Received: 11 June 2021 | Revised: 25 August 2021 | Accepted: 2 September 2021

DOI: 10.1111/mec.16180

FROM THE COVER

MOLECULAR ECOLOGY WILEY

Genetic trade-offs underlie divergent life history strategies for local adaptation in white clover

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Funding information
Division of Environmental Biology, Grant/Award Number: 0845497 and 1601641; Division of Integrative Organismal Systems, Grant/Award Number: 1557770; Division of Graduate Education, Grant/Award Number: 1745038

Abstract

Local adaptation is common in plants, yet characterization of its underlying genetic basis is rare in herbaceous perennials. Moreover, while many plant species exhibit intraspecific chemical defence polymorphisms, their importance for local adaptation remains poorly understood. We examined the genetic architecture of local adaptation in a perennial, obligately-outcrossing herbaceous legume, white clover (*Trifolium repens*). This widespread species displays a well-studied chemical defence polymorphism for cyanogenesis (HCN release following tissue damage) and has evolved climate-associated cyanogenesis clines throughout its range. Two biparental F_2 mapping populations, derived from three parents collected in environments spanning the U.S. latitudinal species range (Duluth, MN, St. Louis, MO and Gainesville, FL), were grown in triplicate for two years in reciprocal common garden experiments in the parental environments (6,012 total plants). Vegetative growth and reproductive fitness traits displayed trade-offs across reciprocal environments, indicating local adaptation. Genetic mapping of fitness traits revealed a genetic architecture characterized by allelic trade-offs between environments, with 100% and 80% of fitness QTL in the two mapping populations showing significant QTL×E interactions, consistent with antagonistic pleiotropy. Across the genome there were three hotspots of QTL colocalization. Unexpectedly, we found little evidence that the cyanogenesis polymorphism contributes to local adaptation. Instead, divergent life history strategies in reciprocal environments were major fitness determinants: selection favoured early investment in flowering at the cost of multiyear survival in the southernmost site versus delayed flowering and multiyear persistence in the northern environments. Our findings demonstrate that multilocus genetic trade-offs contribute to contrasting life history characteristics that allow for local adaptation in this outcrossing herbaceous perennial.

KEYWORDS
adaptive polymorphism, antagonistic pleiotropy, chemical defence, cyanogenesis, local adaptation, QTL×E interactions

1 | INTRODUCTION

Adaptation to local conditions often occurs when species have broad geographic distributions and experience spatially-heterogeneous environments (Hereford, 2009). Species with locally-adapted populations carry a pool of adaptive variation that can facilitate persistence through periods of environmental change (Hedrick, 1986). This may be a particularly important adaptive strategy for plants and other sessile species during the current period of rapid climate change (Bradshaw & Holzapfel, 2001; Leimu & Fischer, 2008; Thompson et al., 2013); however, it may also come at a cost (Aguirre-Liguori et al., 2019; Kooyers et al., 2019; Wilczek et al., 2014; Anderson & Wadgymar, 2020). Identifying the most relevant traits and the underlying genetic variation that contribute to local adaptation are therefore key goals in evolutionary, agricultural, and conservation biology research programmes (Anderson et al., 2011).

Widely distributed plant species encounter myriad selective pressures across their ranges, involving both biotic and abiotic stressors. For plants, herbivores are a pervasive biotic stressor, and plant evolution has been marked by a tremendous diversification in specialized metabolites that function as chemical defences against herbivory (Mithöfer & Boland, 2012). Such defences can be energetically expensive, however, and the costs of producing them might outweigh their benefits in some environments (Züst & Agrawal, 2017). When a chemical defence occurs as a polymorphism across a species range, it is often assumed that trade-offs exist across populations, such that the benefit of the defence for overall fitness varies depending upon environmental context; in these cases, chemical defence polymorphisms may be important for local adaptation (e.g., Kerwin et al., 2015; Prasad et al., 2012). However, the assumption that chemical defence polymorphisms contribute substantially to fitness differences within or across environments is rarely tested empirically (Erb, 2018). Thus, the relative contribution of chemical defence polymorphisms for local adaptation, compared to other factors, is not well understood.

A classic approach to test for local adaptation in plant species is through reciprocal common garden experiments, whereby the same set of genotypes is grown under different environmental conditions (Clausen et al., 1941). Local adaptation is demonstrated when native genotypes exhibit high relative fitness in local environments and reduced fitness in foreign environments (i.e., genotypic fitness trade-offs across environments, quantifiable as fitness G×E interactions) (Kawecki & Ebert, 2004). When these experiments are performed using genetic mapping populations derived from parents that originated in the reciprocal environments, the genetic architecture of local adaptation can be characterized by mapping fitness-related quantitative trait loci (QTLs) in each parental environment (Ågren et al., 2013; Anderson et al., 2014). This combined field-experiment and QTL-mapping approach allows researchers to identify genetic trade-offs at the level of QTLs in non-model organisms (Rausher & Delph, 2015). Moreover, for species with geographically structured chemical defence polymorphisms, the fitness contribution of a chemical defence trait to local adaptation can be directly assessed by determining the extent to which chemical defence loci colocalize with fitness-related QTLs.

For a given fitness-related QTL, alternate alleles that show fitness trade-offs across reciprocal environments are said to show a pattern of antagonistic pleiotropy (Anderson et al., 2011; Hall et al., 2010). Despite the prediction that antagonistic pleiotropy should be common for locally-adapted QTLs, empirical evidence for this trade-off pattern has been mixed in plants (Wadgymar et al., 2017; but see Troth et al., 2018). A more commonly reported pattern is one of conditional neutrality, whereby alleles that provide a fitness advantage in one environment are neutral in contrasting environments (Anderson et al., 2013; Ellis et al., 2021; Hämälä & Savolainen, 2019). This may partly reflect statistical limitations in detecting significant fitness differences between parental genotypes in two environments (Anderson et al., 2013) or "gene swamping," which can occur when gene flow overwhelms selection, potentially leading to the fixation of conditionally neutral alleles and limiting local adaptation (Wadgymar et al., 2017). However, it may also reflect a bias in study systems, as work to date has largely focused on species with high rates of self-fertilization owing to their experimental tractability (Anderson et al., 2011; Ellis et al., 2021; Savolainen et al., 2013). Antagonistic pleiotropy may be more common in outcrossing species, where higher effective recombination and associated reductions in linkage disequilibrium and population structure could lead to a more efficient response to selection and the elimination of conditionally neutral alleles. Simulation studies have suggested that relative to outcrossing, self-fertilization leads to adaptive genetic architectures characterized by larger numbers of loci that have lower effect sizes and increased genomic distance between them (Hodgins & Yeaman, 2019). This pattern may also contribute to statistical limitations in detecting antagonistic pleiotropy in the most tractable plant systems (Mee & Yeaman, 2019; Yoder & Tiffin, 2018). However, restricted gene flow that leads to moderate population structure may be enough to limit genetic trade-offs, even in predominately outcrossing species (Lowry et al., 2019).

In this study, we use QTL mapping of fitness-related traits to assess the genetic architecture of local adaptation in a geographically widespread, obligately-outcrossing species with a well-characterized a chemical defence polymorphism. Our focal species, white clover (*Trifolium repens* L.), is a perennial herbaceous legume that is native to Europe but naturalized in mesic temperate regions worldwide. In North America, where it was introduced within the last 500 years, it is widely distributed across much of the continent and can be found in climates ranging from boreal to subtropical (USDA, 2002). White clover shows evidence of local adaptation across this range. A recent fitness study using genotypes sampled from across North America demonstrated strong associations between climate-of-origin and plant performance in a central U.S. common garden (Wright et al., 2018). In addition, white clover populations have recurrently evolved climate-associated clines in cyanogenesis (the production of HCN following tissue damage; described below), which suggests that this chemical defence polymorphism could play an important role in local adaptation (de Araújo, 1976; Caradus et al., 1990; Daday,

TABLE 1 Climatic information for the three common garden sites.

Site	Location	Cyanotype of local parent	Mapping population(s)	Number of plants	USDA zone*	Average temperature (°C)**			Total precipitation (mm)**	
						Annual (Bio 1)	Daily minimums of coldest month (Bio 6)	During driest quarter (Bio 9)	Annual (Bio 12)	During driest quarter (Bio 17)
DMN	Duluth, MN	acli	DG	1,506 (502 x 3)	4b	3.6	−18.6	−11.2	757	77
STL	St. Louis, MO	acli	SG	1,500 (500 x 3)	6b	13.1	−6.4	0.4	964	178
GFL	Gainesville, FL	AcLi	DG, SG	1,506, 1500	9a	20.4	5.8	17.4	1,334	189

*USDA plant hardiness zones are based on average annual extreme minimum temperatures (https://planthardiness.ars.usda.gov/PHZMWeb/).; **Bioclimatic variables related to temperature and precipitation were provided by BIOCLIM (Hijmans et al., 2005).

1954b, 1958; Kooyers et al., 2014; Kooyers & Olsen, 2012, 2013; Till-Bottraud et al., 1988). As a common, obligately-outcrossing species, white clover is characterized by very large effective population sizes and little population structure on continental or global scales (George et al., 2006; Kooyers & Olsen, 2012, 2013; Wright et al., 2018); these features could promote local adaptation via antagonistic pleiotropy (Anderson et al., 2011). White clover thus provides a useful complement to existing studies for examining the genetic architecture of local adaptation and the relative importance of chemical defence polymorphisms versus other genetic factors in this process.

1.1 | Cyanogenesis in white clover

Cyanogenesis is an antiherbivore defence that has evolved convergently across the plant kingdom and occurs in >3,000 species (Gleadow & Møller, 2014). The cyanogenic response occurs when tissue damage triggers the mixing of two chemical precursors, cyanogenic glucosides and their hydrolysing enzyme, leading to the liberation of toxic HCN. This chemical defence is polymorphic in white clover, with both cyanogenic and acyanogenic plants found in natural populations. At the genetic level, the polymorphism is controlled by two unlinked Mendelian polymorphisms, *Ac/ac* and *Li/li*; these control the presence/absence of cyanogenic glucosides and the hydrolysing enzyme, linamarase, respectively (Olsen et al., 2007, 2008). For each gene, recessive alleles correspond to gene deletions, and homozygous recessive genotypes lack the corresponding precursor (Olsen et al., 2013; Olsen & Small, 2018). Thus, four cyanogenesis phenotypes or "cyanotypes" occur in wild populations: AcLi (cyanogenic, containing both precursors); and Acli, acLi, and acli (acyanogenic, lacking one or both precursors).

The potential adaptive function of the white clover cyanogenesis polymorphism has been studied for more than 65 years (Daday, 1954a, b). Latitudinal and elevational clines in the frequency of cyanogenesis have been documented worldwide, with higher frequencies of cyanogenic (AcLi) plants consistently found in warmer and drier climates and higher frequencies of acyanogenic plants in colder climates (de Araújo, 1976; Caradus et al., 1990; Daday, 1954b, 1958; Kooyers et al., 2014; Kooyers & Olsen, 2012, 2013; Till-Bottraud et al., 1988). Factors that probably maintain the polymorphism include geographically-varying herbivore pressure, energetic costs and trade-offs associated with the defence, and abiotic stress adaptation (Gleadow & Møller, 2014; Hughes, 1991; Kakes, 1989; Kooyers et al., 2014; Kooyers & Olsen, 2013; Kooyers et al., 2018; Kooyers, 2015). While this extensive body of accumulated research provides strong evidence that natural selection acts on the cyanogenesis polymorphism, the importance of this variation for local adaptation, relative to other fitness-related genetic factors, has not been assessed.

In this study we used two white clover F_2 mapping populations in reciprocal common garden experiments spanning the latitudinal climatic gradient across the United States to address the following questions: (1) Does white clover display local adaptation, as

evidenced by genotypic fitness trade-offs across reciprocal environments? (2) If local adaptation occurs, to what extent is it attributable to variation at the *Ac/ac* and *Li/li* cyanogenesis loci, relative to the overall genotypic effect? (3) What is the genetic architecture of local adaptation in white clover, and to what extent does it occur through allelic trade-offs across environments (antagonistic pleiotropy)?

2 | MATERIALS AND METHODS

2.1 | Study system

Native to southern Europe, *Trifolium repens* is one of the most important temperate forage crops and is commonly grown in mixed pastures with grasses (Abberton & Thomas, 2010; Andrae et al., 2016; Kjærgaard, 2003). It has also become widely naturalized in lawns and other mowed or grazed areas. White clover is primarily bee-pollinated; it also spreads vegetatively with lateral stolons, allowing it to form dense clonal patches and providing the opportunity to clonally replicate genotypes for field experiments. The species is allotetraploid (2n = 4x = 32) (Griffiths et al., 2019).

2.2 | F_2 mapping populations

The common garden experiments used two biparental F_2 mapping populations generated from three wild North American plants originating from geographical locations that span the latitudinal and temperature range of white clover populations in the United States: Duluth, MN (DMN), St. Louis, MO (STL), and Gainesville, FL (GFL) (Table 1). The three parental genotypes were selected such that the *Ac/ac* and *Li/li* genetic polymorphisms would be segregating to create all four cyanotypes in the F_2 mapping populations: DMN_010 (*ac/ac*, *li/li*); STL_0701 (*ac/ac*, *li/li*); and GFL_007 (*Ac/Ac*, *Li/Li*) (Table 1). The plant from Gainesville served as a parent in both mapping populations (DG population = DMN × GFL, SG population = STL × GFL), yielding two F_2 populations with all four cyanotypes present. Reciprocal hand crosses were performed between parents to generate 50–100 F_1 genotypes per population. Within each F_1 population, random cross-pollinations were performed by hand or using bee cages to generate 502 and 500 F_2 genotypes in the DG and SG populations, respectively (Olsen et al., 2021). All F_1 and F_2 genotypes were planted from seed and grown in the Washington University greenhouse facilities (see Supporting Information).

Genome-wide SNP markers for the two mapping populations were generated with genotyping-by-sequencing (GBS) using the ApeKI restriction enzyme for digestion (Elshire et al., 2011; Huang et al., 2014). Details on sequencing, processing of raw reads, and SNP filtering are described in Olsen et al. (2021). Cyanotypes for all plants were determined using Feigl-Anger cyanogenesis assays on fresh leaf tissue and by PCR-genotyping the *Ac/ac* and *Li/li* polymorphisms using established protocols (Feigl & Anger, 1966; Olsen et al., 2007, 2008).

Linkage maps for genetic mapping were constructed independently for each F_2 population using a modified version of the createLG function in GUSMap v1.0 (Bilton et al., 2018), with SNP markers that were homozygous in both parents and that had >0.7 heterozygosity in the F_1 population. SNPs were filtered if they did not meet any of the following criteria: a minor allele frequency (MAF) >0.05, missing data <0.1, average read depth >5X, or a $p < 0.01$ in a genotype frequency test (indicating deviations from 1:2:1 segregation in the F_2 generation). Full details of linkage map construction are described in Olsen et al. (2021). Final linkage maps included 2,575 and 2,437 SNPs for the DG and SG populations, respectively, with total lengths of 5057.6 cM (2.0 cM average marker interval) and 5815.6 cM (2.4 cM average marker interval) for the two data sets Olsen et al. (2021).

2.3 | Reciprocal common garden experiments

Common garden experiments were performed for each mapping population in the two regions where the parent plants originated, with all F_2 genotypes grown in both parental environments ($N = 500$ and 502 genotypes for the DG and SG populations, respectively). Thus, both the DG and SG populations were planted at the GFL site, with the DG population also grown at the DMN site and the SG population also grown at the STL site. The DG mapping population was planted in Duluth, MN at the University of Minnesota-Duluth's Research and Field Studies Centre (46.866 °N, −92.048 °W) on 14 June 2016; the SG population was planted in Eureka, MO at Washington University's Tyson Research Centre (38.527 °N, −90.562 °W) on 11 June 2016; and both mapping populations were planted at the University of Florida's Plant Science Research and Educational Unit (PSREU) in Citra, FL (29.409 °N, −82.171 °W) on 12 October 2016. Planting dates were selected such that plants would become established during the main growth season at each site; the DMN and STL common gardens were planted in the late spring and early summer, while the GFL site was established in the fall.

Three replicate stolon cuttings of each F_2 genotype were made 2–4 weeks prior to planting in each common garden (Table 1; see also Supporting Information). All stolon cuttings were initially ~10 cm in length, with 5–15 leaves and nodulated roots present at one or more nodes; rooting hormone was applied to encourage additional root formation. Cuttings were planted in Metro-Mix 360 soil in 2-inch (~5 cm) square pots (Hummert International, Earth City, MO) and were grown in the Washington University greenhouses for 3–4 days on mist benches, then for 1–2 weeks under standard greenhouse conditions to allow for further establishment before being transplanted in the field.

Full sets of F_2 genotypes were planted within three fully randomized blocks for each mapping population at each site (Table 1). Supplemental watering, fertilizer and *Rhizobium* inoculum, as determined by each site's field coordinator (see Acknowledgements) and depending on the condition of the plants, was provided to prevent high mortality from transplantation in the first two months of each

common garden experiment. Parental genotypes were not included in the common gardens due to substantial decline from viral infections in the greenhouse. Some F_2 plants that were suspected to have died from transplanting stress were replanted from new stolon cuttings within the first two months; replanted individuals were random with respect to genotype and constituted less than 1%, 15%, and 10% of all plants at the DMN, STL, and GFL sites, respectively (see Supporting Information). Because white clover spreads laterally through stolon growth, it was important to keep individual plants from intermingling throughout the experiments for accurate fitness measurements. Thus, plants at all gardens were kept trimmed to 930 cm^2 (1 ft^2), following protocols for a previous white clover common garden experiment (Wright et al., 2018). Ad hoc removal of weeds from the common garden plots was performed for the duration of the experiments (see Supporting Information).

2.4 | Fitness measurements

Vegetative area, survival, and reproductive fitness measurements were recorded for all plants in each common garden over a period of two years (2016–2018) (Table S1). Data collection was performed blind with respect to the genotype and cyanotype of each plant. Trait measurement procedures followed protocols used in a previous white clover common garden experiment at the STL site (Wright et al., 2018), as described below.

2.4.1 | Vegetative surface area

Digital photographs were taken directly over individual plants once per month, with red-painted pennies used for scale and color contrast. No photographs were taken at DMN or STL during winter months when plants were dormant and frequently covered in snow. After the experiments concluded, time points that were comparable across reciprocal sites in terms of days into the experiment, or that reflected key seasonal and mortality events, were selected for further digital analysis, in which vegetative surface area (cm^2) was estimated for individual plants using Easy Leaf Area (ELA) software and previously described methods (Table S1) (Easlon & Bloom, 2014; Wright et al., 2018).

2.4.2 | Survival

In GFL, where mortality was high, the presence/absence of living plant material was assessed monthly beginning in the second month of the experiment; total lifespan was calculated by summing the number of months each plant was recorded alive. For the DMN and STL gardens, Year 1 survival was assessed in a binary fashion in the spring following the first winter. Mortality was low throughout the experiment at these sites, but genotypes exhibited variation in their response to winter; final (Year 2) survival measurements following the second winter season were therefore recorded in an ordinal fashion to capture additional variation (0 = dead; 1 = <25% of allotted 930 cm^2 space filled with living plant material; 2 = between 25%–50% of allotted space filled; 3 = 50%–90% filled; 4 = >90% filled).

2.4.3 | Reproductive output

Floral counts were performed by counting maturing inflorescences (identifiable as those with senescent, downturned basal florets) every ~3–7 days throughout the flowering seasons. Inflorescences were removed to prevent seed dispersal and seedling recruitment within the common gardens. Floral counts were used as a proxy for seed set, as the two are correlated in white clover ($R^2 = 0.51$, $p < .0001$, $N = 67$) (Wright et al., 2018).

Flowering duration was calculated by subtracting the dates of the first and final recorded inflorescences for each plant at each site and for each growing season. Flowering time, which is often measured as days to the first flower in common garden experiments, was not recorded because stolon cuttings were planted in gardens, as opposed to seeds (see Supporting Information).

2.4.4 | Combined fitness metric

In addition to individual measurements of vegetative area, survival, and reproductive output, we calculated a composite fitness measure (λ) which is the dominant eigenvalue of a projection matrix (A) calculated for each plant in each environment (Hall et al., 2010; Hall & Willis, 2006):

$$A = \begin{pmatrix} F_1 & F_2 \\ P_1 & P_2 \end{pmatrix}$$

In this matrix, F_1 and F_2 refer to inflorescence counts in Years 1 and 2, respectively, while P_1 and P_2 are equal to 1 or 0, depending on whether plants were living or dead at the end of each growing season. We found that λ was highly correlated with Year 1 floral count (F_1) for both populations in all sites (Figure S1). All subsequent analyses were performed with λ, but the results are included only in Supporting Information Tables, as we found comparisons of vegetative growth versus reproductive output fitness measures to be more revealing (see Results).

2.5 | Quantitative genetic analyses

Prior to statistical analysis, all fitness measurements within each common garden site and year were evaluated for normality using a Shapiro-Wilk test and by visual assessment with histograms and Q-Q plots. Square root transformations were applied, and all analyses were completed using both transformed and nontransformed (raw) data to verify that results did not qualitatively change. Variance components for fitness traits were estimated using both within-site

and across-site linear models using restricted maximum likelihood (REML) in *lme4* (Bates et al., 2015). All analyses were carried out using *R* statistical software (*R* Core Team, 2017).

We first evaluated the extent to which fitness variation was heritable. Using each trait within each common garden site and in each year as response variables, we independently fit within-site linear models to partition trait variance among genotype and block, which were included as random effects. Variance estimates from within-site models were then used to calculate broad-sense trait heritability ($H^2 = V_G/V_P$) (Haselhorst et al., 2011).

To assess the extent to which the different fitness measurements were correlated with one another within sites, we used the genotypic predictions (best linear unbiased predictors =BLUPs) generated from within-site trait models to calculate pairwise trait correlations among all fitness traits within each common garden site (Pearson correlation coefficients, *r*). The false discovery rate (FDR) was adjusted for multiple comparisons using a Bonferroni-type procedure (Benjamini & Hochberg, 1995). We also performed complementary principal component analyses (PCA) to identify major axes of fitness variation within each site. Based on our findings, we performed a post-hoc Bayesian analysis using the traits that appeared to show trade-offs within sites and the *MCMCglmm* package. Using the bivariate animal model, we compared both Year 1 and Total Floral counts to Vegetative Area (day ~120) and assessed genetic correlations. We performed this analysis for each mapping population separately in each common garden site. Runs included 13,000 iterations with a 3,000 burnin, sampled every 10th iteration. We used values from the variance-covariance matrix to estimate the genetic correlation between traits (r_G) (de Villemereuil, 2012).

To evaluate whether white clover displayed local adaptation, we independently fit across-site linear mixed models in each mapping population using all traits that were comparably measured in both reciprocal environments as response variables. Across-site models included the fixed effect of environment (E; common garden site) and the random effects of genotype (G), block nested within site, and G×E. Using genotypic predictions (BLUPs) from the genotype-by-environment interaction, we evaluated genotype-environment correlations (r_{GE}), again with correction for multiple comparisons (FDR). In these tests, negative r_{GE} indicates genotypic trade-offs across reciprocal sites that correspond to local adaptation (i.e., genotypes with high fitness in one environment experience reduced fitness in the reciprocal comparison) (Haselhorst et al., 2011).

For reproductive traits only, we constructed additional multiyear across-site trait models; these models included an added fixed effect of year (Y) and random effects of G×Y and G×E×Y. For all across-site trait models (with and without year effects), we tested the significance of fixed effects using a mixed-model analysis of variance (ANOVA), and the significance of random effects was evaluated using likelihood ratio tests with *lmerTest* (Kuznetsova et al., 2017).

To assess the extent to which local adaptation in white clover was attributable to variation at the *Ac*/*ac* and *Li*/*li* cyanogenesis loci, relative to the overall genotypic effect, we re-constructed all within- and across-site trait models described above, and we replaced Genotype with Cyanotype in all terms where it appeared. For within-site models, we calculated the proportion of phenotypic variance explained by variation among cyanotypes (V_C/V_P), which we compared to H^2. For across-site models, we again evaluated significant fixed and random effects using mixed-model ANOVAs and likelihood ratio tests.

2.6 | QTL mapping analysis

To characterize the genetic architecture of local adaptation, we performed genetic mapping (single marker analysis) and identified quantitative trait loci (QTLs) associated with fitness trait variation in each common garden site. We used genotypic predictions (BLUPs) generated by across-site trait models without year effects as the response variables in our genetic models. We also included as response variables the genotypic predictions from within-site models for traits that were not comparably measured across reciprocal sites (e.g., survival traits); these traits were not used for subsequent QTL×E analysis because they were only measured in one location. We did not perform QTL mapping analysis for the earliest vegetative area measurements at any site because these measurements occurred during the acclimation period. Genotypes with >75% missing SNP data were removed from each mapping population prior to QTL mapping analysis; the DG sample size was reduced from 502 to 423, while all 500 SG F_2 genotypes remained.

QTL mapping analysis was performed with *R*/*qtl* using the scanone function and the Haley-Knott algorithm (Broman et al., 2003; Haley & Knott, 1992). QTLs were considered significant if their LOD score exceeded a $p = 0.05$ confidence threshold that was determined independently for each trait from 1,000 permutations. Significant QTLs for each trait were then incorporated into a multiple QTL model and their positions were refined using the refineqtl function. The refined LOD score and effect size of each QTL were calculated using a drop-one analysis within the fitqtl function. The 1-LOD Drop support intervals for fitness trait QTLs were calculated and visualized with jtlovell/qtlTools using the calcCis and segmentsOnMap functions, respectively (Campitelli et al., 2018).

For each mapping population, we identified colocalization among QTLs as overlap in their refined 1-LOD Drop intervals. Within genomic regions of QTL colocalization, we evaluated the direction of allelic effects to assess allelic trade-offs that may emerge for different aspects of fitness (e.g., growth or survival alleles vs. reproduction alleles that act in opposite directions on their respective traits).

2.7 | Permutation analysis

Because we noted high mortality at the GFL site for both populations, we used permutation analyses to test for strong selection on genotype (e.g., Anderson et al., 2013). We assessed allele frequency change after the first 12-month period of growth at GFL, given that most of the mortality occurred within that time frame (Figure S2). Genotypes were assigned values of 1 or 0 depending on their survival status. For each population at the GFL site, individuals were

then randomly permuted into survival categories. For each permutation, we calculated the change in allele frequency at each marker in the linkage map and generated a null distribution for all markers in the genome. We then determined whether the highest LOD markers identified in GFL were in the 2.5% tails of the null distribution.

2.8 | QTL×E analysis

We performed a post-hoc QTL×E analysis to test for shifts in the rank order of allelic effects on fitness across environments that would be consistent with antagonistic pleiotropy. For QTLs associated with traits that were comparably measured in both reciprocal sites for a mapping population, we compared the fitnesses of plants that were homozygous for different parental alleles across environments. Using the highest LOD markers for fitness QTLs identified in each environment, we constructed linear mixed-models with genotype, environment, and QTL×E (i.e., G×E at a single marker) as fixed effects. Genotypic predictions (BLUPs) from within-site trait models were used as the response variables in these models. Again, we evaluated the significance of effects in the models using ANOVAs (Haselhorst et al., 2011).

3 | RESULTS

In total, 31,560 digitized images of plants were analysed to assess vegetative growth and survival, and 1,958,736 inflorescences were counted for reproductive fitness measures in the three common gardens. By all fitness measures (vegetative area, survival and reproductive output), plants grown in the two more northerly locations (DMN and STL) showed higher fitness over the duration of the experiments than those in the southernmost location (GFL). This was largely due to differences in multiyear survival across reciprocal environments, which led to more pronounced differences in reproductive output over the full two-year experiment that were not evident after the first year (Figure S2). At the DMN and STL common garden sites, 99.5% of the plants (100% of DG F_2 genotypes) and 97.1% of the plants (99% of SG F_2 genotypes) survived the first year, respectively. In contrast, only 45.5% of the SG plants (84% of genotypes) and 16.3% of DG plants (39% of genotypes) survived the first year at the GFL site (Figure S2B, Table S2). Lower survival rates among DG genotypes relative to SG genotypes at the GFL site are potentially consistent with a greater selective disadvantage for alleles from the northernmost DMN parent in the subtropical GFL environment.

For all quantitative genetic analyses, results were qualitatively the same and quantitatively very similar for raw and square root transformed data (Tables S3–S7). Within each environment, a larger proportion of trait variance was attributable to genotypic variance than to replicate block. The average broad-sense heritability (H^2) across all traits was ~0.3, suggesting a substantial heritable component to the observed fitness variation (Table S3).

3.1 | Correlation between growth and reproduction varies in sign among sites

Within individual common garden sites, the different measurements of fitness were broadly positively correlated with one another. This was

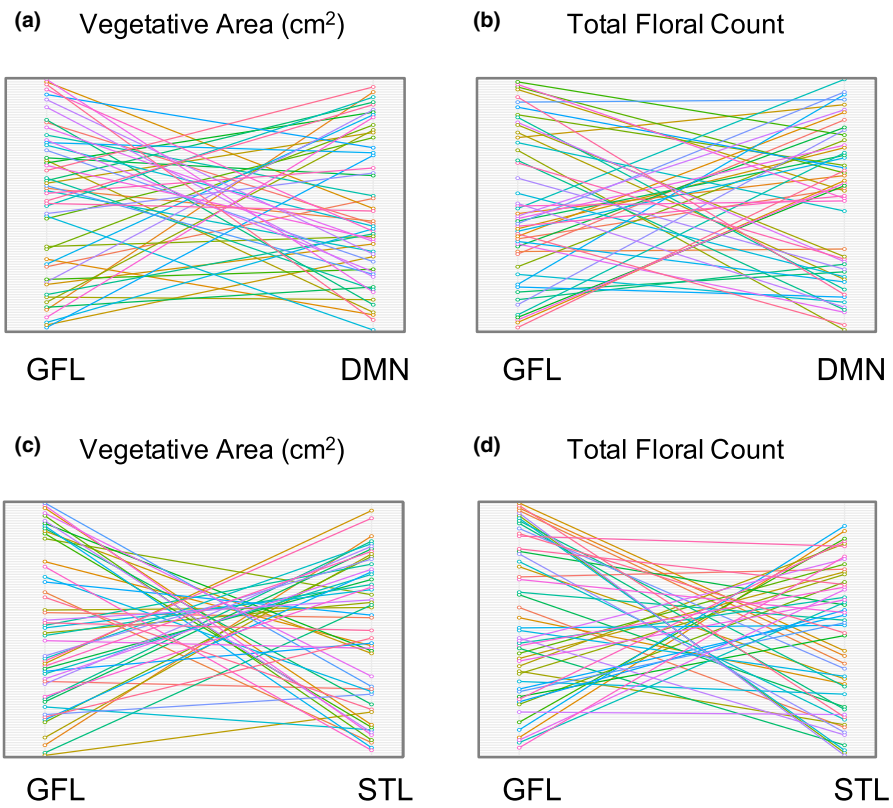

FIGURE 1 Reaction norms for both mapping populations (DG (a, b) and SG (c, d)), including comparisons for vegetative area during the initial 4-month growth period (~120 days) (a, c) and total floral count over the duration of the two-year experiment (b, d). For both traits in both mapping populations, rank-order fitness is generally flipped across reciprocal comparisons. To produce these plots, 50 genotypes were randomly selected for each figure. Data correspond to genotypic predictions (BLUPs) generated by across-site linear mixed models for each trait; these relative trait values are therefore unitless [Colour figure can be viewed at wileyonlinelibrary.com]

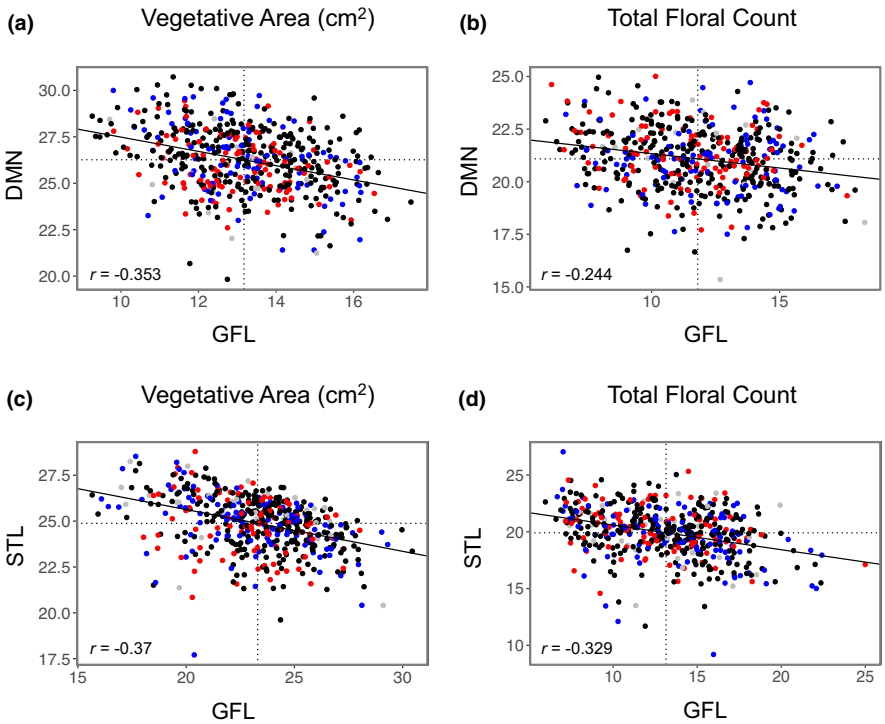

FIGURE 2 Genotype-environment correlations (r_{GE}) for both mapping populations (DG (a, b) and SG (c, d)), including comparisons for vegetative area during the initial 4-month growth period (~120 days) (a, c) and total floral count over the duration of the two-year experiment (b, d). Data correspond to genotypic predictions (BLUPs) generated by across-site linear mixed models that were fit using square root transformed data. Each data point corresponds to a single F_2 genotype coloured by cyanotype (acli (grey), acLi (blue), Acli (red) and AcLi (black)). Dotted axes denote the mean trait values at each site, and solid lines denote lines of best fit (Pearson correlation tests; $p < 0.0001$ in all cases; Table S4). The effects of cyanotype and cyanotype ×E were not significant for any of these traits; thus, variation at the cyanogenesis genes did not significantly account for fitness trade-offs across reciprocal environments (Tables S5, S6)

apparent in the first principal component (PC1) of the PCA for each site, which explained 32.4%–44.3% of the overall fitness variation in common gardens (Figures S3–S6). In particular, positive correlations were observed within the same fitness trait category (e.g., vegetative area measured at different time points). Measurements of vegetative area and survival were also consistently positively correlated at all sites.

Interestingly, the sign and magnitude of the coefficient of correlation between persistence traits and traits related to reproductive output varied depending on the location and the year, such that growth and reproduction were positively correlated in the southernmost GFL site, not correlated at the STL site, and negatively correlated at the DMN site in Year 1 (Figures S3–S6). This pattern potentially suggests that different environments favoured different optimal life history strategies related to investment in vegetative growth versus reproduction (discussed below).

Post-hoc Bayesian analysis further supported these findings. Reproductive traits were negatively correlated with vegetative growth in DMN (r_G = −0.81 and −0.32 for Year 1 and Total Floral counts compared to Vegetative Area (day ~120), respectively), suggesting a trade-off between growth and reproductive output that is strongest in the first year of growth (Wilson et al., 2010). At the STL site, we detected a positive correlation over the entire experiment ($r_G = 0.3$) but a negative correlation between growth and floral counts in year 1 (r_G = −0.41). This result again suggests first-year trade-offs the first year, and as expected, the trade-off is weaker compared to the northernmost site (DMN). In contrast to the two more northern sites, vegetative growth and reproductive traits were positively correlated in GFL (r_G = 0.25 and 0.26 in the DG population, $r_G = 0.44$ and 0.42 in the SG population).

3.2 | White clover displays local adaptation across contrasting environments

Across reciprocal environments, we found strong evidence for genotypic fitness trade-offs, consistent with local adaptation in white clover. Genotype-environment correlations (r_{GE}) were all negative and typically highly significant ($p < 0.0001$) for all comparable traits in both mapping populations; this indicates that both vegetative and reproductive output fitness traits are locally adapted (Figures 1-2, Table S4). Consistent with negative r_{GE} findings, the effects of genotype (G) and G×E interactions were highly significant for nearly all fitness traits in across-site models, while the fixed effect of common garden site (E) was rarely significant (Tables S5, S6). Multiyear analyses further identified highly significant Year (Y) effects and G×E×Y effects for reproductive output traits; these reflect strong differences in flowering across years, which were largely attributable to differences in mortality across reciprocal sites (Figure S2).

TABLE 2 Location and allelic effect for fitness trait QTLs (depicted in Figure 3) for each mapping population at each common garden site. Additional QTLs and results for square root transformed data are located in Table S7

Pop-Site	Fitness trait	Measurement (*Total PVE*[*])	Allele of increased effect[**]	Highest LOD marker[***]	Refined QTL marker	Linkage group	Refined QTL position (cM)	1-LOD drop interval (cM)	PVE[*]	LOD score	Effect size per allele[****]	Mean trait estimate	% effect
DG-DMN	Vegetative area	Day 120 *(10.67%)*	GFL	DG_4_056	DG_4_056	4	141.203	136.60 - 142.82	3.94	5.63	−34.75	746.50	4.66
			DMN	DG_15_084	DG_15_080	15	249.485	211.53 - 319.00	3.56	4.57	30.08	746.50	4.03
			DMN	DG_16_126	DG_16_123	16	223.293	200.50 - 232.63	3.17	5.33	25.04	746.50	3.35
		Day 339 *(9.02%)*	**DMN**	DG_10_127	DG_10_127	10	178.702	153.50 - 239.59	4.92	6.80	43.33	390.35	11.10
			DMN	DG_14_002	DG_14_002	14	5.263	.00 - 35.62	4.10	5.86	32.99	390.35	8.45
	Survival	Winter	**DMN**	DG_14_025	DG_14_025	14	59.920	48.86 - 121.46	4.33	4.83	0.05	1.07	4.52
	Flowering duration	Year 1	GFL	DG_15_070	DG_15_070	15	213.296	207.63 - 260.75	9.68	11.10	−3.76	115.77	3.25
	Floral count	Year 1 *(14.35%)*	**DMN**	DG_5_061	DG_5_061	5	147.083	23.20 - 156.31	2.75	4.53	8.27	173.15	4.77
			DMN	DG_10_143	DG_10_119	10	166.255	156.93 - 214.12	4.03	5.35	10.41	173.15	6.01
			DMN	DG_13_105	DG_13_100	13	276.003	258.18 - 301.29	3.39	4.93	9.03	173.15	5.21
			GFL	DG_15_078	DG_15_076	15	219.377	211.53 - 285.11	4.18	6.44	−9.29	173.15	5.37
		Year 2 *(7.81%)*	**DMN**	DG_11_081	DG_11_081	11	145.788	130.75 - 198.11	4.21	5.17	13.82	287.40	4.81
			DMN	DG_14_003	DG_14_003	14	6.847	5.26 - 22.18	3.60	4.48	13.06	287.40	4.54
DG-GFL	Vegetative area	Day 235	DMN	**DG_15_003**	DG_15_003	15	20.359	.00 - 37.45	12.66	14.79	36.99	201.59	18.35
	Flowering duration	Year 1 *(20.98%)*	**GFL**	DG_10_120	DG_10_120	10	168.000	164.78 - 173.33	9.99	15.28	−10.36	101.13	10.25
			GFL	DG_12_084	DG_12_131	12	333.427	225.23 - 351.83	3.19	4.87	−5.82	101.13	5.75
			GFL	**DG_15_017**	DG_15_017	15	80.604	48.99 - 85.43	4.63	6.94	−4.16	101.13	4.12
			GFL	DG_16_144	DG_16_147	16	267.417	257.82 - 283.42	3.17	5.99	−5.73	101.13	5.67
	Floral count	Year 1 *(23.87%)*	**GFL**	DG_10_120	DG_10_119	10	166.255	164.78 - 239.59	4.33	6.73	−19.36	181.55	10.66
			GFL	DG_12_131	DG_12_131	12	333.427	329.63 - 361.22	4.66	4.75	−19.22	181.55	10.59
			GFL	**DG_15_002**	DG_15_002	15	12.559	.00 - 81.33	11.45	14.07	−26.94	181.55	14.84
			GFL	DG_16_144	DG_16_148	16	269.813	257.82 - 283.42	3.44	6.02	−16.20	181.55	8.92
SG-STL	Vegetative area	Day 362	**STL**	SG_15_106	SG_15_106	15	161.331	119.87 - 175.60	4.29	4.77	52.60	1018.95	5.16
	Survival	Winter	**STL**	SG_15_087	SG_15_087	15	119.214	111.55 - 126.64	3.96	4.38	.09	1.46	6.41
	Flowering duration	Year 1 *(13.21%)*	GFL	SG_13_051	SG_13_051	13	121.105	113.82 - 134.38	4.01	5.82	−4.26	87.89	4.85
			GFL	SG_15_091	SG_15_091	15	126.639	124.09 - 135.94	9.19	11.93	−7.33	87.89	8.34

TABLE 2 (Continued)

Pop-Site	Fitness trait	Measurement (*Total PVE*[*])	Allele of increased effect[**]	Highest LOD marker[***]	Refined QTL marker	Linkage group	Refined QTL position (cM)	1-LOD drop interval (cM)			PVE[*]	LOD score	Effect size per allele[****]	Mean trait estimate	% effect
		Year 2	GFL	SG_15_004	SG_15_004	15	4.818	.00	-	25.65	12.43	14.40	−9.13	173.15	5.27
	Floral count	Year 1 *(12.77%)*	GFL	SG_13_063	SG_13_063	13	140.425	128.67	-	148.88	5.39	7.05	−8.43	48.03	17.55
			GFL	SG_15_104	SG_15_104	15	154.786	124.09	-	170.49	7.39	9.39	−10.27	48.03	21.38
		Year 2	GFL	SG_15_086	SG_15_086	15	117.433	98.72	-	159.63	6.47	7.25	−34.94	385.82	9.06
SG-GFL	Vegetative area	Day 200	STL	**SG_15_112**	SG_15_112	15	170.488	167.24	-	175.60	4.74	5.27	42.89	574.38	7.47
		Day 295 *(12.57%)*	**GFL**	**SG_3_001**	SG_3_018	3	26.086	.00	-	47.75	4.60	4.31	−7.09	29.84	23.76
			STL	SG_15_024	SG_15_024	15	26.789	19.64	-	29.61	7.98	8.19	10.90	29.84	36.52
	Survival	Lifespan *(9.90%)*	**GFL**	**SG_8_036**	SG_8_037	8	105.367	63.38	-	109.59	5.26	5.67	−0.54	12.86	4.23
			STL	**SG_15_024**	SG_15_024	15	26.789	19.64	-	41.04	4.64	4.94	0.55	12.86	4.27
	Flowering duration	Year 1 *(23.81%)*	**GFL**	**SG_7_059**	SG_7_021	7	67.297	61.71	-	79.00	3.54	4.26	−5.14	104.81	4.90
			GFL	**SG_8_045**	SG_8_044	8	140.951	71.00	-	216.26	2.88	4.80	−4.72	104.81	4.50
			GFL	**SG_10_077**	SG_10_089	10	154.732	121.58	-	176.64	3.22	4.71	−5.18	104.81	4.94
			GFL	**SG_15_008**	SG_15_008	15	8.430	0.00	-	17.42	14.17	15.82	−10.55	104.81	10.07
	Floral count	Year 1 *(34.26%)*	**GFL**	SG_1_119	SG_1_119	1	246.700	239.22	-	257.09	2.45	4.53	−17.14	201.39	8.51
			GFL	**SG_6_097**	SG_6_097	6	275.968	190.04	-	284.44	1.88	4.66	−18.67	201.39	9.27
			GFL	SG_10_077	SG_10_104	10	173.517	121.58	-	177.62	5.21	8.56	−29.65	201.39	14.72
			GFL	SG_15_004	SG_15_004	15	4.818	2.56	-	10.32	24.73	32.29	−66.92	201.39	33.23

*PVE is the percent of the phenotypic variation explained by allelic variation at the refined QTL in the full QTL model for that trait at that common garden site. Total PVE is the sum of PVE across multiple QTLs.; **Bold font indicates that the allele of increased effect was the local parental allele.; ***Bold font indicates that the highest LOD marker was in the 2.5% tails of the permuted null distribution of allele frequency changes related to mortality at the GFL site after the first 12 months.; ****A positive value in Effect size represents an increased trait value from the DMN or STL parental allele, while a negative value represents an increased trait value from the GFL parental allele.

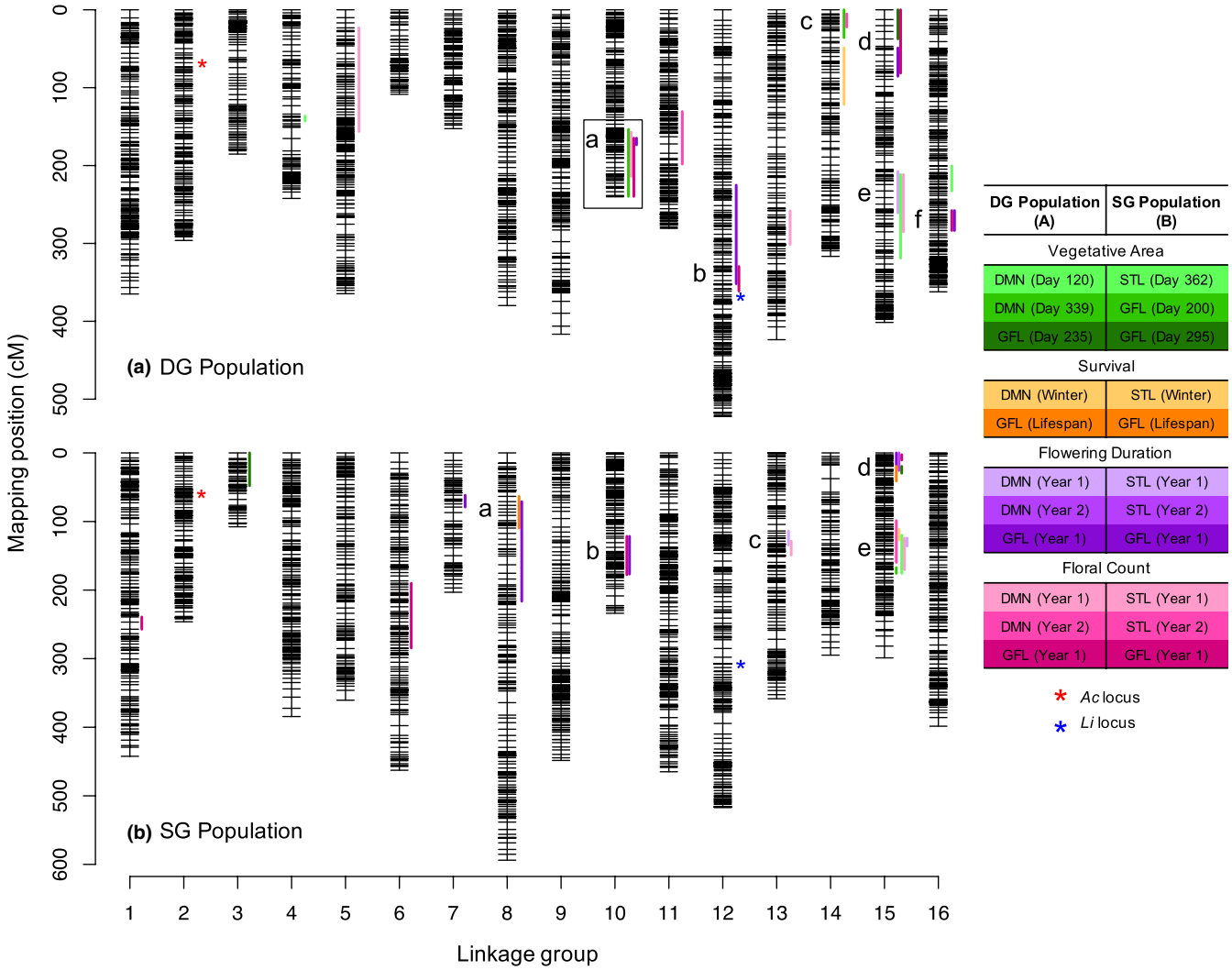

FIGURE 3 Quantitative trait loci (QTLs) associated with fitness traits measured in each common garden for each mapping population ((a) DG and (b) SG). Coloured bars indicate 1-LOD Drop intervals for refined QTLs (Table 2). Coloured asterisks indicate the locations of the *Ac* and *Li* cyanogenesis loci (red and blue, respectively). In each comparison), lower-case letters denote genetic regions where QTLs for multiple fitness traits colocalized (based on overlap in 1-LOD Drop intervals), which are presented in greater detail in Figure 4 (a: a), Figure S7 (a: a–f), and Figure S8 (b: a–e)

3.3 | Local adaptation is not attributable to cyanogenesis variation

Variance analyses indicated that the *Ac*/*ac* and *Li*/*li* cyanogenesis loci accounted for essentially none of the variation in fitness in the common gardens relative to overall genotypic effects. For both mapping populations and within all three common garden sites, cyanotype explained <3% of the variance for all fitness traits (i.e., $V_C/V_P < 0.03$) (Table S3). Cyanotype was never a significant effect in across-site comparisons (Figure 2). In a small number of cases, cyanotype ×E was marginally significant, but this was far exceeded by the significance of the G×E interaction. Cyanotype ×E×Y was the only significant random effect for multiyear floral trait models; however, this was probably driven by the highly significant year effect, or an E×Y effect that was not included in the model, rather than by cyanotype (Tables S5, S6). These patterns are consistent with the conclusion that variation in the chemical defence does not underlie the observed fitness variation between common garden sites.

3.4 | Abundant evidence for genetic trade-offs underlying local adaptation

Genetic mapping analyses revealed many significant fitness-associated QTLs in each common garden. Considering an additive model, QTL effect sizes, as measured by the percent of trait variation explained (PVE), ranged from <2% up to 25% of the total phenotypic variation (Table 2). For reproductive output traits we detected multiple QTLs in all sites, whereas single QTLs were more often identified for vegetative area and survival traits (Table 2, Table S7).

Notably, for traits that were comparably measured across reciprocal sites, the majority of associated fitness QTLs exhibited

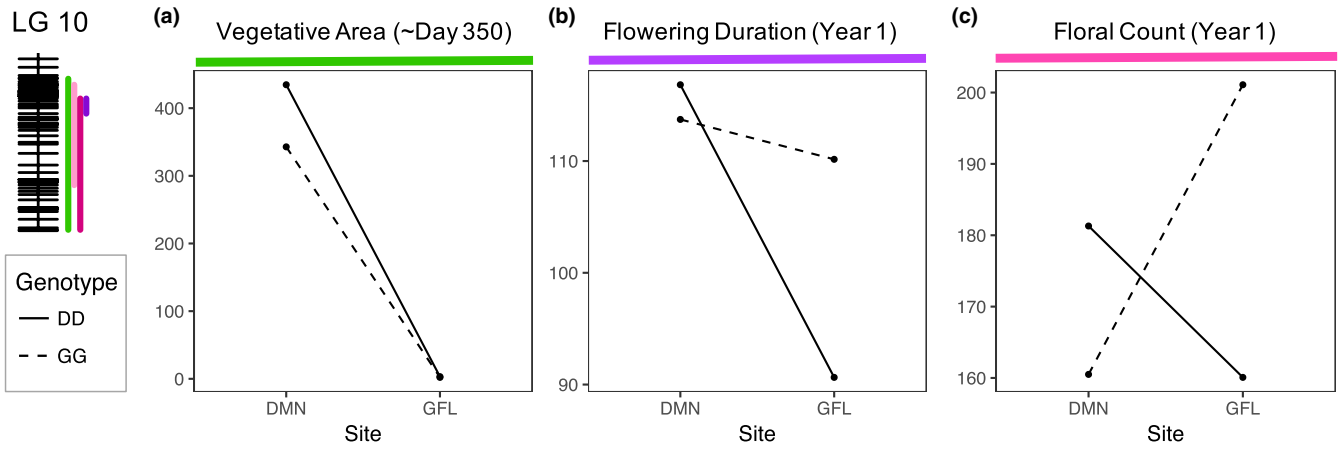

FIGURE 4 One of three hotspots of fitness QTL colocalization was located on linkage group 10. At this hotspot, four QTLs were associated with three fitness traits measured in both the DMN and GFL environments ((a) vegetative area in DMN, (b) flowering duration in GFL, and (c) floral count in both DMN and GFL). Specifically, DD genotypes were (a) 86.7 cm^2 larger (a 25% increase in vegetative area) in Year 2 at the DMN site and (c) produced an average of 20.8 more inflorescences in Year 1 (a 12.8% increase in floral count). Meanwhile, in the GFL environment, GG genotypes (b) flowered 20.7 days longer (a 22.8% increase in flowering duration) and (c) produced an average of 38.7 more inflorescences (a 23.9% increase in floral count) than DD genotypes in Year 1. This hotspot also exhibits antagonistic pleiotropy (Figure S8a); that is, homozygotes of parental alleles (DD vs. GG) exhibited significant fitness trade-offs at the highest LOD markers for these four QTLs (post-hoc QTL×E interactions, $p < 0.0001$; Table S8), with native alleles increasing the trait value in each respective parental environment [Colour figure can be viewed at wileyonlinelibrary.com]

patterns consistent with antagonistic pleiotropy. In post-hoc QTL×E analyses of the DG and SG comparisons, 16 of 16 (100%) and 12 of 15 (80%) of the highest LOD markers displayed significant QTL×E interactions, respectively ($p < 0.05$) (Table S8). The higher proportion of significant QTL×E interactions in the DG population is consistent with greater selection and adaptive differentiation between the two more extreme environments (Figures S7, S8, Table 1).

The strongest evidence of antagonistic pleiotropy was identified in the DG comparison, where QTLs for four different fitness measurements colocalized to the same hotspot on linkage group 10 (Figure 3). Strikingly, genotypes homozygous for the DMN parental allele (DD) at this QTL produced an average of 20.8 more inflorescences in Year 1 (a 12.8% increase in floral count) and were 86.7 cm^2 larger (a 25% increase in vegetative area) in Year 2 (Day 339) at the DMN site compared to GG genotypes. Meanwhile, in the GFL environment, GG genotypes flowered 20.7 days longer (a 22.8% increase in flowering duration) and produced an average of 38.7 more inflorescences (a 23.9% increase in floral count) than DD genotypes in Year 1 (Figure 4, Figure S7a). QTL×E interactions were highly significant for all of the highest LOD markers associated with these traits (Figure 4, Table S8).

While native parental alleles conferred increased fitness for most QTLs, consistent with local adaptation, deviations from this pattern were also observed, where non-native alleles significantly increased fitness. Generally, when such deviations occurred, foreign GFL alleles acted to increase reproductive output at the DMN and STL sites, while foreign DMN and STL alleles acted to increase vegetative area and survival traits at the GFL site (Table 2). This pattern was particularly apparent for two regions on linkage group 15 (Figure 3). For both populations, we identified two nonoverlapping hotspots of QTL colocalization on this linkage group, where parental alleles exhibited trade-offs between persistence and reproductive output. For these QTLs, northern genotypes (DD or SS) displayed increased vegetative area and survival, while southern genotypes (GG) increased reproductive output (Figures S7,S8); this was apparent in all three environments (DMN: Figure S7e; STL: Figure S7d; GFL: Figure S7d, Figure S8d). Permutation analysis performed for both populations in the GFL site suggested that the allele frequency changes for the three significant markers on linkage group 15 were outliers in negative 2.5% tail of a permuted null distribution in the DG population (Table 2; Figure S7d). Additionally, all markers associated with survival and flowering duration in the SG population at GFL were in the 2.5% tails of the permuted null distribution (Table 2; Figure S8a,b,d). Considering both populations together, six of the seven markers that were identified on linkage group 15 were significant in permutation tests, suggesting strong selection at those genomic hotspots, in particular. These results are consistent with strong selection and trade-offs between investment in vegetative growth and reproduction. Moreover, they provide additional evidence that selection may favour different life history strategies in the northern sites compared to the subtropical GFL site. Whereas the DMN and STL sites appear to favour investment in vegetative growth and multiyear survival, fitness at the GFL site investment appears to be optimized by investment in flowering and seed production during the first growing season at the expense of long-term persistence.

Consistent with the variance analyses of fitness traits, none of the significant QTLs for fitness traits mapped to the genomic regions containing the cyanogenesis genes. For the *Ac* locus, there were no significant fitness QTLs on the entire linkage group containing the gene in either population (linkage group 2, Figure 3). For *Li*, QTLs for reproductive output in GFL were located near, but did not overlap with, the locus on linkage group 12. To further test the possibility

that *Li/li* variation might be correlated with reproductive fitness at the GFL site, we directly compared reproductive output between plants with and without the functional *Li* allele; this comparison confirmed that no significant fitness variation was attributable to *Li/li* variation (Figure S7b).

4 | DISCUSSION

Although intraspecific chemical defence polymorphisms are common in plant species and are known to evolve in response to heterogeneous environmental selection (Moore et al., 2014), the relative importance of chemical defence variation for local adaptation has rarely been explicitly examined. Moreover, while it is well established that local adaptation is common in plants (Leimu & Fischer, 2008), the genetic mechanisms underlying this process have been studied in limited detail. Notably, outcrossing herbaceous species have been especially underrepresented in studies characterizing the genetic basis of local adaptation (Savolainen et al., 2013), despite the expectation that outcrossers are more likely to exhibit predicted genetic trade-offs than highly selfing plant species (Hodgins & Yeaman, 2019; Wadgymar et al., 2017). Here, we have examined local adaptation in a perennial, obligately outcrossing herbaceous plant species, white clover, with a well-studied cyanogenesis polymorphism that shows climate-associated clinal variation throughout the species range. Using reciprocal common garden experiments that span a U.S. latitudinal gradient, we demonstrate clear evidence of local adaptation (Figures 1–2), with an underlying genetic architecture characterized primarily by allelic trade-offs at fitness QTL hotspots (i.e., antagonistic pleiotropy) (Figure 4, Figures S7, S8, Table 2, Table S8). We find no evidence that cyanogenesis variation contributes to this local adaptation. Instead, divergent life history strategies — specifically, year 1 investment in earlier and greater amounts of flowering versus delayed flowering and long-term vegetative persistence — appear to be the primary determinants of locally-adaptive fitness, as evidenced by trait correlations and allelic trade-offs. Below we discuss these findings and their implications for understanding local adaptation in herbaceous plants.

4.1 | Strong evidence for local adaptation related to multiple fitness traits

For both mapping populations, genotypic fitness trade-offs across reciprocal common garden environments were evident for both vegetative growth and reproductive output fitness traits (Figures 1–2). This result indicates that multiple aspects of life history contribute to local adaptation in white clover. Moreover, it underscores the previously recognized need to consider more than just the reproductive component of fitness in studies of local adaptation, especially for perennial species (Friedman et al., 2015; Hereford, 2009; Wadgymar et al., 2017). We believe that our findings are conservative and probably underestimate the magnitude of fitness trade-offs in white clover, given the necessarily limited two-year time frame of our experiment and other constraints of our experimental design (e.g., limits on vegetative area differences imposed by trimming; see Supporting Information).

4.2 | Abundant genetic trade-offs underlie local adaptation in an obligate outcrosser

Our study documented more evidence for allelic trade-offs at fitness QTLs across reciprocal environments (i.e., antagonistic pleiotropy) than has been seen in many previous studies in plants (Figures 3-4, Figures S7, S8, Table S8) (Savolainen et al., 2013; but see Price, 2018; Troth et al., 2018). Moreover, allelic trade-offs were more prevalent for the reciprocal comparison representing the greater difference in common garden environments (DG) than for the climatically less diverged comparison (SG). These results are predicted for an outcrossing species with high levels of gene flow, although genetic trade-offs have rarely been empirically demonstrated to this extent in reciprocal common garden studies (Lowry et al., 2019; Wadgymar et al., 2017). Our results therefore contribute compelling empirical evidence supporting the theoretical expectations that antagonistic pleiotropy should underlie local adaptation (Anderson et al., 2011).

We found that fitness QTL hotspots of colocalization were associated with multiple aspects of life history. That is, both vegetative growth/survival traits and reproductive output traits often colocalized to the same QTL regions (Figures 3-4). Similar results have been found in recent studies with annual and perennial *Mimulus guttatus*, where both size/survival and flowering traits have been measured (Friedman et al., 2015; Hall et al., 2010). We were able to further show that for QTL hotspots associated with multiple fitness traits, the effects of alleles that were inherited from different parents acted antagonistically for vegetative growth and reproductive traits in all three common garden environments: alleles from northern parents (DMN and STL) increased growth and survival in all three common garden environments, while alleles from southern parents (GFL) increased reproductive output (Table 2, Figures S7d,e, S8d,e). These striking results may reflect selection for divergent life history strategies in contrasting environments as discussed below.

We identified a wide range of effect sizes among fitness QTLs in each of our common garden environments, suggesting that local adaptation in white clover occurs through the action of both small- and large-effect loci (Table 2). We detected some loci with relatively small effects, despite known statistical limitations of QTL mapping approaches (Beavis, 1998). Simulation studies suggest that local adaptation occurs readily with QTLs of large effect but can also be achieved through the action of many small-effect loci, which may be more common in self-fertilizing species (Hodgins & Yeaman, 2019; Whitlock, 2015; Yeaman, 2015). While our results are similar to empirical studies in other plant species (Ågren et al., 2013; Savolainen et al., 2013), we acknowledge that there are probably additional locally-adaptive small-effect QTLs that we could not detect given the limits of our statistical power (Anderson et al., 2013; Mee & Yeaman, 2019).

High levels of gene flow are predicted to lead to genomic clustering of small-effect loci that are locally adaptive; such clustering can create the appearance of single large-effect QTLs in mapping studies (Yeaman & Whitlock, 2011). Recent studies in sunflowers have demonstrated that large differences in flowering time, which contribute to ecotypic differentiation, are controlled by massive nonrecombining haplotypes (Todesco et al., 2020). For the present study, we cannot be sure whether the effect sizes of single QTLs were the result of single large-effect genes with pleiotropic effects or clusters of small-effect genes that may remain tightly linked in adaptive haplotypes. This lack of genetic resolution has been previously acknowledged to be a limitation of QTL mapping (Rockman, 2012).

Further, we cannot be certain whether our QTL hotspots of colocalization indicate pleiotropically acting genes or physically linked clusters of adaptive genes that affect different traits. Clusters are predicted for outcrossing species like white clover (Hodgins & Yeaman, 2019). On the other hand, evidence for antagonistic pleiotropy has been found in species that display a range of mating systems: ragweed (highly outcrossing) (Hämälä et al., 2020), canola (predominately selfing with some outcrossing) (Raman, 2019), and *Arabidopsis thaliana* (highly selfing) (Auge et al., 2019). Future experiments in white clover using more advanced generations would allow for finer genetic mapping that could resolve these uncertainties.

4.3 | A chemical defence paradox?

More than 60 years of ecological genetic studies have provided evidence that selection acts on the cyanogenesis polymorphism in white clover (reviewed in Hughes, 1991; Kooyers et al., 2018). While, to our knowledge, no study has assessed fitness variation among cyanotypes at different locations along a cyanogenesis cline, the fact that clines have evolved repeatedly along climatic gradients worldwide strongly suggests that one or more climate-associated selective factors act on this chemical defence polymorphism (de Araújo, 1976; Daday, 1958; Hughes, 1991; Kooyers & Olsen, 2012). Nonetheless, we found no evidence that variation for any fitness trait measured was attributable to cyanotype within or across the three climatically-distinct common garden environments (Figure 2, Tables S3, S5, S6). These results are partially consistent with recent findings in *M. guttatus*, which detected mixed evidence for pleiotropy or genetic linkage between correlated life history and defence traits. In that study, growth rate and chemical defence (phenylpropanoid glycoside) traits had differing genetic architectures, but colocalization occurred in a single QTL region for both flowering time and PPG concentrations (Kooyers et al., 2020).

A possible explanation for the lack of a significant cyanogenesis contribution to fitness in this study is that the conditions plants experienced in the common gardens did not accurately capture all selective pressures in local wild populations — for example, the full range of herbivore intensity experienced over a lifetime. To assess this possibility, we compared rates of leaf herbivore damage within our common gardens and in natural plant communities near but outside of the common gardens (see Supporting Information). Measured rates of herbivory were low, both inside and outside of gardens; the lowest rates of herbivory were measured in the southernmost GFL environment, counter to expectations. Additionally, we found no differences in the rate of herbivory experienced by cyanogenic versus acyanogenic plants within gardens (Table S11; Supporting Information). It is possible that experiments performed during natural episodes of more intense herbivory would reveal greater evidence for cyanogenesis-related fitness variation.

Another possibility is that cyanogenesis variation may be most important for survival during the earliest life stages, when loss of vegetative tissue could have a greater negative effect on survival. To achieve genotypic replication, our common garden experiments necessarily utilized stolon cuttings of F_2 individuals planted from seed grown in a greenhouse, where germinant and seedling mortality is essentially 0%. Common garden fitness measurements therefore did not assess potentially important fitness variation that is known to be important for local adaptation during germination and seedling developmental stages (Postma & Ågren, 2016, 2018). A set of complementary germination experiments that we performed at the three common garden sites suggested that selection at the seedling or juvenile life stage may impact local cyanotype frequencies in white clover populations (Wright & Olsen, unpublished observations). Ongoing experiments and analyses will assess this possibility.

4.4 | Divergent life history strategies promote local adaptation in herbaceous species

Local adaptation via differential investment in sustained growth versus early reproduction in contrasting environments has been documented in recent studies of several other well-studied herbaceous species; these include the model annual species *Arabidopsis thaliana* (Debieu et al., 2013; Fournier-Level et al., 2013); two related perennial species, *A. lyrata* (Leinonen et al., 2009; Quilot-Turion et al., 2013; Hämälä et al., 2018) and *Boechera stricta* (Wadgymar et al., 2017); and annual and perennial populations of *M. guttatus* (Friedman et al., 2015; Peterson et al., 2016; Troth et al., 2018). These studies generally suggest that alternate life history strategies may be a common evolutionary strategy for local adaptation in herbaceous plant species.

In the case of white clover, the life history trade-offs we observed were most evident between investment in vegetative growth and reproductive output in the first year. Specifically, in the environments with low overall mortality (DMN and STL), greater investment in vegetative growth came at the cost of year 1 reproductive output but provided the benefit of high reproductive output in year 2. In the northernmost DMN site, negative correlation between growth and year 1 reproduction was captured by PC2 of the within-site principal component analysis, which accounted for 27% of the total variation in measured fitness traits (Figure S3). In particular, we detected a significant negative correlation between vegetative growth in the first 120 days and year 1 floral count at the DMN site. While

this correlation was not statistically significant in the STL common garden (Figure S4), PC2 for the STL site (explaining 27.9% of fitness variation) suggested that growth and reproductive output traits were negatively correlated and post-hoc Bayesian analysis detected a negative correlation in year 1 in STL, albeit to a lesser extent than at the DMN site. Unlike in the northern environments, plants with longer lifespans at the GFL site did not achieve substantial reproductive gains in year 2 due to high year 1 mortality. Thus, in a southern U.S. environment with harsh summer conditions for white clover, rapid growth and early reproduction appears to be selectively advantageous. Taken together, differences in trait correlations across sites strongly suggest that divergent optimal life history strategies are favoured in different environments and contribute to local adaptation in white clover. At the genetic level, opposing allelic affects at fitness QTLs bolster this argument: northern alleles favour growth and survival, while southern alleles favour reproduction (Figures S7a,d,e, S8d,e).

We propose that these divergent optimal life history strategies across environments may be directly tied to the intensity of heat stress exposure in each location. Heat stress can be a major limiting factor for vegetative growth and survival in plants and can favour earlier investment in reproduction and more rapid life cycles (Ågren & Schemske, 2012; Moles et al., 2014; Preite et al., 2015; Kooyers, 2015; Wright et al., 2018; Lowry et al., 2019; Ehrlén & Valdés, 2020; Hamann et al., 2021). In line with these findings, patterns in the present study suggest that heat stress led to vegetative tissue loss and mortality. Plants in the DMN common garden did not appear to suffer from heat stress at any point, whereas plants in both the STL and GFL environments displayed tissue loss and leaf senescence indicative of heat stress. The duration of heat stress lasted only 2–4 months each year at the STL site and was followed by a recovery period of two or more months before winter. In contrast, heat stress due to elevated temperatures was more intense and prolonged at the GFL site (5–6 months) and was associated with periods of massive tissue loss and high mortality (Figure S2, Table S10). As a result, hotter conditions in the southernmost common garden environment (GFL) favoured genotypes that invested in reproduction earlier in the first growing season, prior to mortality during the summer months. In contrast, the two more northerly common garden environments (DMN and STL) favoured genotypes with early and ongoing investment in vegetative growth over the two-year experiment; this investment came at the expense of floral production in the first year.

For widespread North American plant species, southern populations currently and increasingly experience natural selection due to climate change-associated heat stress (and potentially associated drought stress). Thus, life history variation is more likely to be a major contributor to local adaptation than chemical defence variation in white clover, as our results support, and also more broadly in herbaceous plant species. Specifically, a heat/drought escape strategy involving rapid life cycles and early flowering, as opposed to the evolution of physiological tolerance, appears to be locally adaptive for herbaceous populations that experience prolonged periods of stress (Hamann et al., 2021; Kooyers, 2015). Over time, one might predict natural selection to favour the evolution of annuality in populations of perennial herbaceous species that occur in hotter and more stressful environments.

ACKNOWLEDGEMENTS

We thank Drs Justin Fay (U Rochester), Christy Edwards (Missouri Botanical Garden), Stephanie Spielman (Rowan U), Nicholas Kooyers (U Louisiana-Lafayette), and Ken Quesenberry (U Florida) for experimental design suggestions, statistical advice, and manuscript feedback. Special thanks to Mike Dyer and the Washington University greenhouse staff for supporting plant breeding, maintenance, and propagation. Thank you to Linda Small, Samantha Myers, and Maya Dutta for assistance with DNA extractions, genotyping, and phenotyping. Field experiments were made possible by the help of numerous field coordinators and assistants. In particular, we thank Haley Reeves and María José Gómez-Quijano (DMN field coordinators); Kenneth Wright, Julien Weinstein, Amy Kuhle and Bailee Warsing (STL assistants); Dr Yolanda Lopez (GFL assistant); and Lindsey Brush and Dr Luis Inostroza (GFL field coordinators). We also thank two anonymous reviewers for feedback. This work was supported by the following funding sources: NSF CAREER Award to KMO (DEB-0845497); NSF Integrated Organismal Systems award to KMO, BLG and PRM (IOS-1557770); NSF Graduate Research Fellowship to SJW (DGE-1745038); NSF Doctoral Dissertation Improvement Grant (DDIG) to SJW and KMO (DEB-1601641); and William H. Danforth Plant Science Fellowship to DMG.

AUTHOR CONTRIBUTIONS

Sara J. Wright[1] – designed research, performed crosses, coordinated DNA extractions and cyanotyping, prepared GBS libraries, STL field coordinator, analysed all data (except raw GBS reads, which were handled by David M. Goad), and wrote the manuscript. David M. Goad – GBS bioinformatics, linkage map construction, QTL mapping guidance. Briana L. Gross – directed research at the DMN site. Patricio R. Muñoz – directed research at the GFL site. Kenneth M. Olsen – designed research, contributed to manuscript writing, directed research at the STL site, mentored Sara J. Wright and David M. Goad.

DATA AVAILABILITY STATEMENT

The following are available on Dryad: https://doi.org/10.5061/dryad.j0zpc86f7

- Raw phenotype data used for all quantitative trait and genetic mapping analyses (four.csv files for the two reciprocal comparisons: DMN, GFL-DG, STL, and GFL-SG).
- Full results from QTL mapping analysis (R printouts from all models as.txt files)
- R scripts for various analyses:
- λ calculations and correlations with Year 1 floral count (Figure S1)
- within-site trait variance and broad-sense heritability (Table S3)
- within-site trait correlations and PCAs (Figure S3,S6)

- across-site trait variance (Figure 1, Table S5, Table S6)
- *rGE* correlations (Figure 2, Table S4)
- QTL mapping analysis (Figure 3, Table 2, Table S7)
- QTL×E post-hoc analysis (Figure 4, Figure S7,S8, Table S8)

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How to cite this article: Wright, S. J., Goad, D. M., Gross, B. L., Muñoz, P. R., & Olsen, K. M. (2022). Genetic trade-offs underlie divergent life history strategies for local adaptation in white clover. *Molecular Ecology*, 31, 3742–3760. https://doi.org/10.1111/mec.16180