correlation between lamina mass and petiole mass. Each leaf lamina and petiole was weighed on a sensitive balance (Mettler Toledo, XS105 DualRange Analytical Balance). Specific leaf area (SLA - mm²/mg) was calculated for each of three leaves per individual by dividing the leaf area to leaf dry mass.

SLA was used as a trait associated with a strategy of resource acquisition since under competition light, water, and nutrients are limited, and an increase in SLA can allow better growth in plants by maximizing resource acquisition. Moreover, we recorded flowering time (as a phenological indicative of genetic differences between populations); allocation to stolon, leaf, and inflorescences; and the total mass of leaves, stolons, and reproductive mass

(inflorescences mass). Leaf accumulation rate per day was calculated by dividing the leaf number increase between weeks 3 and 6 to the number of days between these weeks.

We used linear mixed model analysis of variance to test for differences in performance and functional traits between high and low altitude populations across competition treatments. Altitude, treatment, and altitude by treatment interaction were fixed effects. Population (nested in altitude) was a random effect. Results of the model were analyzed by using Tukey's HSD test to detect significant differences between the factors. When Tukey's test was not possible due to the limitation of the model (i.e. binary comparisons), t-tests were used. ANOVAs were conducted using JMP version 10 (SAS Institute, Cary, NC, USA). We fit bivariate data to regression lines to test for a significant size-dependent relationship between lamina mass and petiole mass for each ratio. Significant differences in slope or shifts along a common slope between high and low elevation populations were analyzed using SMATR software (Standardised Major Axis Tests and Routines Version 2.0, 2006).

3. Results
We observed a significant competition treatment effect in performance traits such as total biomass, stolon mass, leaf mass, reproductive mass (inflorescences mass), inflorescence number, and leaf accumulation rate (leaf number increase per day) (Table 1). Under high competition, grass seedlings grew up to 50 cm in height and covered about 70%–80% of the pot surface. Total biomass values of individuals from both low and high altitude populations under high competition were significantly lower than those of the control and low competition (Figure 1; Table 1). Total biomass and reproductive mass of low altitude populations were greater than high altitude populations in the low competition treatment (a significant altitude × treatment interaction).

We found no significant effect of altitude, treatment, and altitude × treatment interaction on functional traits such as petiole length, SLA, or lamina area (Table 1). There was no significant shade avoidance response and no treatment effect on functional traits (Figure 2). However, allocation to leaves under high competition was greater than under low competition for both high and low altitude populations (Table 1). The difference between slopes of lamina mass to petiole mass relationship in low versus high altitude populations was significant under high competition treatment where high altitude populations had a greater slope (greater increase in lamina mass with increasing petiole mass) (Table 2). Both under control and high competition treatments, slopes of lamina mass to the petiole mass relationship were significantly <1 for low altitude populations. Therefore, increasing petiole mass decreased relative investment to the lamina.

The competition treatments also had a significant impact on reproductive traits. As a general trend, low altitude populations allocated more biomass for reproduction, produced more inflorescences, and started flowering earlier (46 ± 2 days) than high altitude populations (57 ± 3 days) under competition (Table 1; Figure 3).

Table 1. Linear mixed effects model ANOVA results (P-values) for low and high altitude populations across competition treatments. All traits were square-root transformed except petiole length, leaf accumulation rate, and flowering time to meet assumptions of statistical analyses. Ave. = average, ind. = individual. Treatment refers to low versus high competition treatment.

Source of variation	d.f.	Total biomass	Stolon mass	Leaf mass	Reproductive mass	Inflorescence number	Allocation to reproduction	Allocation to leaves	Leaf accumulation rate	Flowering time
Altitude	1	0.07	0.38	0.04	<0.001	<0.001	0.002	0.84	0.02	0.01
Treatment	2	<0.001	<0.001	<0.001	0.002	<0.001	0.91	0.04	<0.001	0.75
Altitude × treatment	2	0.04	0.20	0.01	0.05	0.06	0.72	0.33	0.19	0.23

Table 1. Continued.

Source of variation	d.f.	Petiole length	SLA	Ave. of ind. lamina area	Ave. of ind. lamina mass	Ave. ind. petiole mass	Ave. ind. lamina mass to petiole mass
Altitude	1	0.62	0.51	0.90	0.59	0.74	0.96
Treatment	2	0.12	0.89	0.88	0.73	0.46	0.38
Altitude × treatment	2	0.12	0.13	0.47	0.02	0.07	0.80
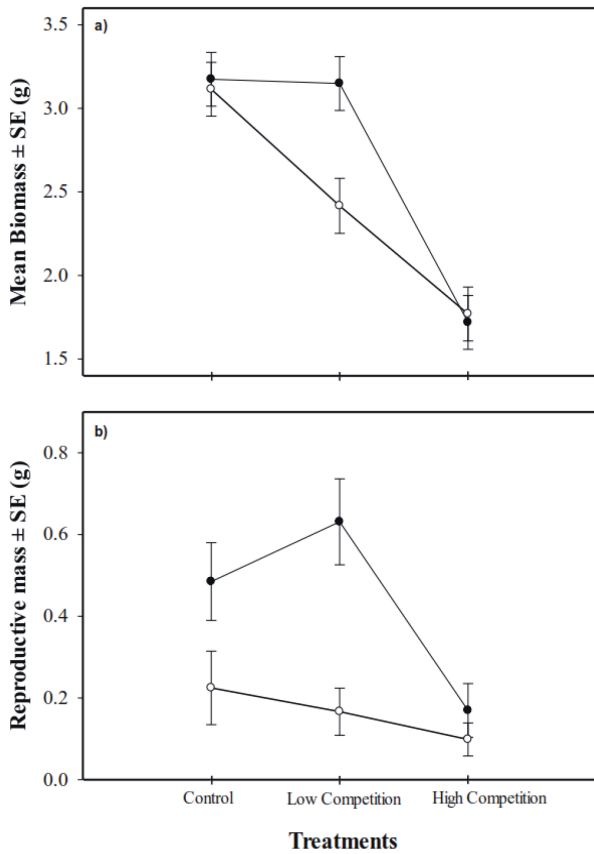
Figure 1. Mean (±SE) of the performance traits a) biomass and b) reproductive mass for low altitude (●) and high altitude (○) populations across competition treatments. Biomass and reproductive mass were square-root transformed.

4. Discussion
We found that low and high altitude populations experienced a significant loss of performance under high competition (high plasticity in performance traits) and the expression of performance plasticity was not consistent with the specialization hypothesis (Lortie and Aarssen,

1996). Competition treatments did not induce significant shade avoidance responses and plants from both low and high altitude populations expressed low functional trait plasticity (i.e. petiole length, SLA, lamina area) across competition treatments. Lower plasticity in petiole length and SLA in response to lower light conditions can be regarded as a specialization to homogeneous light habitats (Balaguer et al., 2001) because these traits become less sensitive to the changes in light. Therefore, ecological expansion of *T. repens* populations from low stress to high stress environments is consistent with a shift from good to bad environment specialists (scenario a, see Introduction) since low stress and high stress populations expressed low adaptive plasticity in functional traits, and both populations experienced a steep drop in performance under high competition (Figures 1 and 2). That is, specialization is associated with a general loss in functional trait plasticity.

There was no apparent difference between low and high altitudes in terms of performance traits (i.e. total mass, stolon mass, leaf mass, reproductive mass, leaf accumulation rate per day) and functional traits (petiole length, SLA, lamina area) under control and high competition (Table 1; Figures 1 and 2). Shade avoidance is a typical response of plants to shading from competing plants (Huber and Wiggerman, 1997; Schmitt, 1997; Weijschedé et al., 2006). The lack of shade avoidance responses observed in our study was unexpected, although it is possible that *T. repens* seedlings experienced intense competition under high competition treatment, preventing these individuals from expressing a typical shade avoidance response. Indeed, individuals suppressed by superior competitors would not likely achieve competitive superiority simply through shade avoidance responses (Fazlioglu et al., 2016). Moreover, increasing the sample size (replicate number per population) would perhaps increase our power to detect differences across treatments. Surprisingly, under low competition, there was a difference between low and high altitude populations in terms of performance traits. That is, low altitude populations maintained their

Table 2. Slopes of lamina mass to petiole mass relationship across competition treatments. Traits were log-transformed. n = number of individual leaves measured, Low CI = lower confidence interval, Upp C = upper confidence interval. Treatment refers to low versus high competition treatment.

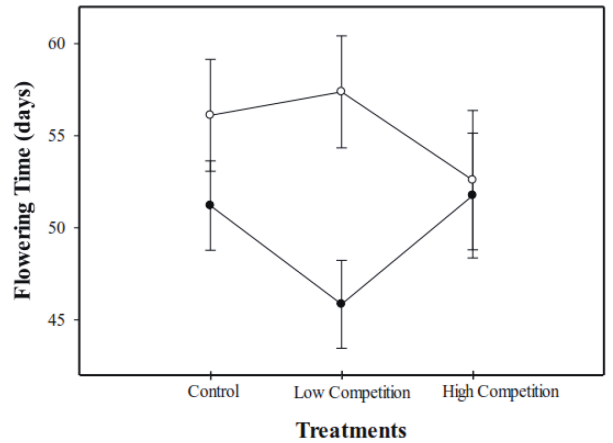| Treatment	Altitude	n	Slope	Low CI	Upp CI	P
Control	Low	237	0.824	0.752	0.903	0.147
	High	237	0.904	0.823	0.992	
Low competition	Low	239	0.953	0.876	1.037	0.481
	High	230	0.912	0.833	0.997	
High competition	Low	236	0.825	0.773	0.880	0.035
	High	238	0.921	0.846	1.001	
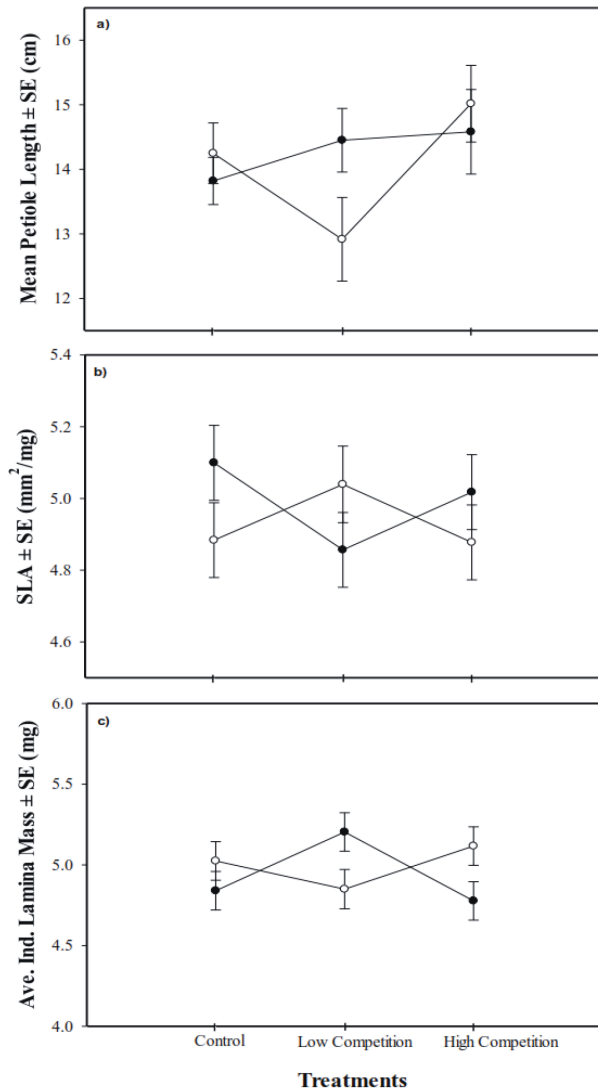
Figure 2. Mean (±SE) of the functional traits a) petiole length, b) SLA, and c) individual lamina mass for low altitude (●) and high altitude (○) populations across competition treatments.

Figure 3. Mean (±SE) of flowering time for low altitude (●) and high altitude (○) populations across competition treatments.

total performance under low competition while high altitude populations had a significantly lower performance compared to the control treatment, which might suggest that low altitude populations are more generalist.

The ability to survive in more stressful environments in high elevations was not associated with performance differences between high and low altitude populations. However, adaptation to the more stressful high altitude was associated with a shift in reproductive strategy. High altitude populations expressed lower allocation to reproduction and reproduced later than low altitude populations, which suggests the presence of genetic differentiation in those traits since phenology is genetically controlled (e.g., Elzinga et al., 2007). Low allocation to

reproduction is predicted under intense competition as a strategy to invest more in traits associated with resource capture and increasing competitive ability (Grime, 1979 but see Bonser, 2013). Hence, competition can be a major driving force even in stressful habitats (e.g., Taylor et al., 1990). The differences in reproduction allocation are also generally consistent with the evolution of a stress-tolerant strategy in high altitudes. Moreover, under low competition, low altitude populations reproduced earlier, which is consistent with a strategy of reproduction before the onset of competition.

We found significant differences in leaf growth form under competition between low and high altitude populations. The slope of lamina mass to petiole mass relationship was greater for high altitude populations under high competition (Table 2). In other words, increases in petiole size were related to greater increases in lamina mass in high altitude populations when compared to low altitude populations. While slopes of this relationship were <1 (an increase in petiole mass was associated with a disproportionately lower increase in lamina mass), this decrease was lower in high altitude populations compared to low altitude populations. Expressing larger lamina biomass per petiole can be advantageous under a shade environment to harvest more light and increase carbon capture (Li et al., 2008). In our study, high altitude populations seemed to be more adapted to heterogeneous light conditions compared to low altitude populations (Table 2). The presence of trees at the edge of habitat patches and fewer sunny days in high altitudes could lead to this dissimilarity in harvesting light. Interestingly, this lamina-petiole relationship implies an advantage under shading in these high altitude populations relative to low altitude populations.

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The fact that neither low nor high altitude populations expressed shade avoidance perhaps suggests an important cost to specialization: the loss of plasticity even when the benefits may be very high. Alternately, a short leaf with low costs (in terms of support) may be more advantageous than a tall high-cost leaf with a relatively low lamina area. For example, increased allocation to structurally expensive longer petioles can outweigh the benefits of adaptive leaf placement, and efficient carbon capture is still possible even at the bottom of canopy in herbaceous layer (Vermeulen et al., 2009). Therefore, expression of a tall petiole under shade may not always be adaptive. Perhaps a low-cost leaf strategy returns more benefits than a shade avoidance strategy for habitat specialists.

Contrasting selective forces acting in different habitats can shape the evolution of adaptive plasticity or specialization in different genotypes and populations of the same species. The ability to adapt changing environments via adaptive plasticity and/or specialization is crucial for populations to persist and flourish under new and changing environmental stresses. In this study, expansion of *T. repens* populations from low to high altitudes during the colonization of Australia was associated with a general loss of adaptive plasticity (nearly a flat response) in functional traits under competition – a different type of stress than the stresses associated with differences in elevation. Therefore, our results suggest that specialization may constrain the ability of populations to react to future environmental changes. Habitat disturbance, climate fluctuations, loss of biodiversity, and invasive species all induce novel stresses for plants (Matesanz et al., 2010; Clavel et al., 2011) and these new environmental stresses may threaten the persistence of specialist populations if specialization is constantly associated with the loss of adaptive plasticity.

Acknowledgment

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