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Aridity shapes cyanogenesis cline evolution in white clover (*Trifolium repens* L.)

NICHOLAS J. KOOYERS,*[1] LILY R. GAGE,† AMAL AL-LOZI* and KENNETH M. OLSEN*

**Department of Biology, Washington University in St. Louis, St. Louis, MO 63130, USA, †Department of Biology, Carleton College, Northfield, MN 55057, USA*

Abstract

Adaptive differentiation between populations is often proposed to be the product of multiple interacting selective pressures, although empirical support for this is scarce. In white clover, populations show adaptive differentiation in frequencies of cyanogenesis, the ability to produce hydrogen cyanide after tissue damage. This polymorphism arises through independently segregating polymorphisms for the presence/absence of two required cyanogenic components, cyanogenic glucosides and their hydrolysing enzyme. White clover populations worldwide have evolved a series of recurrent, climate-associated clines, with higher frequencies of cyanogenic plants in warmer locations. These clines have traditionally been hypothesized to reflect a fitness trade-off between chemical defence in herbivore-rich areas (warmer climates) and energetic costs of producing cyanogenic components in areas of low herbivore pressure (cooler climates). Recent observational studies suggest that cyanogenic components may also be beneficial in water-stressed environments. We investigated fitness trade-offs associated with temperature-induced water stress in the cyanogenesis system using manipulative experiments in growth chambers and population surveys across a longitudinal precipitation gradient in the central United States. We find that plants producing cyanogenic glucosides have higher relative fitness in treatments simulating a moderate, persistent drought stress. In water-neutral treatments, there are energetic costs to producing cyanogenic components, but only in treatments with nutrient stress. These fitness trade-offs are consistent with cyanogenesis frequencies in natural populations, where we find clinal variation in the proportion of plants producing cyanogenic glucosides along the precipitation gradient. These results suggest that multiple selective pressures interact to maintain this adaptive polymorphism and that modelling adaptation will require knowledge of environment-specific fitness effects.

Keywords: adaptation, cyanogenic glucosides, drought tolerance, ecological genetics, parallel evolution, plant–animal interaction

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Introduction

Clines, gradients in phenotypes or genotypes over space, are frequently used to study the process of adaptive differentiation between populations (Haldane 1948; Antonovics & Bradshaw 1970; Kettlewell 1973; Slatkin 1973; Endler 1977; Barton & Hewitt 1985). Studies documenting adaptive clines frequently interpret clinal variation as the product of a linear (or logistic) selection gradient interacting with interpopulation gene flow, with deviations from optimum phenotypic or genotypic frequencies in a given location reflecting gene flow or dispersal patterns (Endler 1977; Barton & Hewitt 1985). In species with restricted contact between different portions of the species range, parallel clines are predicted to form along parallel environmental gradients in different regions (e.g. Oakeshott *et al*. 1982; Mallet *et al*. 1990;

Correspondence: Kenneth M. Olsen, Fax: +1-314-935-4432; E-mail: kolsen@wustl.edu

[1]Present address: Department of Biology, University of Virginia, Charlottesville, VA 22904, USA

Szymura & Barton 1991; Huey *et al.* 2000). However, in parallel clines observed in nature, there are often notable differences among regions in cline shapes or patterns (e.g. Keller *et al.* 2009; Steiner *et al.* 2009; Samis *et al.* 2012). One possible explanation for this variability is that there may be regional differences in selective pressures and in the specific traits that are subject to selection (Endler 1986; Reznick & Travis 1996). Additionally, multiple different selective pressures may act on a single trait, and these forces could interact in different ways in independent clines (Jones *et al.* 1977). Modelling clinal variation in these systems requires knowledge of the relationships between phenotypes, fitness and the geographical context of varying selective pressures. Despite a well-developed body of theory to describe the dynamics of cline evolution (Haldane 1948; Endler 1977; Barton & Hewitt 1985), relatively few studies have empirically examined the roles of multiple interacting selective factors in this process.

Cyanogenesis, the ability to release hydrogen cyanide (HCN) after tissue damage, occurs as a discrete polymorphism in white clover (*Trifolium repens* L.), with cyanogenic and acyanogenic plants co-occurring in many natural populations (Armstrong *et al.* 1913; Ware 1925). The evolution and ecology of this polymorphism has been studied for over seven decades (reviewed by Hughes 1991; Kooyers & Olsen 2012), and it is considered a classic example of selectively maintained adaptive variation (Olson & Levsen 2012). The polymorphism is spatially distributed as a series of parallel adaptive clines across the species range, with cyanogenic plants occurring at higher frequencies in warmer locations. Both latitudinal and altitudinal clines have formed in native Eurasian populations (Daday 1954a,b; De Araujo 1976; Till-Bottraud *et al.* 1988; Caradus & Forde 1996; Majumdar *et al.* 2004), as well as in regions where white clover has been introduced and naturalized, such as the central United States (Daday 1958; Kooyers & Olsen 2012), New Zealand (Kooyers & Olsen 2013) and the U.S. Pacific Northwest (Ganders 1990; Kooyers & Olsen 2013). For example, in the central United States, we have documented that the frequency of cyanogenic plants steadily decreases over a ~900km latitudinal transect from 86% in southern Louisiana to 11% in northern Wisconsin (Kooyers & Olsen 2012).

The white clover cyanogenesis polymorphism arises through two independently segregating presence/absence polymorphisms for two cyanogenic components, cyanogenic glucosides (stored in the vacuoles of photosynthetic tissue) and their hydrolysing enzyme, linamarase (present in the cell wall). Both components must be present for a plant to be cyanogenic. In cyanogenic plants, tissue damage that causes cell rupture leads to the hydrolysis of the cyanogenic glucosides and ultimately the liberation of free HCN, a potent toxin. The inheritance of the cyanogenic glucosides and linamarase can be described by two Mendelian genes, *Ac/ac* and *Li/li*, respectively (Coop 1940; Melville & Doak 1940; Corkill 1942; reviewed by Hughes 1991). For both genes, dominant alleles correspond to the presence of the cyanogenic component, and a recessive homozygote for either gene (*acac* or *lili*) will lack cyanogenic glucosides or linamarase, respectively. Thus, four cyanogenesis phenotypes (or 'cyanotypes') are possible: plants with a least one *Ac* and one *Li* allele are cyanogenic and have the AcLi cyanotype, while the acLi, Acli and acli cyanotypes lack one or both components and are acyanogenic. The molecular bases of the *Ac/ac* and *Li/li* biochemical polymorphisms have been characterized; a gene deletion polymorphism at the *CYP79D15* locus underlies the *Ac/ac* polymorphism, while an unlinked gene deletion polymorphism at the *Li* locus is responsible for the *Li/li* polymorphism (Olsen *et al.* 2007, 2008, 2013).

Selective factors in cyanogenesis cline evolution

Several different selective pressures have been proposed to account for the fitness trade-offs leading to the evolution of cyanogenesis clines. One well-supported hypothesis proposes that cyanogenesis serves as a feeding deterrent to small generalist herbivores, including insects (Dritschilo *et al.* 1979; Mowat & Shakeel 1989; Pederson & Brink 1998), slugs and snails (Angseesing 1974; Dirzo & Harper 1982a,b; Horrill & Richards 1986; Burgess & Ennos 1987; Kakes 1989) and small mammals (Corkill 1952; Saucy *et al.* 1999; Viette *et al.* 2000); cyanogenesis would therefore be expected to provide a fitness benefit in areas with high herbivore richness or abundance (but see also Bishop & Korn 1969; Angseesing 1974; Miller *et al.* 1975; Dirzo & Harper 1982b; Hruska 1988). Correspondingly, production of the cyanogenic components may be energetically costly and lead to a competitive disadvantage for cyanogenic plants in areas of low herbivore pressure (Ennos 1981; Kakes 1989). This proposed trade-off corresponds well with the observed clines if there is greater herbivore pressure in warmer areas, a pattern that has been observed in other systems (Pennings & Silliman 2005; Schemske *et al.* 2009; Salazar & Marquis 2012).

Besides herbivore pressure, other environmental stresses have also been proposed to play a role in cyanogenesis cline evolution. Hydrogen cyanide is regularly produced at subtoxic levels in many plant tissues (e.g. during ethylene production) and has been implicated as a signalling compound that facilitates stress resistance (reviewed in Siegień & Bogatek 2006). Thus,

plants that can actively synthesize cyanogenic components could potentially have a fitness advantage under stressful conditions, such as those caused by pathogens or abiotic stress. However, empirical studies in white clover and other species have suggested that cyanogenic individuals may actually be less fit in the face of fungal infection (Dirzo & Harper 1982b; Lieberei *et al.* 1989; Ballhorn *et al.* 2010), drought stress (Foulds & Grime 1972; Foulds 1977) and freezing damage (Brighton & Horne 1977). Nutrient availability could be an additional factor contributing to cyanogenesis fitness tradeoffs. Investigations in several cyanogenic species have found that cyanogenic glucosides can function as nitrogen storage or transport molecules (Lieberei *et al.* 1985; Selmar 1993; Gleadow *et al.* 1998; Jenrich *et al.* 2007; reviewed by Møller 2010), and these nutrient cycling functions could interact in complex ways with other selective pressures in shaping patterns of clinal variation. For example, usable plant nitrogen declines soon after the initiation of drought stress (Nilsen & Orcutt 1996; Foyer *et al.* 1998; Larcher 2003), so that environments with frequent moisture limitation might select for plants with increased nitrogen storage capability. In the context of herbivore defence, energetic costs of cyanogenesis as a feeding deterrent might only become apparent under nutrient stress conditions (see reviews by Strauss *et al.* 2002; Agrawal *et al.* 2010). The importance of nutrient limitation, drought and other abiotic stresses in shaping cyanogenesis clines remains unclear.

Although the general pattern of cyanogenesis clines is the same for white clover populations worldwide, with cyanogenic plants increasing in frequency with warmer locations, there is marked variation among clines in the distributions of the two underlying cyanogenic components. In the central United States, we have observed a significant latitudinal clinal variation in both the *Ac* and *Li* genes (Kooyers & Olsen 2012). In contrast, an altitudinal transect in southern New Zealand has revealed a significant cline in the *Li* gene, but not the *Ac* gene; and an altitudinal transect along Mt. Baker (in the U.S. Pacific Northwest) shows a significant cline for the *Ac* gene only (Kooyers & Olsen 2013). A recent analysis of these geographically disparate clines suggests that the different clinal patterns can be accounted for if white clover populations are showing adaptive responses to aridity variation, with Acli or AcLi cyanotypes differentially favoured in drier locations (Kooyers & Olsen 2013). Interestingly, this apparent correlation between habitat aridity and cyanogenesis has not been reported in most previous studies that have examined drought stress (or precipitation) as related to the cyanogenesis polymorphism (Daday 1954a; Foulds & Grime 1972; Foulds 1977; but see Caradus *et al.* 1990).

The present study investigates potential fitness tradeoffs among white clover cyanotypes in environments that differ in water availability. To examine this potential selective factor, we manipulated moisture levels in growth chamber experiments, and we also conducted a spatial survey of cyanotype frequencies along an environmental gradient in annual precipitation and aridity. We tested whether: (i) the presence of cyanogenic glucosides and/or the cyanogenic phenotype is beneficial in a controlled environment where there is persistent drought stress; (ii) there is a cost to producing cyanogenic components under standard or low-nutrient growth chamber conditions; and (iii) fitness differences observed in growth chamber experiments translate into clinal patterns along an aridity gradient in nature. We find that plants that produce cyanogenic glucosides have higher fitness in a growth chamber environment with moderate but frequent drought stress. Costs of producing cyanogenic glucosides or linamarase are observable in the water-neutral treatment only when there is nutrient stress. These patterns are consistent with a cline in cyanogenic glucoside production that we detect across a longitudinal precipitation gradient in the central United States, suggesting a key role for aridity in the selective maintenance of this long-studied adaptive polymorphism.

Materials and methods

Trifolium repens is an obligately outcrossing perennial legume that is native to Eurasia. It spreads vegetatively through stolons. In the last 500 years, white clover has been introduced into mesic, temperate regions worldwide as a crop cultivated for forage agriculture and nitrogen fixation (Kjærgaard 2003). In many areas, the species has escaped cultivation and become naturalized in disturbed areas such as roadsides, athletic fields and lawns. As a species that thrives in human-disturbed habitats, white clover has very large populations in both its native and introduced range, and there is little evidence of population structure within or among continents (George *et al.* 2006; Olsen *et al.* 2007; Kooyers & Olsen 2012). Here, we used two complementary approaches to examine the relationship between water availability and cyanotype fitness: growth chamber and greenhouse experiments in which we manipulated both water stress and nutrient stress, and a survey of cyanotype frequencies along a gradient in annual precipitation and aridity within the introduced North American species range.

Growth chamber experiments

We investigated the relationship between cyanotype, temperature, fertilizer and fitness by conducting a

manipulative experiment using Conviron growth chambers (Model PGW36) in the greenhouse facilities of Washington University in St. Louis. We used plants derived from crosses designed to reduce the effects of different genetic backgrounds. Because white clover has gametophytic self-incompatibility, we used half-sib crosses to create F_2 populations. Three parental plants were used with the following cyanogenesis genotypes: *acac lili* (parent G), *AcAc Lili* (parent H) and *Acac Lili* (parent I); all three parents were originally collected from wild populations in St. Louis, MO. Each parental cross had one *Ac_ Lili* parent (H or I) and one *acac lili* parent (G) to create two F_1 populations (HG and IG). F_1 plants were phenotyped using a modified Feigl-Anger HCN assay (Feigl & Anger 1966; details in Olsen *et al.* 2007, 2008) to confirm cyanotype, and genotypes were confirmed by PCR assays for the cyanogenesis loci (*CYP79D15* and *Li*) using previously described primers (Olsen *et al.* 2007, 2008). Mating pairs consisting of one GI F_1 plant and one HG F_1 plant were crossed, and seeds from each of these pairs were considered a single family. Seeds (total $N = 329$, across nine families) were scarified and planted individually into 4″ pots filled with MetroMix 360 soil. Pots were placed on the mist bench; initial germination rates were relatively low, so seeds were re-scarified after 10 days. Germination dates for 184 plants were recorded; germination date was not a significant predictor of fitness measures (described below). Plants were removed from the mist bench after establishment and watered as needed until all plants were moved into the chambers twenty days into the experiment (days after scarification of seeds). All plants were watered to saturation before being placed in growth chambers. Germinated plants from each family were evenly divided into treatment groups (described below). Flats of plants were rotated within the chamber every 10 days for the duration of the experiment to minimize the effects of any environmental heterogeneity within the growth chamber.

We used two treatments to manipulate the levels of nutrient and drought stress. We manipulated nutrient levels (N, K, P) through fertilizer treatments. We assigned half of the plants to a standard greenhouse fertilizing regime (treatment with 125–150 ppm Peter's PEAT-LITE Special 20-10-20 fertilizer every 2 weeks) and the other half to a low-fertilizer treatment where plants were fertilized half as often (every 4 weeks). To manipulate drought stress levels, we used two chambers with different temperature regimes, where we watered all plants in a chamber only when >50% of the plants in the lower-temperature chamber were dry. Soil moisture was determined by assessing friability by touch at 1 cm under the soil surface. At the point of watering, most plants in the warm chamber treatment had typically begun wilting. This approach exposed plants in the warmer chamber to frequent periods of water stress without inducing mortality. Temperature regimes in the two chambers were selected to mimic those observed in natural populations within a previously described North American cyanogenesis cline (Kooyers & Olsen 2012). The warmer chamber maintained a high temperature of 26 °C and a low temperature of 16 °C, while the cooler chamber had a high temperature of 20 °C and a low of 8 °C. Both chambers were on a 14-h light/10-h dark daily cycle with relative humidity of 60% during the day and 70% at night (±5%). Light intensity was similar between chambers. Both chambers had a 1-h ramp at dawn and dusk where both light intensity and temperature gradually decreased or increased. Watering consisted of three inputs of ~200 mL of water per pot spaced out over 30 min, ensuring that each pot was brought to saturation.

To ensure that the temperature manipulation had the desired effect on water stress, we also examined the rate of water loss through evaporation in each chamber by watering 30 soil-filled 4″ pots to saturation, placing 15 pots in each chamber and recording the mass of each pot at 24, 48, 72 and 96 h. This experiment indicated that, as expected, pots of soil in the warmer chamber lost water more quickly than those in the cooler chamber, thereby confirming that the temperature treatment was also a water-stress treatment (Fig. S1, Supporting Information). The moisture difference experienced by plants growing in the chambers was likely magnified over the difference observed in this experiment, as plants in the warmer chamber would be expected to undergo higher transpiration rates than those in the cooler chamber.

Because the growth chamber experiment confounded water stress with elevated temperature, we also conducted a parallel greenhouse experiment where we varied water availability by manipulating soil moisture retention levels (through manipulations of soil composition) rather than temperature manipulation; that experiment confirmed that differences observed between cyanotypes in the growth chamber experiment are not dependent on temperature-induced aridity (see Appendix S1, Supporting Information). We did not directly manipulate watering regimes in either experiment, as preliminary trials indicated that this approach yielded inconsistent and unreliable levels of aridity due to interactions between plant size, rates of development and water requirements.

For each plant, we measured a number of growth and reproductive traits. For growth measurements, we counted the number of leaves on days 30, 60, 90 and 120 and the number of stolons on day 120; we also

measured the longest stolon on days 60, 90 and 120 and the length of each stolon on day 120. We did not measure total biomass because we needed to destructively sample some leaf tissue for cyanogenesis phenotyping (described below). For reproductive traits, we measured the time to first flower and the total number of flower heads produced over the course of the experiment. We also dried and weighed the vast majority of flower heads (3481 in total) to assess reproductive output in terms of total floral mass. Not all flower heads were used because some had not senesced completely by the end of the experiment. Flower heads were dried at ~65 °C and weighed after 24 and 48 h to ensure complete water loss. For each plant, we calculated average flower head mass. In addition, 'total reproductive investment' was calculated for each individual by multiplying average flower head mass by the total number of flower heads produced by that individual. Average values for total reproductive investment were calculated across individuals within each moisture and fertilizer treatment. Plants that had flowered but did not have a fully senesced flower head at the end of the experiment were excluded from total reproductive investment calculations ($N = 8$).

Between days 83–87 after the start of the experiment, fresh leaf tissue (four leaves per plant) was collected for Feigl-Anger HCN assays to determine the cyanotype of each plant. The procedure for HCN assays has been described in detail elsewhere (Olsen *et al.* 2007, 2008). Briefly, a set of three wells of a 48-well Costar polystyrene plate (Corning) was filled with tissue from one plant; tissue was frozen, thawed and then crushed before being exposed to one of three treatments. One treatment consisted of leaf tissue alone (to identify AcLi cyanotypes); exogenous linamarase was added to wells of the second treatment (to identify Acli cyanotypes); and exogenous cyanogenic glucosides were added to wells in the third treatment (to identify acLi cyanotypes). Sample wells were then covered with Feigl-Anger test paper and incubated for 4 h at 37 °C; a blue spot on the test paper over a well indicates the presence of HCN and a positive reaction. Plants that showed no response under any of the three treatments correspond to acli cyanotypes. Any ambiguous assay results were repeated. In order to determine whether cyanogenesis components were segregating in crosses as predicted for biallelic Mendelian polymorphisms, observed frequencies of cyanotypes in each family were compared to expected frequencies using chi-squared goodness-of-fit tests along with randomization tests with 2000 replicates (McDonald 2009). Families were also pooled, and observed frequencies of cyanotypes were compared to expected frequencies via chi-squared goodness-of-fit tests.

All statistical analyses were conducted in R 2.13.2 (R Foundation for Statistical Computation, Vienna, Austria) using the lme4 package (Bates *et al.* 2011). Statistical tests were designed to assess whether fitness measures were affected by cyanotype, presence of cyanogenic glucosides or presence of linamarase in either the fertilizer or temperature treatments. General linear mixed models were constructed for each fitness measure independently. Each model included the fixed effects of cyanotype, temperature treatment and fertilizer treatment and the random effect of family. Models with the additional random effects of plant flat (i.e. starting location in the chamber) and germination time were not used in reported models because inclusion of these variables did not improve the fit of models for any response variable (based on AIC and likelihood ratio criteria). An additional random effect of observation date was included for response variables with repeated measures (i.e. number of leaves and length of longest stolon). Additional models were constructed with the presence of cyanogenic glucosides, linamarase or both components replacing cyanotype as a fixed effect. In order to better understand how cyanotypes behaved in each temperature treatment and to avoid three-way interactions, we created a separate general linear model for each temperature treatment and report summary statistics for these models. Statistical significance of models was analysed with univariate ANOVAs using either type II or type III sum of squares from the *car* package depending on whether there were statistically significant interaction terms (Fox & Weisberg 2011). Deviations from normality were rare; when present, log transformations were used to improve normality of model residuals.

Clinal variation along a precipitation gradient

In order to further test the hypothesis that plants with cyanogenic glucosides have a fitness advantage in more arid natural populations, we surveyed cyanotype frequencies along a longitudinal precipitation transect ranging from Memphis, TN, to Oklahoma City, OK. This transect was chosen to maximize differences in aridity while minimizing the differences in photoperiod and temperature, a distinction from all previously surveyed cyanogenesis transects we are aware of. We collected stolons from 20 plants apiece from each of four subpopulations (sampling locations) per population, with four populations sampled along the transect (Memphis, TN; Little Rock, AR; Fort Smith, AR; and Oklahoma City, OK) (320 plants in total; Fig. 1; Table S1, Supporting Information). Subpopulations were typically located within ~5 km of each other, and previous population structure analyses with similar sampling

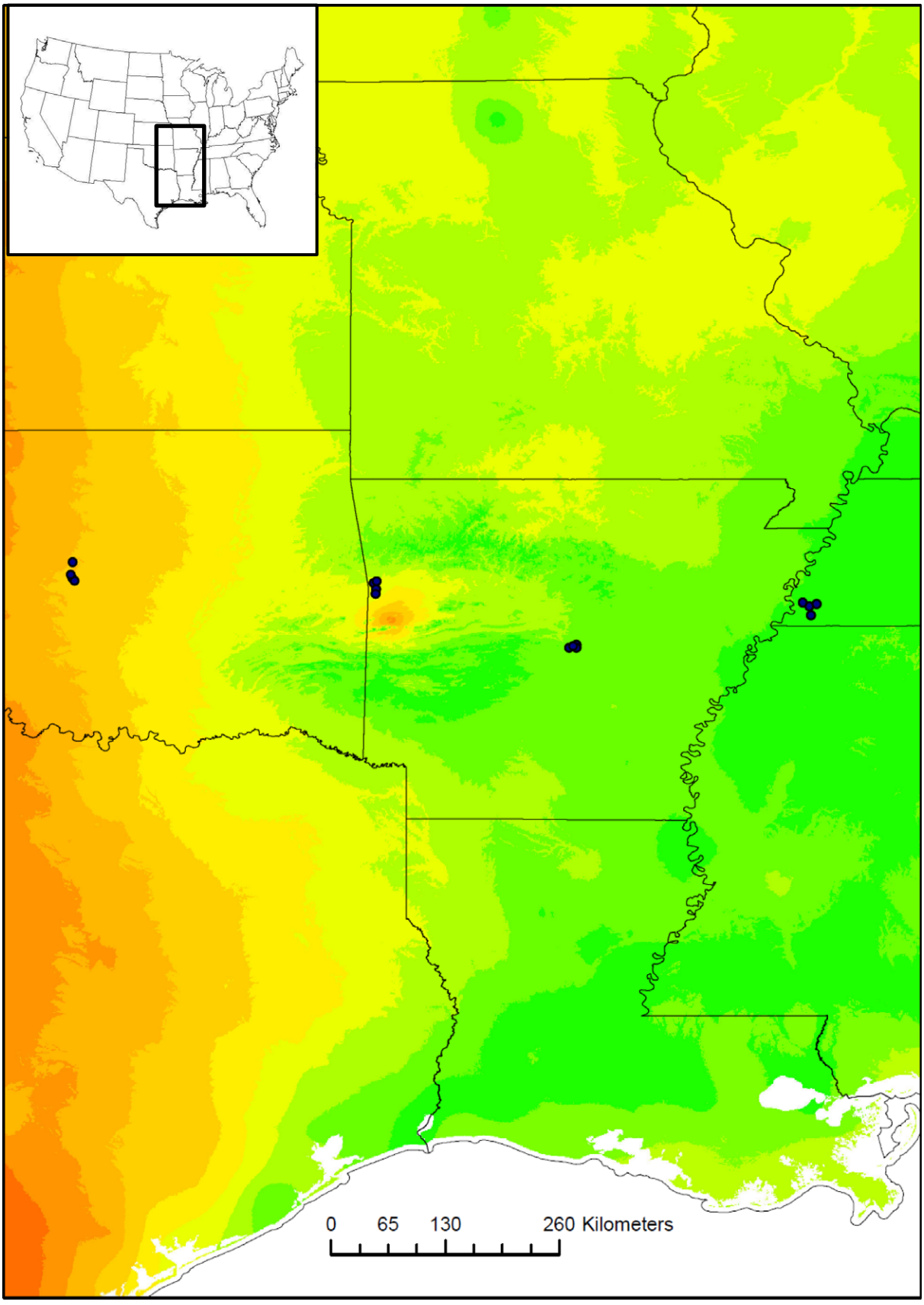

Fig. 1 Sampling locations across a longitudinal precipitation gradient in the central United States. Green to red gradient represents the annual aridity index (extracted from CGIAR-CSI; Trabucco & Zomer 2009) from mesic to xeric, respectively. Dots represent each subpopulation location; there are four subpopulations in each population. Populations (east to west) are Memphis, TN; Little Rock, AR; Fort Smith, AR; and Oklahoma City, OK.

schemes have indicated no significant genetic differentiation among white clover subpopulations within populations (Kooyers & Olsen 2012). The annual aridity index (defined as the quotient of annual precipitation and potential evapotranspiration; Trabucco & Zomer 2009) decreased along the transect from 1.034 (average of 1292 mm annual rainfall) in Memphis to 0.653 (average of 844 mm of annual rainfall) in Oklahoma City, indicating increasingly xeric environments. Whereas white clover is nearly ubiquitous in lawns and mown areas at the eastern end of this transect, plants are scarce and restricted to locations near water sources at the western end of the sampled range. Stolon cuttings were brought back to the Washington University greenhouse and planted in 4″ pots filled with MetroMix 360. Plants were grown to maturity and phenotyped using the Feigl-Anger HCN assay as described above. We statistically assessed the correlations between the frequency of the *Ac* allele, *Li* allele and the AcLi cyanotype with minimum winter temperature, aridity index and annual precipitation using general linear models in R 2.13.2.

We conducted an assessment of population structure to test whether any observed clinal variation at the cyanogenesis genes might be an artefact of neutral demographic processes. Six microsatellite loci that have been used in a previous study of white clover (Kooyers & Olsen 2012) were genotyped in a random subset of individuals (190 individuals genotyped of 307 individuals in total; Table S2, Supporting Information). At least 11 plants were genotyped from each subpopulation (Table S3, Supporting Information). Identical methods to those of Kooyers & Olsen (2012) were used for genotyping, including use of a PIG-tail (Brownstein *et al.* 1996) and M13 fluorescently labelled primers (Schuelke 2000); further amplification details are provided for each locus in Table S2 (Supporting Information). All genotyping was performed on an ABI 3130 sequencer at Washington University in St. Louis. All genotype calls were made and hand-checked in GeneMapper version 3.7 (Applied Biosystems, Foster City, CA). There was <6% missing data for every locus except one (TRSSRA01G05; Table S2, Supporting Information), which had 15.7% missing data. GenAlEx 6 (Peakall & Smouse 2006) was used to provide summary statistics for each subpopulation (Table S3, Supporting Information) and locus (Table S2, Supporting Information) as well as to provide a measure of pairwise population differentiation (Φpt) that could be used to explicitly compare measures of population differentiation between codominant markers (the microsatellites) and dominant markers (the *Ac*/*ac* and *Li*/*li* genes). Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004) was used to test for the presence of null alleles; there was little evidence for null alleles (Table S2, Supporting Information).

The microsatellite data were used to create an estimate of neutral population structure and subsequently used as a control matrix in partial Mantel tests assessing cyanogenesis clinal variation. To detect signatures of isolation by distance from microsatellite data, Mantel tests were performed in the program zt (Bonnet & Van de Peer 2002). This program was also used to run partial Mantel tests that compared a matrix of linearized pairwise population differentiation (Φpt) at either *Ac* or *Li* with a matrix of pairwise population differences in geographical distance, aridity index, annual precipitation or minimum winter temperature while controlling for population structure (measured as a matrix of linearized pairwise population differentiation from microsatellite markers). Significance of Mantel coefficients was determined through Fisher's exact tests following 10 000 permutations in zt. Because we did not obtain microsatellite genotypes for every individual, we only used samples for which we had both cyanotype and microsatellite data. Cyanogenesis gene regressions for this subset of samples were similar to those for the full data set (Table S4, Supporting Information), indicating that the subset can be considered representative of the total population set. If cyanogenesis clines have formed by adaptive processes, a signature of clinal variation should still be present after accounting for neutral population structure. Because the results of partial Mantel tests have recently come into question due to inflated type 1 error (Balkenhol *et al.* 2009; Cushman & Landguth 2010), we also used an ANCOVA-based approach where we conducted a principle coordinate analysis on the microsatellite data set using the adegenet package in R (Jombart 2008) and used subpopulation averages for PCo1 and PCo2 as covariates in an ANCOVA examining the relationship between the frequency of the *Ac* allele, *Li* allele and the AcLi cyanotype with minimum winter temperature, aridity index and annual precipitation. Because the conclusions from these tests did not differ from the partial Mantel results, we report only the partial Mantel results.

To assess whether any observed correlation between cyanotype and water availability might be reflecting a correlation between water availability and levels of

herbivory, we surveyed herbivore damage on the field-collected plants across the sampling transect. The amount of herbivore damage was assessed for each leaflet on each collected stolon as a ratio of damaged to total leaflets. Damaged leaflets were categorized as having <25% damage, 25–75% damage or >75% damage (see Dirzo & Harper 1982a,b). For analysis, we created two different herbivore damage metrics. The first we refer to as 'total damage'; this term refers to the number of leaflets with any damage divided by the total number of leaflets surveyed. The second we refer to as 'high damage'; this term refers to the number of leaflets with >25% damage divided by total number of leaflets surveyed. General linear mixed models were created in R 2.13.2 using the lme4 package (Bates *et al.* 2011) with phenotype and population as fixed effects and subpopulation as a random effect nested within population. ANOVAS employing type III sum of squares were used to assess statistical significance.

Results

We examined the fitness of all cyanotypes in a growth chamber experiment that manipulated temperature/aridity and fertilizer. The temperature treatment had significant effects on most growth and reproductive measures. Plants in the cooler (more mesic) treatment tended to flower later ($\chi^2 = 20.4$, $P < 0.001$) and produce fewer flower heads ($\chi^2 = 7.01$, $P = 0.008$) that were heavier ($\chi^2 = 4.24$, $P = 0.039$) than plants in the warmer treatment (Table 1). Plants in the warmer chamber initially grew faster, produced longer stolons and more leaves than plants in the lower-temperature treatment; however, differences disappeared over the course of the experiment, and by the end of the experiment, plants in the cooler temperature chamber were larger in terms of total stolon length and number, and they showed greater total reproductive investment. The fertilizer treatment had less of an effect on plant fitness measures than the temperature treatment; however, plants in the standard fertilizer treatment produced more flower heads on average in both the warmer chamber (means: 38.5 and 32.9 for standard and low-fertilizer treatments, respectively) and the cooler chamber (means: 31.4 and 26.2 for standard and low-fertilizer treatments, respectively).

To ensure that all cyanotypes were represented in proportion to Mendelian expectations, we compared the observed cyanotype frequencies in each family to expected frequencies assuming Mendelian inheritance. The frequencies of cyanotypes did not differ significantly from expectations in any family or when families were pooled together, consistent with Mendelian segregation and independent assortment (Table S5, Supporting Information). However, including family as a random factor in data analysis did significantly improve model fit for many of the fitness variables and was thus included in all models. This suggests that there could be effects of the genetic background of each cross or that there may be functional differences among the

Table 1 Comparison of average fitness measures for plants grown in well-watered and water-restricted conditions, as well as standard and low-fertilizer treatments

	Standard water/cooler		Restricted water/warmer	
Fitness measure	Standard fertilizer	Low fertilizer	Standard fertilizer	Low fertilizer
Sample size (*N*)	43	46	45	44
AcLi (*N*)	18	23	19	16
Acli (*N*)	7	10	5	11
acLi (*N*)	11	8	14	9
acli (*N*)	7	5	7	8
Number of leaves – day 30	11.7 (5.2)	13.6 (6.9)	15.6 (7.2)	16.7 (7.1)
Number of leaves – day 60	58.3 (16.2)	46.0 (12.8)	62.5 (13.2)	53.2 (10.0)
Number of leaves – day 90	151.4 (46.3)	134 (39.6)	109.9 (36.4)	109.4 (39.7)
Number of leaves – day 120	162.7 (38.0)	147.2 (35.9)	117.0 (57.0)	128.9 (67.4)
Total stolon length (cm)	111.7 (34.8)	105.1 (29.7)	74.7 (38.8)	81.3 (34.4)
Flowering time (days)	50.0 (9.0)	50.7 (12.2)	39.2 (4.7)	39.5 (5.5)
Number of flower heads	31.4 (16.8)	26.2 (16.6)	38.5 (13.9)	32.9 (13.3)
Flower head mass (mg)	46.8 (26.4)	46.2 (19.5)	23.2 (10.6)	23.8 (8.2)
Total reproductive investment (mg)	1545.9 (928.0)	1320.2 (905.4)	818.8 (383.5)	739.1 (327.6)

Values indicate means across individuals in each treatment, with standard deviations in parentheses. Total reproductive investment for each individual was calculated as the product of its mean flower head mass and the number of flower heads produced; the average values for each treatment exclude any individuals for which no flower heads were weighed.

dominant alleles (e.g. quantitative differences in cyanogenic component production) that are segregating in the different families. Including germination date or plant flat as random factors did not significantly increase the fit of models for any fitness variables.

Fitness advantage for plants with cyanogenic glucosides under water-stressed conditions

In the warmer chamber treatment, cyanogenic (AcLi) plants had significantly longer total stolon length ($\chi^2 = 11.15$, $P = 0.008$; Fig. 2A) and more stolons ($\chi^2 = 8.77$, $P = 0.003$) than acyanogenic (Acli, acLi and acli) plants, suggesting that cyanogenic plants grow faster than acyanogenic plants under warmer or water-limited conditions. Plants that do not produce linamarase (Acli and acli cyanotypes) flowered an average of 2.07 days earlier after germination than plants that produce the enzyme ($\chi^2 = 3.868$, $P = 0.049$; Fig. 2B). There were no significant differences between cyanotypes in the average number of flower heads produced in the warmer temperature treatment ($\chi^2 = 2.344$, $P = 0.50$); however, cyanogenic plants had marginally greater flower head mass than acyanogenic plants ($\chi^2 = 2.99$, $P = 0.084$; Fig. 2C). Overall, plants producing cyanogenic glucosides (AcLi and Acli cyanotypes) had significantly greater total reproductive investment (measured as the product of an individual's average flower head mass and the number of flower heads it produced) than plants not producing cyanogenic glucosides ($\chi^2 = 4.73$, $P = 0.03$; Figs 2D and 3). All cyanotype–fertilizer interactions in the warmer chamber were not statistically significant (data not shown).

Costs of cyanogenesis depend on nutrient availability when water is abundant

In the cooler (more mesic) chamber, there was no significant difference between cyanotypes in total reproductive investment ($\chi^2 = 2.58$, $P = 0.46$; Figs 2D and 4A). However, there appear to be strong effects of nutrient availability on cyanotype performance. There were significant cyanotype-by-fertilizer treatment interactions for days to flowering ($\chi^2 = 10.26$, $P = 0.02$) and for total number of flower heads produced ($\chi^2 = 14.66$, $P = 0.002$). Under standard fertilizer conditions, cyanogenic plants flowered an average of 6.46 days earlier (Fig. 4B) and produced an average of 14.32 more flower heads (Fig. 4C) than acyanogenic plants. Interestingly, the opposite is true under low fertilizer conditions;

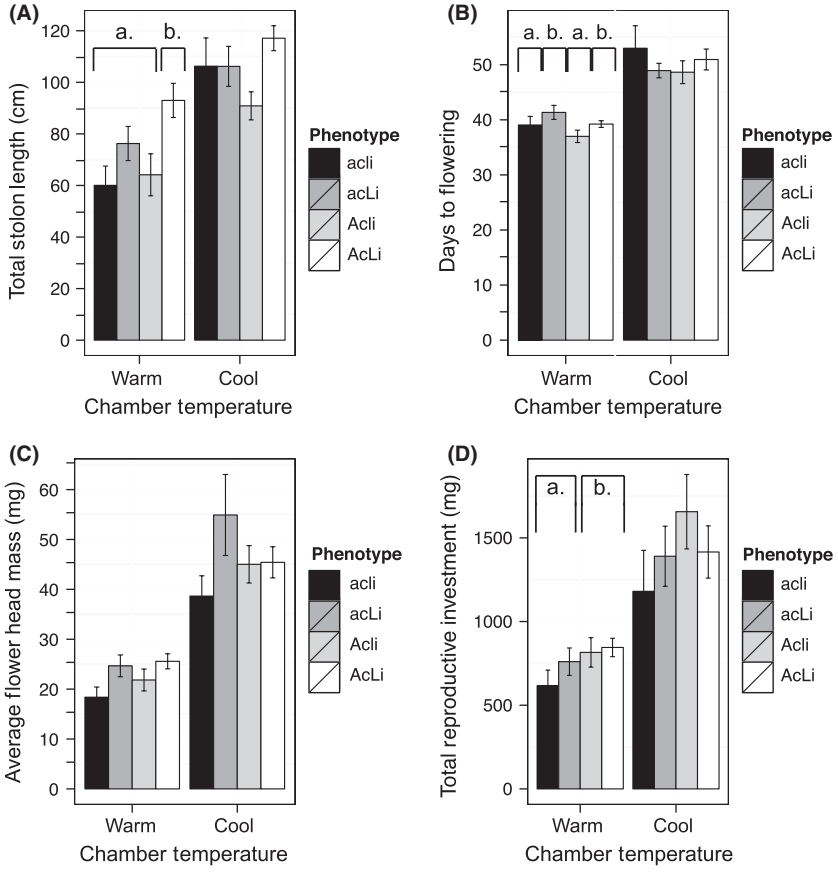

Fig. 2 Bar graph comparisons of fitness measures between cyanotypes in each temperature treatment (warmer temperature corresponds to limited water availability). Four different measures of fitness are shown: (A) total stolon growth at end of the experiment, (B) flowering time from germination date, (C) average flower head mass and (D) total reproductive investment, calculated per individual as the product of mean flower head mass and total number of flower heads. Statistical significance is shown only for the warmer temperature chamber because there were significant interactions between cyanotype and fertilizer treatments in the low-temperature treatment (see Fig. 4). Error bars correspond to standard errors.

cyanogenic plants flowered later by an average of 7.74 days (Fig. 4B) and produced an average of 11.91 fewer flower heads (Fig. 4C) than acyanogenic plants. This pattern suggests that the cost of producing cyanogenic glucosides and linamarase may only be apparent when plants are under nutrient stress and that cyanogenic plants may have some physiological advantage when well watered and well fertilized.

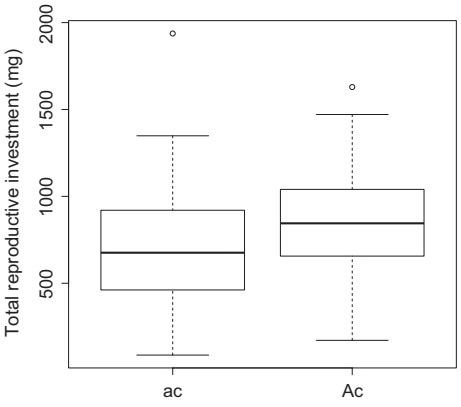

Fig. 3 Box plot comparing the total reproductive investment for plants with cyanogenic glucosides (AcLi, Acli) and plants without cyanogenic glucosides (acLi, acli) in the warmer temperature chamber. Boxes correspond to the interquartile range for each category, intersecting lines are medians, and whiskers show the minimum and maximum values for each category. Outliers on this graph were included in analyses. The total reproductive investment is significantly greater for plants with an *Ac* allele ($\chi^2 = 4.73$, $P = 0.03$).

Clinal variation in cyanogenic glucosides along a precipitation gradient

We tested whether there was a relationship between aridity and frequency of the *Ac* allele, the *Li* allele or the AcLi cyanotype along a natural precipitation gradient in the central United States. We collected stolon cuttings from a total of 320 plants, and 307 of these survived to be tested for cyanotype. Frequencies of cyanogenic plants within populations ranged from 43.7% in Fort Smith, AR, to 69.2% in Oklahoma City, OK (Table 2). Every sampled subpopulation but one (Yale Ave., in Memphis, TN; see Table S1, Supporting Information) was polymorphic at both cyanogenesis genes. The aberrant subpopulation has been surveyed three times in the last 5 years and has undergone a transition from old field to mowed lot within the last year; it has also undergone a change in cyanogenesis frequency from 33% AcLi cyanotypes in 2008 to 100% AcLi plants in the 2012 survey conducted for the present study. These changes suggest that the 2012 cyanotype frequencies may be an artefact of recent habitat disturbance. With this subpopulation included in the analysis, we found no significant correlations between climatic variables and the frequency of the *Ac*

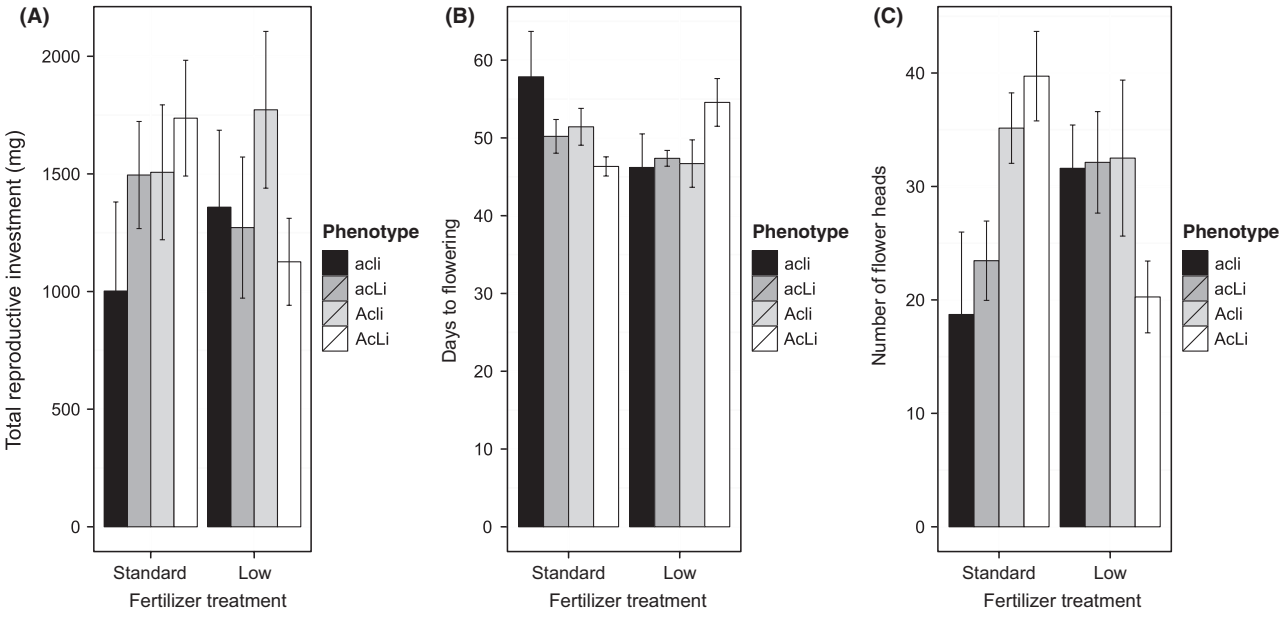

Fig. 4 Comparisons between cyanotypes in the cooler temperature treatment for (A) total reproductive investment; (B) flowering time from germination date; and (C) total number of flower heads in each fertilizer treatment. There were significant cyanotype-by-fertilizer treatment interactions for flowering time ($\chi^2 = 10.26$, $P = 0.02$) and for total number of flower heads produced ($\chi^2 = 14.66$, $P = 0.002$).

allele, *Li* allele or AcLi cyanotype. However, if the subpopulation is excluded as an outlier (or if previous years' data from this location are used in place of the 2012 cyanotypes; data not shown), we find a significant correlation between the frequency of the *Ac* allele and increasing aridity, as measured by annual precipitation ($r^2 = 0.353$, $P = 0.020$) and aridity index (the quotient of annual precipitation and potential evapotranspiration; $r^2 = 0.376$, $P = 0.015$; Fig. 5A). Similarly, there are marginally significant correlations for the frequencies of the *Li* allele and the AcLi phenotype with annual precipitation (*Li*: $r^2 = 0.24$, $P = 0.064$; AcLi: $r^2 = 0.219$, $P = 0.079$) and aridity index (*Li*: $r^2 = 0.2272$, $P = 0.072$; AcLi: $r^2 = 0.198$, $P = 0.096$; Fig. 5B,C).

Besides aridity variables, there is a marginally significant correlation for the frequencies of both *Ac* and *Li* alleles with minimum winter temperature (*Ac*: $r^2 = 0.213$, $P = 0.083$; *Li*: $r^2 = 0.214$, $P = 0.083$), with a higher frequency of the dominant alleles in cooler areas. Curiously, this correlation is in the opposite direction than observations in previously documented climatic transects (e.g. Daday 1954a,b; Ganders 1990; Kooyers & Olsen 2012). However, given that minimum winter temperate shows relatively little variation across the longitudinal transect sampled in the present study (ranging from −1.25 °C in Memphis, TN, to −3.15 °C in Oklahoma City, OK; Table 2) and that these values are correlated with aridity index ($r^2 = 0.81$), this marginal correlation is unlikely to be of any biological significance.

Clinal variation can be formed through either neutral or adaptive processes. In order to test whether the observed longitudinal variation in cyanotype frequencies could be explained by neutral population structure, we compared the spatial pattern of variation in the cyanogenesis genes to the pattern of population structure inferred from six neutrally evolving microsatellite loci. Clines evolving from adaptive processes should have a significant signature of isolation by distance even after neutral population structure is statistically accounted for. Patterns of variation at the microsatellite loci indicated that there was a detectable signature of isolation by distance across the longitudinal cline (Mantel's

Table 2 Summary of cyanogenesis polymorphism data by population

	N	AcLi cyanotype freq.	*Ac* allele freq.	*Li* allele freq.	Avg. annual precipitation (mm)	Avg. minimum winter temperature (°C)	Latitude (°N)	Longitude (°W)
Memphis, TN*	61 (81)	0.492 (0.617)	0.347 (0.433)	0.472 (0.542)	1293	−1.25	35.187	−89.906
Little Rock, AR	77	0.519	0.453	0.408	1271	−1.30	34.782	−92.326
Fort Smith, AR	71	0.437	0.407	0.372	1141	−2.90	35.392	−94.356
Oklahoma City, OK	78	0.692	0.642	0.547	844	−3.15	35.526	−97.458

*Numbers in parentheses indicate values with the inclusion of the outlier subpopulation described in the text. Allele frequencies for *Ac* and *Li* were calculated with the assumption of Hardy–Weinberg equilibrium within populations. Annual precipitation and minimum winter temperature values are the averaged values from the four subpopulations within each population.

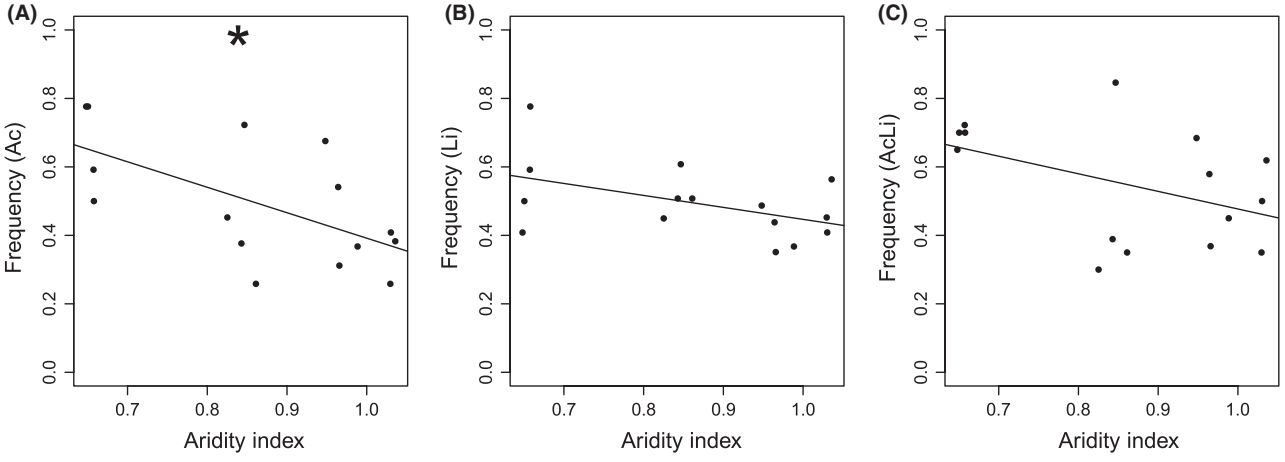

Fig. 5 Regression of subpopulation frequencies of the *Ac* allele (A), *Li* allele (B) and the AcLi cyanotype (C) against the annual aridity index where samples were collected. Higher annual aridity index values indicate more mesic populations. The asterisk indicates statistical significance of the linear regression at $P < 0.05$.

$r = 0.246$, $P = 0.016$, Table 3). Nonetheless, after accounting for this population structure, a strong correlation remains between *Ac* allele frequency and both annual precipitation (Mantel's $r = 0.434$, $P = 0.003$) and aridity index (Mantel's $r = 0.424$, $P = 0.002$) if the outlier Memphis subpopulation is excluded (Table 3). Likewise, accounting for population structure does not alter the conclusions for the *Li* gene, where there is still a marginal correlation between the frequency of the dominant *Li* allele and either annual precipitation (Mantel's $r = 0.266$, $P = 0.051$) or aridity index (Mantel's $r = 0.191$, $P = 0.092$) (Table 3). However, accounting for population structure eliminates the marginal correlation observed for AcLi cyanotype frequency with aridity parameters. Taken together, these results suggest that components of the cyanogenesis clinal variation detected by regression analysis are not caused by neutral processes, but instead have an adaptive basis.

To assess potential variation in herbivore pressure across the sampled transect, we examined herbivore damage on 5438 leaflets from 307 different plants in the sampled populations (Fig. S2, Supporting Information). There was no significant relationship between cyanotype and either the total damage or high damage metrics (total damage: $\chi^2 = 5.15$, $P = 0.1614$; high damage: $\chi^2 = 2.50$ $P = 0.48$; Figs S3 and S4, Supporting Information). There was also no significant relationship between population location and either damage metric (total damage: $\chi^2 = 3.34$, $P = 0.343$; high damage: $\chi^2 = 5.10$, $P = 0.16$). These findings suggest that the observed cyanogenesis cline is not attributable to variation in herbivore pressure across the aridity gradient.

Discussion

Cyanogenesis in white clover has been a long-standing model for understanding the adaptive processes that maintain polymorphism within and among natural populations. In this study, we have investigated the relationship between abiotic stress, including temperature-induced drought stress and nutrient limitation, and fitness effects as manifested in white

Table 3 Results of Mantel and partial Mantel tests comparing differentiation at the cyanogenesis genes or microsatellite loci (SSRs) with geographical and climatic data for sampled populations

Matrix 1	Matrix 2	Control matrix	All populations		Without Memphis outlier	
			Mantel's *r*	*P*	Mantel's *r*	*P*
SSRs	Geographical location	—	**0.246**	**0.016**	**0.263**	**0.015**
Ac/ac	Geographical location	—	**0.222**	**0.016**	**0.363**	**0.004**
Ac/ac	Minimum winter temperature	—	0.132	*0.057*	**0.253**	**0.007**
Ac/ac	Aridity index	—	**0.231**	**0.018**	**0.422**	**0.002**
Ac/ac	Annual precipitation	—	**0.222**	**0.032**	**0.433**	**0.003**
Li/li	Geographical location	—	**0.177**	**0.049**	0.156	0.115
Li/li	Minimum winter temperature	—	0.038	0.314	0.007	0.466
Li/li	Aridity index	—	0.169	*0.060*	0.203	*0.078*
Li/li	Annual precipitation	—	0.174	*0.071*	**0.275**	**0.044**
AcLi	Geographical location	—	0.094	0.171	0.112	0.137
AcLi	Minimum winter temperature	—	0.008	0.457	0.001	0.472
AcLi	Aridity index	—	0.058	0.268	0.156	*0.082*
AcLi	Annual precipitation	—	0.024	0.353	**0.209**	**0.050**
Ac/ac	Geographical location	SSRs	**0.224**	**0.017**	**0.366**	**0.003**
Ac/ac	Minimum winter temperature	SSRs	0.131	*0.059*	**0.250**	**0.009**
Ac/ac	Aridity index	SSRs	**0.233**	**0.019**	**0.424**	**0.002**
Ac/ac	Annual precipitation	SSRs	**0.222**	**0.034**	**0.434**	**0.003**
Li/li	Geographical location	SSRs	**0.185**	**0.046**	0.141	0.153
Li/li	Minimum winter temperature	SSRs	0.040	0.310	−0.003	0.479
Li/li	Aridity index	SSRs	0.175	*0.058*	0.191	*0.092*
Li/li	Annual precipitation	SSRs	0.179	*0.065*	0.266	*0.051*
AcLi	Geographical location	SSRs	0.100	0.175	0.078	0.222
AcLi	Minimum winter temperature	SSRs	0.010	0.449	−0.019	0.432
AcLi	Aridity index	SSRs	0.062	0.259	0.129	0.120
AcLi	Annual precipitation	SSRs	0.026	0.349	0.187	*0.070*

Bold text indicates statistical significance at $P < 0.05$; italics indicate marginal significance at $P < 0.10$.

clover plants that either do or do not produce the components required for cyanogenesis. Our data suggest that plants that produce cyanogenic glucosides have higher fitness in populations that experience frequent water stress, but not in populations that are not water limited. The energetic costs of producing cyanogenic components become detectable specifically under conditions of nutrient limitation. We further find that these fitness differences could explain the adaptive differentiation we detect in natural populations along a longitudinal aridity gradient. Below, we discuss the implications of these findings for the cyanogenesis system and more broadly in the context of how multiple interacting selection forces can affect models of clinal variation.

Fitness trade-offs under water and nutrient stress

The growth chamber experiments indicate that plants that produce cyanogenic glucosides have a fitness advantage when experiencing moderate, frequent drought conditions associated with elevated temperature (Fig. 2). The drought stress imposed by the warmer growth chamber was sufficient to induce wilting in most plants every 3–4 days, and almost all plants showed evidence of drought-induced developmental changes by the end of the experiment (e.g. altered leaf morphology, desiccated leaves, stunted stolon development). These differences could also be associated with the differences in temperature rather than the amount of water stress imposed by the temperature. However, we found similar results from our parallel greenhouse experiment manipulating water availability via difference in soil composition (see Appendix S1, Supporting Information). This suggests that water availability rather than temperature causes the difference in fitness. It should be noted that all plants survived the experiment in the warmer chamber, indicating that the treatment created moderate-intensity stress that was not catastrophic; this stands in contrast to some previous studies of drought stress in white clover, where cyanotype fitness has been measured as percent mortality (e.g. Foulds & Grime 1972; Foulds 1977). Under higher-intensity drought stress treatments in the greenhouse, we have observed little evidence for a competitive advantage for plants with cyanogenic glucosides (N. Kooyers, unpublished).

The fitness advantage of the plants with cyanogenic glucosides was not caused by drought escape (i.e. earlier flowering to secure reproductive success before severe water stress), but rather reflects these plants producing more and larger flower heads than plants that did not produce cyanogenic glucosides (Fig. 2C,D). This result is interesting in that it stands in contrast to previous hypotheses regarding fitness trade-offs and cyanogenic glucoside production. Early studies in white clover proposed that cyanogenic plants may be at a disadvantage under drought conditions because desiccation could result in incidental release of HCN in amounts that could be toxic to the plant (Daday 1965; Foulds & Grime 1972). To test this hypothesis, Foulds and Grime (1972) imposed drought stress at lethal and sublethal levels in experimental field plots. Under conditions that resulted in the mortality of >33% of their test plants, they found that survivorship of plants producing cyanogenic glucosides was approximately one-third that of plants lacking these compounds; however, no fitness differences were apparent at sublethal drought stress levels. In a larger-scale follow-up experiment, Foulds (1977) found no clear evidence that differential mortality under drought is attributable to differences in cyanotype. Given that these studies used experimental field plots rather than controlled growth chamber conditions, some of the variability in their results may reflect environmental influences other than drought stress.

The physiological mechanism that produces a fitness advantage for plants with cyanogenic glucosides in our experiments is unknown. However, studies in other cyanogenic species, including rubber tree (*Hevea brasiliensis*), almond (*Prunus dulcis*) and *Eucalyptus cladocalyx*, have found that cyanogenic glucosides can function as nitrogen transport or storage molecules (Kongsawadworakul *et al.* 2009; Sánchez-Pérez *et al.* 2008; reviewed by Møller 2010). This function may be particularly beneficial during periods of drought stress, when nitrogen availability becomes limited due to decreased nitrate reductase activity (Nilsen & Orcutt 1996; Foyer *et al.* 1998; Larcher 2003). Thus, cyanogenic glucosides could act as a buffering mechanism against drought-induced nutrient stress (see also Siegień & Bogatek 2006). In sorghum, the molecular mechanism and biochemical pathway for the hydrolysis of the cyanogenic glucoside dhurrin into usable NH_3 has been described (Jenrich *et al.* 2007); this catabolic pathway could provide potentially interesting homologs for studying mechanisms of drought stress adaptation in white clover. Future research is also needed to address how variation in drought tolerance relates to quantitative variation in cyanogenic glucoside production, and how drought tolerance might be related to levels of expression and activity of genes that function in cyanogenic glucoside catabolism.

The energetic costs of producing linamarase and cyanogenic glucosides have been hypothesized to be severe enough to cause plants with either one or both of these components to be less fit when few herbivores are present (Daday 1965; Ennos 1981; Dirzo & Harper 1982b;

Kakes 1989). We find little evidence for any cost to these components under standard growth chamber conditions; however, under conditions of nutrient limitation, there do appear to be fitness costs to producing cyanogenic glucosides and linamarase – both in terms of the number of flower heads produced and in days to flowering (Fig. 4B,C). While this effect does not translate into statistically significant differences between cyanotypes in total reproductive investment, we suspect that this is because some plants had no weighable flower heads (i.e. they were not mature by the end of the experiment) and so could not be included in the data. A trend is evident in total reproductive investment that corresponds to patterns seen for flowering time and flower head production, with AcLi plants showing a notable drop in total reproductive investment between standard and low-nutrient treatments (Fig. 4A). These findings from growth chamber experiments are also consistent with common garden studies that have been conducted in the field. Using experimental field plots, Kakes (1989) found that plants without cyanogenic glucosides had significantly greater dry mass and flower head production than plants with cyanogenic glucosides and that there was a trend indicating a cost to linamarase production as well. Taken together with our findings, these observations suggest that under biologically realistic conditions in nature, the production of cyanogenic components comes at an energetic cost to the plant.

Clinal variation in relation to water availability

Cyanogenesis clines have evolved on multiple continents, including in areas where white clover has been introduced from Europe within the last 500 years (reviewed by Kooyers & Olsen 2012). Until recently, these clines were thought to be primarily correlated with winter climatic variables, such as minimum winter temperature, which are believed to be good proxies for the abundance and richness of herbivores (e.g. Pennings & Silliman 2005; Salazar & Marquis 2012). We recently proposed that aridity may also be a factor in the formation of cyanogenesis clines; based on a correlational analysis of environmental variables in three world regions, we found that regional differences in cline shapes can potentially be explained by taking into account differences in aridity gradients (Kooyers & Olsen 2013). In the present study, we tested this hypothesis by sampling white clover along a longitudinal transect that spans a sharp aridity gradient with little variation in temperature variables. Our results provide clear evidence of an aridity-associated cyanogenesis cline, particularly for cyanogenic glucoside production (Fig. 5). This clinal variation does not appear to be attributable to variation in herbivore pressure, at least when herbivory is assessed by measuring damage to adult leaf tissue (Figs S2–S4, Supporting Information). We find that using the aridity index alone, we can explain 36.6% of the variation between subpopulations in frequency of cyanogenic glucosides. We also find some evidence for clinal variation in the frequency of cyanogenic plants and plants producing linamarase (Fig. 5B,C; Table 3). This weaker signal may indicate some advantage to being cyanogenic (i.e. possessing both cyanogenic components) in areas with low water availability, as was previously suggested by Caradus *et al.* (1990).

Our finding that multiple selective factors affect the cyanogenesis polymorphism suggests that predicting cyanotype frequencies in natural populations will be extremely challenging. The difficulty stems primarily from a lack of information on how the known selective forces – herbivore abundance, water availability and energetic costs – interact to create fitness differences between cyanotypes in any given location, and what unknown selective factors may also be at play. Even under highly controlled growth chamber conditions, we find that interactions between just two abiotic factors, water availability and soil nutrient levels, profoundly influence the relative fitness of the different cyanotypes (Figs 2 and 4). It seems likely that the complexity of selective interactions will be greatly amplified in nature, where both abiotic and biotic factors may be highly variable over space and time and may interact to varying degrees. As one example, plants that withstand drought better may become nutrient-rich targets for grazing by herbivores, a pattern that has been observed in other systems (e.g. Quiroga *et al.* 2010).

There is also little understanding of the role that quantitative variation in the expression of the cyanogenic phenotype may play in these interactions. Several studies in other species have documented that among cyanogenic plants, herbivores prefer those with lower amounts of HCN production over those that produce higher quantities (Patton *et al.* 1997; Gleadow & Woodrow 2002; Ballhorn *et al.* 2010). In white clover, quantitative variation in cyanogenic component production has a heritable component (Hughes *et al.* 1984; N. Kooyers unpublished), but it is also highly plastic (Vickery *et al.* 1987; Hayden & Parker 2002). Vickery *et al.* (1987) report that levels of HCN production in cyanogenic white clover can change dramatically in high vs. low water conditions; this variability holds true in other cyanogenic species as well (Gleadow & Woodrow 2002; Ballhorn *et al.* 2010). Thus, environmental variability is likely to directly affect the expression of the cyanogenic phenotype, adding further complexity to the dynamics of cyanotype fitness in a given location.

Multiple selection pressures and estimation of clinal parameters

While it is apparent that multiple selective forces are acting to create and maintain cyanogenesis clines in white clover, the generalizability of this finding to other instances of cline evolution is less clear. There are several other examples of clines that have evolved in response to multiple selective pressures; these include classic discrete polymorphisms such as shell colour and banding patterns in *Cepaea* snails (Cain & Sheppard 1954; Jones *et al.* 1977) and *Adh* enzyme variation in *Drosophila melanogaster* (Oakeshott *et al.* 1982; Geer *et al.* 1993), as well as more complex traits such as flowering time in *Arabidopsis thaliana* (Stinchcombe *et al.* 2004; Samis *et al.* 2008). However, theoretical work is scarcer. Models of cline evolution have typically assumed that there are only two opposing selective pressures whose relative strengths are inversely related across an environmental gradient (e.g. Endler 1977). Violations of this assumption will be context dependent: the relative strengths of different selective pressures may be overestimated or underestimated depending on how they covary over space and time. If selective pressures are tightly correlated across an environmental gradient and favour the same phenotype, clines will become steeper and the strength of individual selective pressures may be overestimated. Alternatively, if there is little correlation between them, cline shape may flatten and significant clinal variation may not be detected. Understanding how multiple selection pressures influence clinal evolution will require determining how these selection pressures interact within populations.

The most general conclusion of the present study and our other analyses of the selective factors underlying cyanogenesis clines (Kooyers & Olsen 2012, 2013) is that simple trade-offs do not exist in this system despite a remarkable convergence in spatial patterns of parallel cline evolution. While the cyanogenesis polymorphism has long been used as an example demonstrating how adaptive polymorphism is maintained in natural populations, it is clear that much work remains to understand how multiple selective pressures interact to determine population allele frequencies.

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N.J.K. and K.M.O. conceived and designed the experiments. N.J.K., L.R.G., and A.A.L. performed the experiments. N.J.K. and K.M.O. analyzed the data. K.M.O. contributed reagents/materials/analysis tools. N.J.K. and K.M.O. wrote the article.

Data accessibility

All data from growth chamber experiments and microsatellite data: Dryad: doi:10.5061/dryad.j7q43.

Subpopulation information and cyanogenesis polymorphism data: Table S1 (Supporting Information).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Graph indicating the proportion of water lost in from soil in 4″ pots after saturation over a 96 h period.

Fig. S2 An example of herbivore damage and damage scoring system on white clover leaves.

Fig. S3 Bar graphs indicating the average ratio of leaflets damaged ('total damage') for each cyanotype in each population.

Fig. S4 Bar graphs indicating the average ratio of leaflets highly damaged ('high damage') for each cyanotype in each population.

Table S1 Subpopulation information and cyanogenesis polymorphism data.

Table S2 Details of microsatellite markers and observed polymorphism data.

Table S3 Summary of microsatellite polymorphism data per subpopulation.

Table S4 Comparison of regression model results using all samples and only those genotyped at SSR loci.

Table S5 Parent cyanogenesis genotypes and progeny cyanotypes from F_2 crosses.

Appendix S1 Methods.