






RESEARCH ARTICLE

Phenotypic plasticity masks evolutionary change in grassland plant traits in response to land use abandonment

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Abstract

1. Traditionally managed grasslands are among the most biodiverse habitats in Europe, but are threatened by land use abandonment. While the negative impacts of grazing and mowing cessation on species richness are well documented, little is known about potential evolutionary changes within species. Yet, intraspecific functional diversity is critical for successful grassland restoration and ensuring adaptive potential to future climate change.
2. To disentangle the heritable and non-heritable components of population-level response to land use change in semi-natural grasslands, we examined 22 populations of a common grass, *Briza media*, from grazed sites and sites where grazing was abandoned, with resulting tree and shrub encroachment. We measured a range of traits under field conditions and following clonal propagation under common garden conditions.
3. Field surveys revealed that abandonment resulted in litter accumulation, greater shading by woody and herbaceous vegetation, and lower temperature and moisture fluctuations compared with grazed sites. Plants responded to conditions at abandoned sites with phenotypic plasticity in traits that enhanced competitive ability for light (greater height and specific leaf area) but reduced tissue protection against stress (lower dry matter content). However, when the same genotypes were measured after clonal propagation in common conditions, counter-gradient variation was apparent, with heritable shifts towards increased tissue protection in populations from abandoned sites.
4. When measured under field conditions, trait diversity was higher in sites with higher levels of shading and productive sites with higher variation in light conditions. However, high heritable trait diversity was instead characteristic of grazed sites with high densities of flowering *B. media* individuals.
5. *Synthesis.* These findings demonstrate that land use change can cause evolutionary shifts and changes in heritable trait diversity that are masked in the field by phenotypic plasticity. Hence, the assessment of functional trait variation based

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on field observations is not a reliable way to assess the genetic variation essential for population adaptive potential.

KEYWORDS

adaptive potential, counter-gradient variation, grazing abandonment, heritable change, land use change, local adaptation, phenotypic plasticity, population size

1 | INTRODUCTION

Ancient semi-natural grasslands represent local biodiversity hotspots and are targets for conservation (Buisson et al., 2022; Habel et al., 2013; Nerlekar & Veldman, 2020; Subedi et al., 2022). However, abandonment of grassland management, including cessation of grazing and mowing, is widespread in regions with low soil fertility and challenging topography and is a major cause of biodiversity loss (Estel et al., 2015; Habel et al., 2013). Semi-natural grasslands that are no longer grazed or mown often experience declines in plant species richness due to the encroachment of shrubs and trees and increasing dominance of highly competitive herbaceous species, which outcompete species characteristic of open grasslands (Louault et al., 2005; Pykälä et al., 2005; Ratajczak et al., 2012). Abandonment also causes shifts in plant taxonomic and functional trait composition (Aldezabal et al., 2015; Janeček et al., 2013; Johansson et al., 2011; Neuenkamp et al., 2016) and deterioration of wider ecosystem functions (Peco et al., 2017; Prangel et al., 2023; Quintas-Soriano et al., 2022; Söber et al., 2024). In addition to eliciting community-level changes, land use change can potentially lead to evolutionary changes within populations. Evidence is accumulating about the impacts of grassland abandonment and fragmentation on population size and genetic diversity (Aavik et al., 2019; Johansson et al., 2011; Reinula et al., 2021); yet, the consequences of grassland abandonment for plant functional traits known to be key in regulating ecosystem functions remain unknown. Whether land use abandonment and the environmental changes that occur alongside it drive heritable evolutionary change in plant populations, or simply trigger trait changes via phenotypic plasticity, is unclear. A better understanding of the eco-evolutionary dynamics of plant functional traits under land use change is key for assessing the adaptive and restoration potential of remnant populations in abandoned grasslands.

Traits such as specific leaf area (SLA), leaf dry matter content (LDMC), and root tissue density (RTD) reflect the fundamental trade-off between investment into fast resource acquisition (high SLA) and resource conservation (high LDMC and RTD) (Reich, 2014; Weigelt et al., 2021; Wright et al., 2004). These traits are of key importance for ecosystem level processes, as they are known to have cascading effects on other trophic levels, resulting in an overall gradient between so-called 'fast' and 'slow' ecosystems (Neyret et al., 2024). Land use change may affect these traits by altering the selective environment for grassland plants. Plant populations subjected to chronic vertebrate herbivory are known

to exhibit more acquisitive leaf traits, which enhance grazing tolerance by enabling fast leaf regrowth (Rotundo & Aguiar, 2008; Gorné & Díaz, 2022; though selection for higher leaf protection can be observed in arid ecosystems, Díaz et al., 2007). Hence, long-term grazing abandonment can reduce selective pressure for fast leaf regrowth and instead favour resource-conservative traits (e.g. high LDMC) that enhance leaf longevity, as leaves remain ungrazed for the entire growing season, besides occasional light grazing by wild herbivores. This should be particularly true in the shady conditions of abandoned grasslands, where carbon conservation might be key to survival (Gommers et al., 2013; Poorter et al., 2009; Valladares & Niinemets, 2008). In addition to the direct impact of grazers, grazing cessation can alter selective pressures by modifying light conditions. First, increased shading by neighbouring herbaceous vegetation can select for fast growth and enhanced elongation to compete for light (i.e. shade avoidance, Gruntman et al., 2017; Nagashima & Hikosaka, 2011). Second, shade from encroaching shrubs and trees favours species with shade tolerance, which is achieved through high SLA, making light capture more efficient (Celis et al., 2017; Semchenko et al., 2011; Sultan, 2000). Hence, increased SLA under shaded conditions may not reflect a fast growth strategy but an adaptation to tolerate shade. Third, the presence of trees and shrubs increases buffering against abiotic stress by reducing wind and evaporation, temperature fluctuations, and UV light exposure, which could lead to reduced selection for abiotic stress tolerance (i.e. lower LDMC; Gruntman et al., 2017; Semchenko et al., 2011). Thus, management abandonment modifies abiotic and biotic conditions in ways that might create opposing selection on traits related to leaf structural protection, with tree shading and reduced abiotic stress selecting for high SLA and low LDMC, but the lack of grazing selecting for enhanced leaf longevity (i.e. high LDMC). Increased shading and associated carbon limitation in abandoned grasslands could also drive shifts in root morphology that reflect weaker associations with arbuscular mycorrhizal (AM) fungi (i.e. higher specific root length; Johnson, 2010; Neuenkamp et al., 2018). However, such shifts within species remain unexplored.

At the intraspecific level, plant populations can respond to land use abandonment via two distinct mechanisms: heritable changes in plant populations (i.e. shifts in the genetic makeup of populations or persistent epigenetic modifications affecting trait expression over generations) and phenotypic plasticity, which describes the ability of a genotype to produce different phenotypes in response to varying environments (Callaway et al., 2003;

Miner et al., 2005; Miryeganeh & Saze, 2019). To date, empirical field studies have reported both increased and decreased SLA and LDMC, as well as no detectable change in these traits, between populations inhabiting managed versus abandoned grasslands (Castro et al., 2010; Celis et al., 2017; Louault et al., 2005; Pakeman, 2013; Targetti et al., 2013; Volf et al., 2016), but we lack comparative studies in common garden conditions, which could disentangle trait changes due to phenotypic plasticity from those driven by evolutionary change (but see Cid et al., 1989; Detling & Painter, 1983 for evidence of evolutionary change detected in experimental grazing exclosures).

Phenotypic plasticity allows plants to adjust their traits in accordance with the prevailing conditions (Miner et al., 2005) and can contribute to population persistence under land use change. However, as light availability and other environmental conditions in abandoned grasslands become suboptimal for grassland specialists, this is known to cause declines in population size and genetic diversity within populations (Aavik et al., 2019; Reinula et al., 2021), which in turn may reduce functional trait diversity. Land use change may also influence trait diversity by affecting environmental heterogeneity within grasslands (Karbstein et al., 2020; Stark et al., 2017). As with shifts in trait means, changes in trait diversity may reflect both plastic responses and changes in genetic variation within populations. So far, land use impacts on functional trait diversity, particularly those associated with heritable change in populations, remain undescribed. However, these changes require investigation as there is accumulating evidence about the importance of genetic and functional diversity within populations in ensuring ecosystem functioning and stability (Prieto et al., 2015; Semchenko et al., 2021; Whitlock, 2014).

Here we evaluated the extent to which phenotypic plasticity and heritable trait changes drive population responses to long-term grazing abandonment. We examined 22 populations of a common grass species, *Briza media*, across sites that had been either continuously grazed or where grazing had been abandoned for at least three decades, resulting in gradual shrub and tree encroachment. For each population, we measured a range of traits (a) in the field where differences in trait expression are a combined result of phenotypic plasticity and evolutionary responses to changes in environmental conditions triggered by grazing abandonment, and (b) after the propagation of clonal offspring in standardised, common conditions, with the aim of highlighting heritable differences in trait expression. The following hypotheses were tested:

Hypothesis 1. Grazing abandonment creates shadier and more heterogeneous light conditions, results in litter accumulation, buffered temperature and moisture conditions and reduced *B. media* population sizes and richness of grassland species, compared with continuously grazed sites.

Hypothesis 2. As a result of environmental changes, grazing abandonment drives shifts in trait means towards:

- (i) Taller phenotypes, reflecting a more competitive strategy to resist light competition from surrounding herbaceous vegetation.
- (ii) Higher SLA, enhancing light capture and tolerance of shade from shrubs and trees.
- (iii) Lower LDMC if selection following abandonment is primarily driven by a reduction in exposure to physical hazards (e.g. wind, UV radiation). Alternatively, if selection in abandoned grasslands is primarily mediated by the absence of grazers and the advantage of enhanced leaf longevity, a shift towards higher LDMC in abandoned grasslands is expected.
- (iv) Thinner roots and higher specific root length (SRL) with lower mycorrhizal colonisation rates, indicating reduced reliance on mycorrhizal symbiosis.

Hypothesis 3. Trait diversity is lower in populations from abandoned sites due to lower genetic diversity stemming from shrinking population sizes. Alternatively, trait diversity within abandoned populations may increase as abandoned grasslands temporarily provide a more heterogeneous environment due to some areas becoming shaded by trees while others remain open.

In the case of both trait means and trait diversity within populations, we expected to observe stronger responses to grazing abandonment via plastic responses to field conditions compared with heritable trait responses detectable under common conditions, as evolutionary responses make take longer to arise. In the case of alternative hypotheses regarding tissue protection and trait diversity, support for one of the hypotheses would indicate which process potentially imposes a stronger selective pressure on trait expression.

2 | MATERIALS AND METHODS

2.1 | Study sites

Twenty-two sites, grouped in 11 pairs, were selected for this study: 11 grasslands that had been continuously grazed and were expected to represent ancient grasslands; and 11 nearby sites that had been grazed historically but grazing had been abandoned several decades prior to the study (at least 35 years previously based on satellite and aerial photos available at Estonian Land Board; Table S1). The sites are located on the western coast of Estonia and on the nearby islands Muhu and Saaremaa (Figure 1; see Table S1 for coordinates). Most of the sites are located close to the coastline and have probably been used for grazing since vegetation established following emergence from the sea due to postglacial rebound (circa 1000–2000 years ago; Aavik et al., 2008). Two sites were located within a nature reserve, and a sampling permit was acquired from the Environmental Board to carry out fieldwork at these sites (permit number 1–3/23/270).

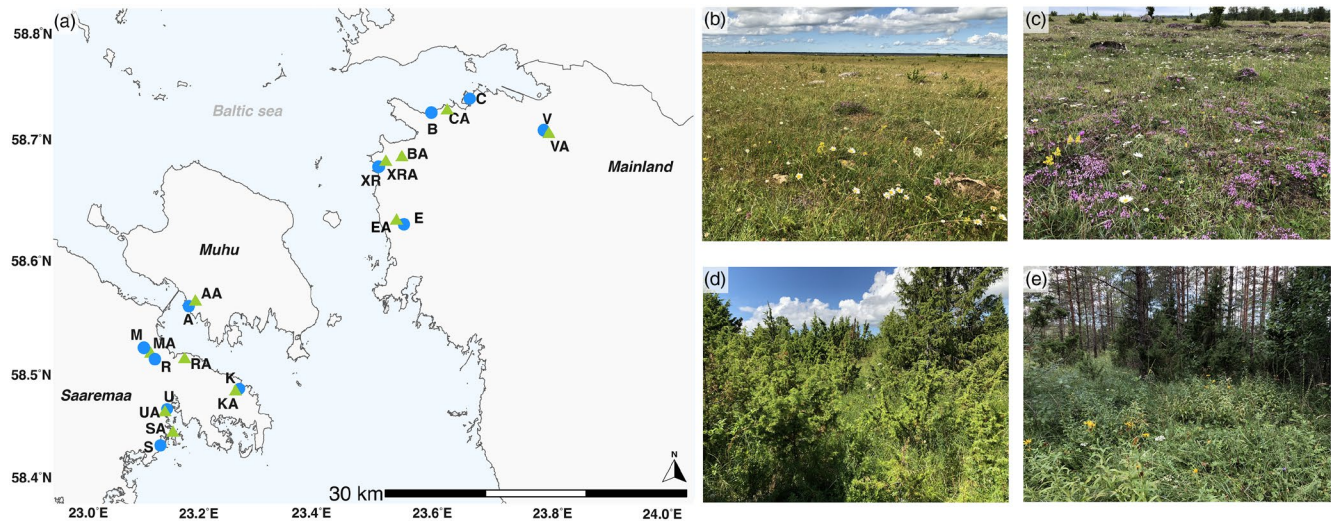


FIGURE 1 Study sites. (a) Map of the western coast of Estonia. Blue points represent continuously grazed sites, and green points show abandoned sites. See [Table S1](#) for precise coordinates and the environmental characteristics of the sites. (b, c) Examples of grazed sites. (d, e) Examples of abandoned sites.

2.2 | Genotype sampling and propagation

In August 2022, 20 individuals of *Briza media* L. were collected from each of the 22 sites, for a total of 440 plant genotypes. Plants were collected at least 2 m away from each other to ensure that each individual represented a unique genotype (the species is self-incompatible and does not spread long distances by clonal reproduction; Prentice et al., 2006). Collected genotypes were extracted from soil and washed, and their root and leaf traits were measured as described in Section 2.3. Clonal offspring of each collected genotype were propagated by transplanting individual cuttings of about 3 cm with intact root and leaf buds into 200 mL pots filled with a sterilised sand and soil mixture (collected from a range of nearby grasslands not used in this study; sterilised with gamma irradiation, dose 25 Gy). Clonal offspring were propagated in a greenhouse for 8 months under common conditions: additional lighting ($50 \mu\text{mol}/\text{m}^2/\text{s}$) was provided for 16 h per day in winter months, plants were watered daily, leaves were trimmed and fertiliser was applied (20 mL of fertiliser solution at the manufacturer's recommended rate, 10 mL fertiliser per 1.2 L solution; 7:4:6 NPK, Biopon, Bros, Poznan, Poland) shortly after trimming every 2 months. The plants were trimmed to remove senescent leaves and promote the production of new ramets. The period of plant propagation (8 months) exceeds a typical growing season in the region (164 days per year above 10°C ; Kollo et al., 2023). The positions of plants were periodically re-randomised during the propagation period.

2.3 | Plant trait measurements

Plant traits were measured for each genotype on two occasions: in the field upon genotype collection and on clonal offspring following propagation in common conditions for 8 months (Figure 2). After

8 months, each genotype had produced several rounds of new ramets since initial planting, and the trait measurements were performed on a freshly produced ramet, 6 weeks after the last trimming and fertilisation round. Hence, we expected that this propagation period under common conditions eliminated plastic responses to environmental conditions at the site of origin and highlighted heritable trait differences among populations. These heritable components can include not only genes coding for a fixed phenotype but also genes that determine the way plants respond plastically to their environment, in this case, the specific propagation conditions in the greenhouse (i.e. genotype-by-environment interactions). Therefore, the extent of detected heritable trait differences might vary depending on the common garden conditions. Plant height, LDMC, SLA, RTD, SRL, and root diameter (RD) were measured. Plant height was measured as the distance between the rooting point and the tip of the longest leaf when fully extended. Three to five leaf fragments (ca 5–10 cm in length, comprising the middle section of fully expanded leaf blades) were sampled per genotype and immediately placed upright into plastic ziplock bags with a few drops of water and stored in the dark at 4°C for 24 h, after which leaf fresh mass was recorded and leaves were scanned using an Epson V370 scanner (Epson America Inc., Los Alamitos, USA). Root samples were extracted from each genotype, washed out of the soil, and kept in 10% ethanol solution at 4°C until analysis. A subsample of fine roots was scanned using an Epson v800 scanner (Epson America Inc., Los Alamitos, USA). Leaf area, root length, root volume, and mean RD were calculated based on scans using RhizoVision Explorer (version 2.0.3; Seethepalli et al., 2021). The scanned leaf and root samples were dried at 40°C till constant weight and weighed separately. LDMC was calculated as the ratio of leaf dry mass to fresh mass, and SLA as the ratio of leaf area to leaf dry mass. SRL was calculated as the ratio of root length to the dry mass of the scanned root sample, and RTD as the ratio of dry root mass to root volume.

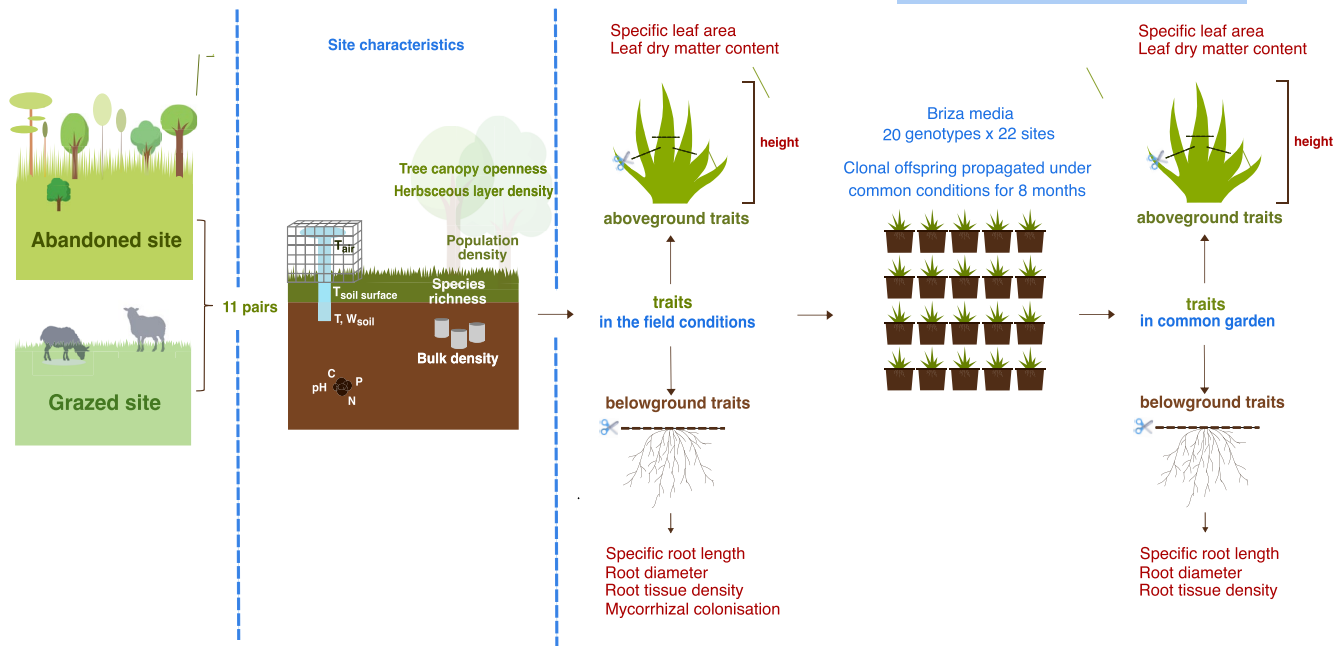


FIGURE 2 Schematic describing the design of the field observational study, genotype sampling, and greenhouse common garden study. Eleven pairs of coastal grasslands were examined, each consisting of one site that had been continuously grazed and a nearby site that had been historically grazed but grazing had been abandoned several decades previously. At each site, temperature and soil moisture probes were installed for the duration of the growing season, soil samples were collected for chemical analysis and bulk density measurements, above-ground productivity and litter accumulation were measured, shading by tree canopy and herbaceous vegetation was quantified, and vegetation composition and the population density and flowering frequency of the focal species *Briza media* were recorded. Twenty genotypes of the focal species were collected from each site and above- and below-ground plant traits were measured in the field and on clonal offspring of each genotype after 8 months of propagation under common conditions.

2.4 | Assessment of root mycorrhizal colonisation

The roots of four randomly selected individuals of *B. media* per population grown in the field were analysed to estimate levels of colonisation by AM fungi, using the ink-vinegar method described in Vierheilig et al. (1998). Roots were soaked in 10% KOH for 24 h and cleared by boiling in 10% KOH for 15 min followed by rinsing several times with tap water. Cleared roots were boiled for 15 min in a staining solution (5% ink, 5% acetic acid) and subsequently destained by rinsing with tap water and soaking overnight in a weak acetic acid solution. Stained roots were mounted on slides and the presence or absence of AM fungal structures (hyphae, arbuscules, and vesicles) was recorded at 100 randomly selected root intersections at 400 \times magnification (Zeiss Microscope Axioscope 5, Germany).

2.5 | Measurement of site characteristics

Soil samples were collected from four random locations within each site that were separated from each other by at least 20 m. The samples were passed through a 2 mm sieve and air-dried. To measure pH, soil samples were mixed with distilled water in a ratio of 1:5 (5 g of soil to 25 mL of water), shaken for 1 h, and pH was recorded using a Hanna HI-98191 Professional Waterproof PH/

ORP/ISE meter (UK). Total P was extracted following the method described in Wuenscher et al., 2015 (calcination for 1 h at 550 $^{\circ}$ C and extraction with 1 M HCl) and quantified using the ammonium molybdate–malachite green assay adapted for microplates (Jeannotte et al., 2004). Absorbance at 600 nm was recorded using a microtiter plate reader (Tecan Sunrise, Tecan Group Ltd., Männedorf, Switzerland). Total C and N were determined using a PerkinElmer 2400 Series II CHNS (Shelton, CT, USA) elemental analyser.

For soil particle size analysis, four soil samples per site were combined, and sand, silt, and clay fractions were determined using the laser diffraction method with a Malvern Mastersizer 3000, Malvern Panalytical B.V., Almelo, Netherlands (Polakowski et al., 2023) after pretreatment with H₂O₂ (Koza et al., 2021). Soils were categorised using the U.S. Department of Agriculture method for naming soils based on clay, silt, and sand content (Groenendyk et al., 2015). A separate set of four samples per site was collected to measure soil bulk density; samples were collected using 100 mL cylinders (5 cm in diameter and 5 cm depth), and soil was dried at 105 $^{\circ}$ C for 4 h and weighed (Al-Shammary et al., 2018).

To describe shading from herbaceous vegetation, 20 measurements of vegetation height and five measurements of the proportion of photosynthetically active radiation (PAR) available above herbaceous vegetation compared with the soil surface (PAR quantum sensor, Skye Instruments Ltd., Powys, UK) were recorded

at random locations within each site. To quantify shading from shrubs and trees, five hemispherical (fish-eye) photos were taken using a Pentax K-S2 camera with a Sigma 4.5 mm F2.8 EX DC circular fish-eye HSM lens. Data on total, diffuse, and direct radiation transmitted through the crown canopy and canopy openness were calculated using a Gap Light Analyser (GLA) 2.0, Version 2.0 (Frazer et al., 1999).

In the period between the beginning of May and the end of September 2023, soil moisture and temperature probes (TMS-4, TOMST, Praha, Czech Republic) were installed at each site. The probe recorded soil volumetric moisture down to 10 cm depth, soil temperature at 6 cm depth, soil surface temperature, and air temperature at 15 cm above the surface every 15 min. The probes were placed within 30 × 30 × 33 cm metal mesh cages (mesh size 2.5 cm) to protect them from damage by grazers. The vegetation inside meshes was cut at 2 cm height at the end of September to estimate above-ground vegetation biomass. At grazed sites, an additional cut was performed at the beginning of August to simulate grazing, and the biomass collected in August and September was summed.

At the beginning of August, vegetation surveys were performed. Four plots of 25 × 25 cm were placed randomly within each site, and species richness and the number of *B. media* shoots were recorded. In addition, four plots of 2 × 2 m were randomly placed within each site, and the number of flowering *B. media* shoots within a plot was recorded.

2.6 | Statistical analyses

2.6.1 | Analysis of site characteristics

Linear mixed-effects models were used to test for the effect of land use (abandoned versus grazed) on each site characteristic with replicated measurements per site (light conditions, soil properties, vegetation characteristics). Site nested within the site pair was included in the models as random factors to account for the spatial pairing of sites. We used principal component analysis (PCA) to identify the main axes of variation in environmental factors across sites, which were subsequently used to explain variation in trait means and trait diversity between sites (see below). PCA included mean values of measured properties per site as well as coefficients of variation (CV) for properties with multiple measurements per site (either spatial replicates or temporal replicates for soil moisture and soil and air temperature) to describe environmental heterogeneity within sites. Soil phosphorus (P) and nitrogen (N) content, CV in soil P content and soil bulk density, the density of *B. media* individuals and litter mass were ln-transformed to achieve normal distributions, and all variables were scaled prior to PCA. Among groups of highly correlated variables, only one variable was included in the PCA: mean soil surface temperature and its variability were included to represent highly correlated temperature dynamics at 6 cm soil depth and 15 cm above soil

surface; vegetation height and its variability were excluded as these were highly correlated with other estimates of light availability; tree canopy openness was included to represent highly correlated estimates of direct and diffuse solar radiation transmission through the canopy; and only sand % was included in the analysis to represent soil texture.

2.6.2 | Analysis of shifts in trait means in response to land use and other environmental factors

To test how land use affected plant traits across the studied sites, linear mixed-effects models were used with land use type (grazed or abandoned) included as a fixed factor and site nested within the site pair included as random factors. Site and/or pair factors were omitted from models if they did not describe detectable variation in the data (random effect variance at or close to zero). The models were applied to each of the six measured traits as a response variable (plant height, LDMC, SLA, RD, RTD, SRL) and two datasets: (a) traits measured in the field (i.e. trait differences between sites reflect the net effect of phenotypic plasticity and heritable differences between populations), (b) traits measured on clonal offspring after propagation in common garden conditions for 8 months (i.e. trait differences reflect heritable differences between populations).

In addition to incorporating land use type as a categorical predictor of trait shifts, we also fitted linear mixed-effects models with the two first PCA axes of site characteristics as predictors of traits across populations. We also fitted models using productivity (above-ground biomass produced during a growth season; not strongly related to the PCA axes of site characteristics) as a predictor of traits, since productivity is known to be a major driver of vegetation assembly (Fraser et al., 2015; He et al., 2023). Site nested within site pair were included in the models as random factors. These models were applied to trait values measured in the field and under common conditions after 8 months of clonal propagation. These models were performed because the binary classification of grazed and abandoned sites probably simplifies several environmental gradients elicited by variation in land use (e.g. variation in grazing intensity or the extent of tree encroachment), with different grazed and abandoned sites potentially being positioned at various points along these gradients. Such variation could be better captured by using PCA axes of site characteristics as continuous predictors instead of the categorical land use factor and therefore provide a more accurate representation of how land use affects plant traits.

To assess how well the measured site characteristics captured environmental variation that mediates the effect of land use on trait expression, we built two models—one including the two PCA axes and productivity as predictors and the other model including an additional categorical factor of land use type—and compared model fit using AIC values and a likelihood ratio test. If the addition of land use type significantly improved model fit, we could conclude that some additional unmeasured environmental factors mediated the effect of land use on trait expression.

2.6.3 | Analysis of shifts in trait diversity in response to land use and other environmental factors

In addition to shifts in trait means, land use can affect genetic and phenotypic variation within populations. Multivariate trait volumes were calculated using Gaussian kernel density estimation as implemented in the R package *hypervolume* (Blonder et al., 2024), using six traits measured in the field (reflecting trait variation caused by a combination of genetic variation and phenotypic plasticity to environmental heterogeneity) and under common conditions following 8 months of propagation (reflecting heritable variation only). For each of the field and common garden trait datasets, PCA was used to reduce the dimensionality of scaled trait data (i.e. to account for correlations between measured traits) prior to *hypervolume* calculation. In each case, four PCA axes were retained, reflecting the number of axes expected to summarise plant form and function (plant height, LDMC and SLA representing the leaf economic spectrum, RTD representing the root economic spectrum, and SRL and RD representing the mycorrhizal collaboration spectrum; Carmona et al., 2021; Weigelt et al., 2021). Linear mixed-effects models were constructed to test the effects of land use and the first two PCA axes of site characteristics, as well as individual site characteristics (productivity, abiotic and vegetation properties), on trait volumes under field and common garden conditions. Site pair was included in the models as a random factor.

All data processing and analyses were conducted using R version 4.3.3 (R Core Team, 2024). For linear mixed models, we used the *lmer* function from the 'lme4' package version 1.1–35.3. The significance of fixed effects was assessed using F-tests (Anova function in the package 'car' version 3.1–2) (Bates et al., 2015; Fox & Weisberg, 2019). Additionally, the proportion of trait variation explained (R^2) by fixed and random effects in the models was assessed using the 'performance' package version 0.11.0 (Lüdtke et al., 2021). The normality of model residuals and the homogeneity of variances across groups were assessed using Q-Q plots and Levene's tests. Figures were generated using the package 'ggplot2' version 3.5.1 (Wickham, 2016).

3 | RESULTS

3.1 | Environmental variation across sites

PCA identified two main axes of variation in site characteristics, explaining 44.5% of total variation. PC1 aligned with differences in land use: grazed sites were characterised by higher tree and shrub canopy openness, lower shading by herbaceous vegetation, higher mean air temperature, lower litter accumulation and lower total soil P than abandoned sites (Figure 3, Table S2). Grazed sites also exhibited higher within-site variability in bulk density, soil surface temperature and soil moisture but lower variability in tree and shrub canopy openness, compared with abandoned sites. Species richness and the density of vegetative and flowering *B. media* individuals were higher

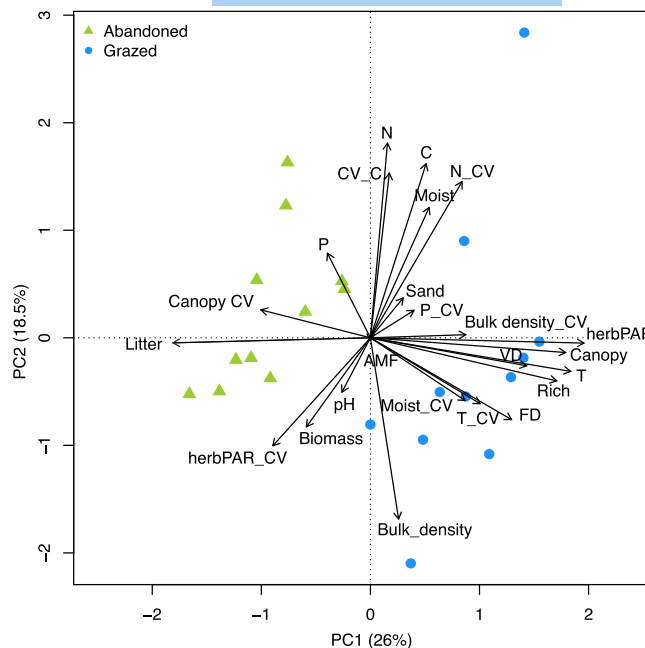


FIGURE 3 PCA plot of site characteristics. Abandoned sites are indicated with green triangles and grazed sites with blue round symbols. CV—coefficient of variation. Soil properties: AMF—mean root colonisation by arbuscular mycorrhizal fungi; Bulk_density—mean soil bulk density, g/cm^3 ; C—mean soil total carbon, %; Moist—mean volumetric soil moisture, %; N—mean soil total nitrogen, %; P—mean soil total phosphorus, $\mu\text{g}/\text{g}$ dry soil; pH—mean soil pH; T—mean air temperature at 2 cm from soil surface; Sand—mean soil sand content, %. Vegetation properties: Biomass—above-ground productivity of herbaceous vegetation, g; Canopy—mean tree and shrub canopy openness, %; FD—mean density of flowering *B. media* individuals in 2×2 m plots; herbPAR—mean proportion of photosynthetically active radiation below compared with above the herbaceous vegetation; Litter—litter dry mass, g; Rich—mean plant species richness in 0.25×0.25 m plots; VD—mean density of vegetative *B. media* shoots in 0.25×0.25 m plots.

in grazed than abandoned sites (Table S2). PC2 reflected other soil conditions, including soil C, N, moisture, and bulk density, that were not related to land management (Figure 3). Meanwhile, soil pH, root AM fungal colonisation and sand content were not strongly related to either PC1 or PC2 (Figure 3). Primary productivity of herbaceous vegetation was positively correlated with variation in shading by herbaceous vegetation, that is, more productive sites exhibited more variable light conditions ($r=0.74$, $p<0.001$), and neither variable was strongly related to PC1 or PC2 (Figure 3).

3.2 | Shifts in trait means in response to land use

Under field conditions, plant individuals from abandoned sites were significantly taller and had lower RTD than individuals from grazed sites ($F_{1,10}=91.5$, $p<0.001$, Figure 4a and $F_{1,20}=7.2$, $p=0.014$, Figure S1, respectively). Non-significant trends towards lower LDMC and higher SLA in abandoned than grazed sites were detected in the field ($F_{1,20}=2.9$, $p=0.107$ and $F_{1,10}=2.3$, $p=0.159$,

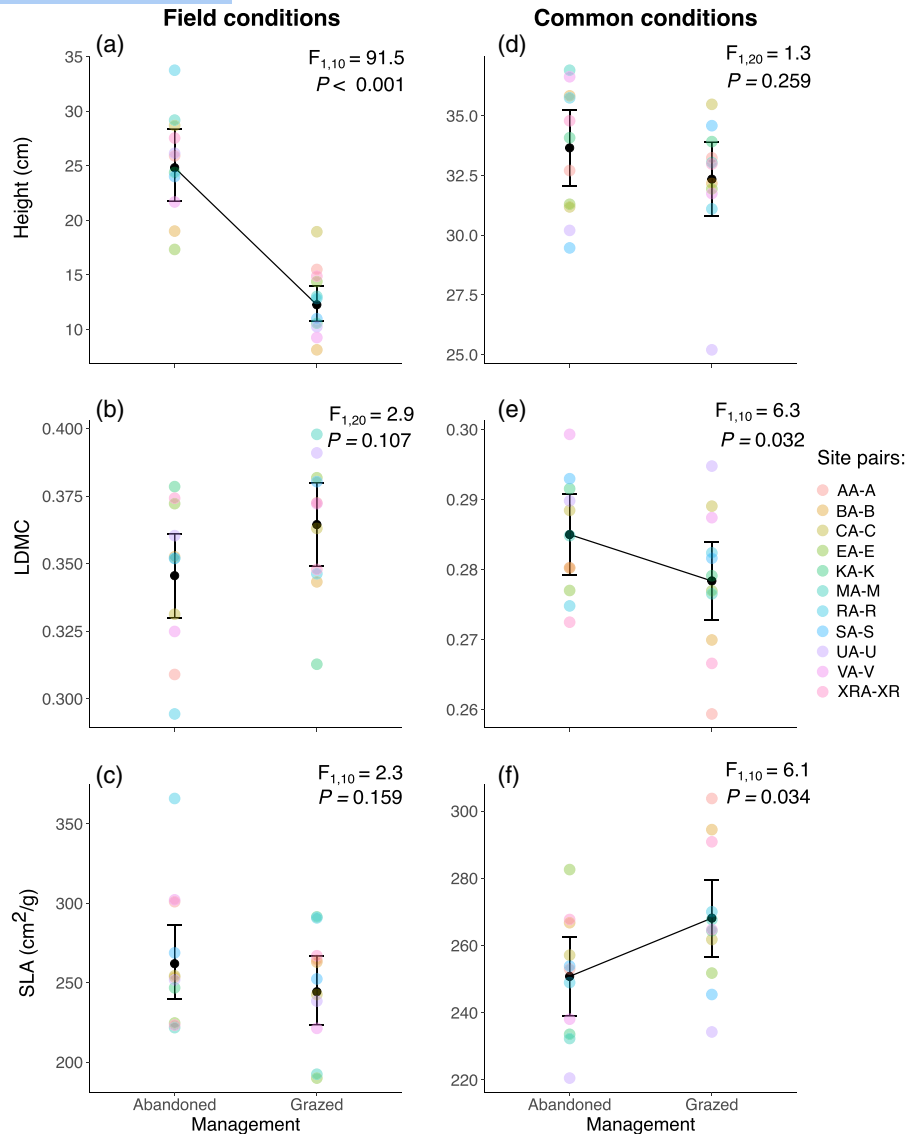


FIGURE 4 Differences in plant functional traits between populations originating from abandoned and grazed sites in the field (a–c) and after 8 months of propagation under common conditions (d–f). LDMC—leaf dry matter content; SLA—specific leaf area, cm^2/g . Site abbreviations correspond to those presented in the map in Figure 1 and Table S1. Each coloured point represents an average trait value per population. Predicted means and their 95% confidence intervals from linear mixed models based on measurements of 20 genotypes per population, and population nested within a site pair as random factors, are shown in black. Solid lines indicate significant relationships ($p < 0.05$).

respectively; Figure 4, Table S3). When clonal offspring of each genotype were propagated under common conditions, populations from different land use regimes no longer differed significantly in plant height and RTD ($F_{1,10} = 1.3$, $p = 0.259$, Figure 4b and $F_{1,10} = 0.1$, $p = 0.736$, respectively; Figures S1 and S2). However, LDMC was significantly higher and SLA significantly lower in abandoned than grazed populations grown under common conditions ($F_{1,10} = 6.3$, $p = 0.032$ and $F_{1,10} = 6.1$, $p = 0.034$, respectively; Figure 4).

When the two first PCA axes of site characteristics were used as predictors of trait values across populations, we found that under field conditions LDMC and RTD exhibited significant positive relationships, and height and SLA negative correlations, with PC1, indicating that LDMC and RTD were significantly lower, and plant height

and SLA were higher, under conditions characteristic of abandoned than grazed sites (Table S4, Figure S3a–d). LDMC and SLA were also significantly related to site primary productivity, with lower LDMC and higher SLA detected at sites with higher productivity (Table S4, Figure S3e–f). When plants were grown under common conditions, no significant relationships were detected between trait values and PC1 of site characteristics (Table S4), while RD and SRL were significantly related to PC2, such that populations from soils with higher moisture, total N, and total C were characterised by thinner roots (Figure S4, Table S4). RD measured under common conditions was also negatively related to the primary productivity of the origin site (Figure S4, Table S4). We found that including land use type as an additional categorical factor in the models described above did

not significantly improve model fit, indicating that environmental PCA axes generally captured the effect of land use type on traits (Table S5). The only exception was LDMC in common conditions, where the addition of land use type significantly improved model fit, suggesting a role of additional unmeasured factors ($\Delta\text{AIC}=2.9$, $p=0.027$; Table S5).

3.3 | Shifts in trait diversity in response to land use

The extent of multivariate trait variability within each population (expressed as trait volumes in multidimensional space defined by six measured traits) was not significantly related to land use type as a categorical variable (trait volume under field conditions $F_{1,20}=1.5$, $p=0.238$; under common conditions $F_{1,10}=2.6$, $p=0.137$; Table S6). However, population trait volumes in the field were significantly

negatively related to PC1 of site properties, indicating that conditions characteristic of grazed sites favoured less variable phenotypes than conditions in abandoned sites ($F_{1,20}=5.1$, $p=0.035$; Figure 5a). However, when using trait volumes measured under common conditions, a non-significant positive relationship was detected, suggesting that the heritable component of trait diversity was driven by other environmental factors ($F_{1,20}=2.6$, $p=0.124$; Figure 5c).

Divergent patterns in field and common conditions were also found for the relationship between trait volume and site productivity: trait volumes were higher in the field at more productive sites ($F_{1,20}=6.3$, $p=0.020$; Figure 5b), but no significant relationship was detected when trait volumes were measured under common conditions ($F_{1,20}=1.5$, $p=0.239$; Table S6, Figure 5d). Likewise, when considering relationships between trait volumes and individual site characteristics, we found significant relationships between population trait volume in the field and light transmission through

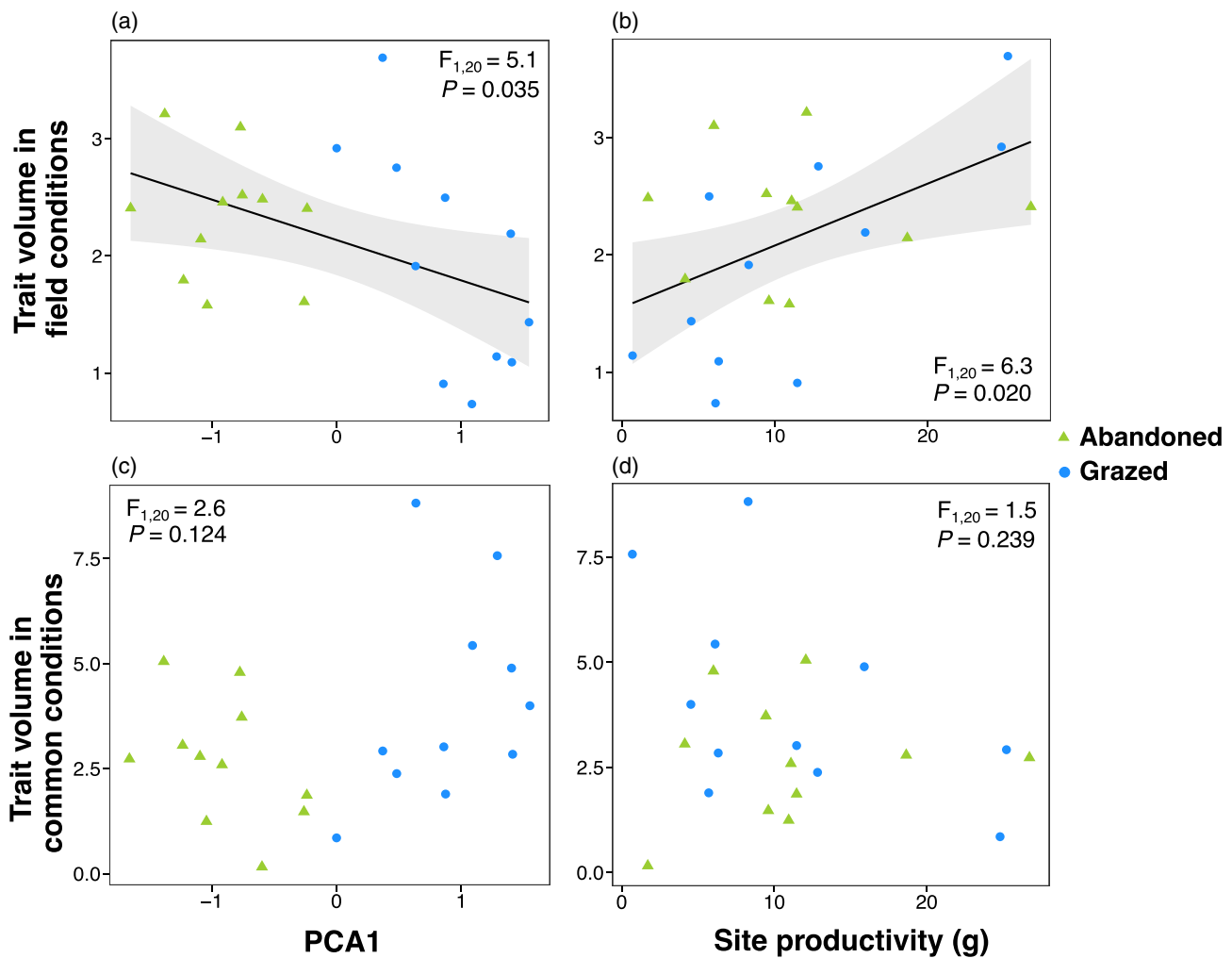


FIGURE 5 Relationships between site characteristics and multivariate trait volumes measured in the field (a–b) and under common growth conditions (c–d). Each point represents a population-level trait volume based on measurements of 20 genotypes. Abandoned sites are indicated with green triangles and grazed sites with blue circles. PCA1 is the first principal component of site characteristics and is strongly related to land management, with more positive values indicating grasslands with higher light availability, lower litter accumulation, and higher species richness and *B. media* population densities (see Figure 3). Solid lines indicate significant relationships ($p < 0.05$). Shaded areas show 95% confidence intervals of the fitted lines.

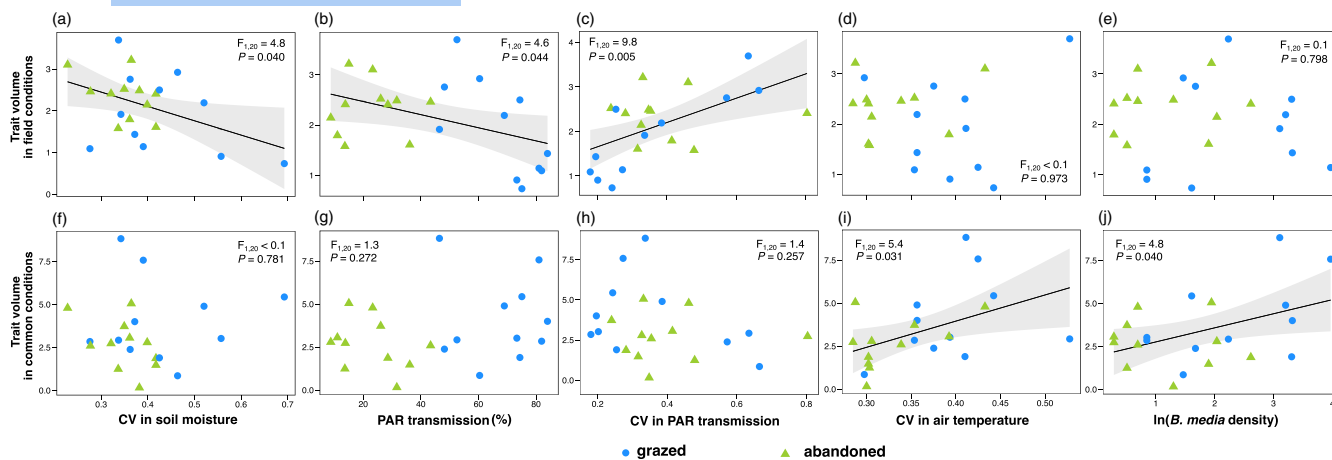


FIGURE 6 Relationships between individual site characteristics and multivariate trait volumes measured in the field (a–e) and under common growth conditions (f–j). Each point represents a population-level trait volume based on measurements of 20 genotypes. Abandoned sites are indicated with green triangles and grazed sites with blue circles. CV in soil moisture—coefficient of variation in soil moisture; PAR transmission—mean percent of photosynthetically active radiation transmitted through herbaceous vegetation; CV in PAR transmission—coefficient of variation in the proportion of photosynthetically active radiation transmitted through herbaceous vegetation (i.e. variation in herbaceous shading); CV in air temperature—coefficient of variation in air temperature at 2 cm above the soil surface; *B. media* density—mean density of flowering *B. media* individuals measured in 2 × 2 m plots. Solid lines indicate significant relationships ($p < 0.05$). Shaded areas show 95% confidence intervals of the fitted lines.

herbaceous vegetation ($F_{1,20} = 4.6$, $p = 0.044$), variation in light transmission ($F_{1,20} = 9.8$, $p = 0.005$), and variation in soil moisture ($F_{1,20} = 4.8$, $p = 0.040$). At the same time, these relationships were non-significant for trait volumes measured under common conditions (Figure 6, Table S7). By contrast, trait volumes measured under common conditions were significantly positively related to mean air temperature variation ($F_{1,20} = 5.4$, $p = 0.031$) and the density of flowering *B. media* individuals ($F_{1,20} = 4.8$, $p = 0.040$), while such relationships were absent for trait volumes measured under field conditions (Figure 6, Table S7).

4 | DISCUSSION

To assess whether trait responses to land use change are driven by phenotypic plasticity or heritable shifts reflecting evolutionary responses, we measured six functional traits in 22 populations of *Briza media* both under field conditions and after propagating clonal offspring of each genotype for 8 months under common conditions. We found that population-level differentiation in above-ground plant traits between grazed and abandoned sites was driven by both phenotypic plasticity in response to divergent environmental conditions in the field as well as by heritable trait shifts, which were detected after propagating all plant genotypes under controlled conditions. Importantly, population trait means shifted in opposing directions as a result of phenotypic plasticity and heritable changes. Plants responded to environmental changes associated with grazing abandonment in the field with changes in leaf traits that are known to increase competitive ability for light at the expense of tissue protection against stress. However, trait measurements after propagation under common conditions revealed opposing heritable

shifts towards higher levels of leaf tissue protection in populations where grazing had been abandoned. These findings indicate that diverging selective pressures may act on the plastic and heritable components of trait variation under land use change, which can be overlooked when traits are only measured under field conditions.

4.1 | Contribution of phenotypic plasticity and heritable trait shifts to population response to land use change

We found that grazing abandonment resulted in extensive changes in abiotic conditions and vegetation, consistent with findings reported elsewhere (Milberg et al., 2020; Pykälä et al., 2005; Ratajczak et al., 2012; Rosset et al., 2001). Long-term grazing cessation resulted in increased light limitation due to reduced tree canopy openness, a denser herbaceous layer, and litter accumulation. On the other hand, the absence of grazers was associated with lower variation in soil bulk density, higher soil P, and greater buffering against extreme temperatures. Grazed grasslands were characterised by higher plant species richness and denser *B. media* populations, suggesting that grazing was essential for maintaining diversity and ensuring favourable growth conditions for this grassland specialist plant (Busch & Reisch, 2016; Johansson et al., 2011; van der Meer et al., 2014).

We hypothesised that increased light limitation in abandoned grasslands, due to the absence of grazers and tree and shrub encroachment, should lead to trait shifts towards phenotypes that are more efficient in light capture either via enhanced vertical growth or shade tolerance. Indeed, we found that plants were considerably taller in abandoned than grazed sites under field conditions.

However, contrary to previous studies (Cid et al., 1989; Detling & Painter, 1983), no significant differences in plant height were detected between the grazed and abandoned populations after clonal propagation under common conditions, indicating that investment into vertical growth was entirely driven by phenotypic plasticity. This agrees with previous work demonstrating that plants possess high plasticity in leaf and stem elongation, which confers an advantage in competition for light (Gruntman et al., 2017; Schmitt et al., 1999).

We found that plants produced leaves with significantly lower LDMC and higher SLA in the field along the environmental gradient of tree shade and other correlated environmental conditions typically observed in abandoned sites (Figure S3). Such phenotypic changes are in agreement with previous studies demonstrating the role of high SLA and low LDMC in enhancing light capture and carbon gain per unit of biomass in light-limited conditions (Reich et al., 2003; Rozendaal et al., 2006). However, populations from abandoned grasslands showed heritable shifts in SLA and LDMC in the opposing direction to patterns observed in response to field conditions—genotypes from abandoned sites were characterised by lower SLA and higher LDMC than genotypes from grazed sites when grown under common conditions, a trait shift indicative of higher tissue protection and longer leaf lifespan (Reich, 2014; Weigelt et al., 2021; Wright et al., 2004). Therefore, land use abandonment resulted in evolutionary change towards more conservative phenotypes and higher levels of leaf tissue protection, which was masked in the field by plastic responses in the opposite direction, a phenomenon known as counter-gradient variation (Albecker et al., 2022; Villellas et al., 2021).

Counter-gradient variation has typically been described along latitudinal or elevational gradients where compensatory mechanisms have evolved to counteract the negative effect of stressful conditions (Albecker et al., 2022; Valdés et al., 2019). For example, it has been demonstrated that plants respond to cold temperatures with suppressed growth rates and delayed flowering as plastic responses, but populations adapt to cold climates by genetic shifts towards higher investment into seed production and earlier flowering (Caignard et al., 2021; Valdés et al., 2019). However, in many cases, the underlying causes of counter-gradient variation remain unknown (Gorton et al., 2018; Madaj et al., 2023; Villellas et al., 2021). Changes in the measured environmental parameters explained the increase in SLA and decrease in LDMC in response to grazing abandonment under field conditions but not the heritable trait shifts in the opposing direction. These may have been driven by additional selective pressures that were not captured in our study. We propose that while frequent defoliation by grazers selects for less protected but fast-growing leaves, the absence of large herbivores extends the potential leaf lifespan and selects for greater protection of tissues against pathogens and insect herbivores. Therefore, it may be that shading primarily triggers phenotypic plasticity towards better shade tolerance, while the absence of grazers pushes the phenotype in the opposing direction and acts on the heritable component of trait variation. Reciprocal transplant experiments and surveys of variation in leaf lifespan and natural enemy pressure across sites

with different land use would be required to test this hypothesis. As we only measured heritable trait differences in a single set of common conditions, additional measurements are needed to test trait expression in multiple settings, potentially simulating different aspects of the environment in grazed and abandoned sites.

We failed to detect any significant differences in below-ground traits between grazed and abandoned grasslands, except for a non-heritable shift towards lower RTD in abandoned sites. Root mycorrhizal colonisation also did not consistently differ between grazed and abandoned sites, which aligns with the variable effects of grazing on mycorrhizal interactions described in other studies (Faghihinia et al., 2020; van der Heyde et al., 2017). Instead, RD and SRL showed heritable differentiation between sites with different soil nutrient levels and productivity, suggesting that the direct impact of soil conditions on root traits is stronger than the indirect impact of changes in light conditions due to grazing abandonment.

4.2 | Response of population-level trait diversity to land use change

We predicted that functional trait diversity within populations would be lower in abandoned than grazed sites due to smaller population sizes and, hence, lower genetic diversity (Busch & Reisch, 2016; Helm et al., 2009; Prentice et al., 2006). When we quantified trait diversity by calculating multivariate trait volumes for each population, we found that within-population trait diversity was not significantly influenced by land use type as a categorical factor but by environmental variables that differed between grazed and abandoned sites, including light availability, temperature, and moisture conditions as well as the population density of flowering individuals. Similarly, to patterns observed for population trait means, we found different relationships between environmental factors and within-population trait variability depending on whether traits were quantified in the field or under controlled conditions. Functional trait diversity under field conditions was higher for populations experiencing conditions typical of abandoned and more productive sites, but these relationships were in the opposite direction or absent for genetically based trait diversity measured under common garden conditions.

Increased trait diversity in productive and abandoned sites under field conditions could be explained by higher variation in light conditions, as such sites were characterised by high spatial variation in shading by herbaceous vegetation and variation in tree canopy openness, respectively. While land use abandonment in general can lead to landscape homogenisation (Rey Benayas et al., 2007), grazing abandonment can temporarily provide a more heterogeneous local environment, as some areas become shaded by trees while others remain open. Indeed, grazing abandonment can result in transient increases in adaptive genetic variation in grassland populations (Träger et al., 2021 preprint). However, as these relationships were no longer detectable for the component of trait diversity attributable to underlying genetic variation, more

heterogeneous light conditions seemed to only cause trait variation via phenotypic plasticity. Instead, the highest genetically based trait diversity was detected in grazed populations, particularly those characterised by high densities of flowering individuals. Such a relationship was not detectable when densities of non-flowering individuals were considered. Although additional studies are needed to confirm links between functional trait diversity and population genetic diversity, sites with large populations of flowering individuals are likely to host the highest genetic variation due to efficient cross-pollination (Aavik et al., 2009; Leimu et al., 2006). Hence, our study indicates that relationships between habitat characteristics and functional trait diversity arising from genetic variation only become evident when traits are measured under common conditions, which removes the masking effect of phenotypic plasticity. While phenotypic variation generated by plasticity is an important mechanism by which plants adjust to short-term environmental variation (Callaway et al., 2003; Nicotra et al., 2010), heritable trait variation is critical for the long-term population survival and adaptive potential, particularly under conditions of current climate change and increasing frequency of extreme weather events (Pearman et al., 2024). Therefore, the restoration and conservation of grassland habitats should aim to preserve and boost genetically based variation in plant traits related to stress tolerance, which cannot be quantified with field surveys but requires assessment under common garden conditions.

5 | CONCLUSIONS AND FUTURE DIRECTIONS

Our findings suggest that grazing abandonment may impose divergent selective pressures on populations of a grassland specialist plant species, leading to counter-gradient variation. In particular, heritable trait shifts towards more protected leaves in response to the absence of grazers, which were detected under common conditions, were masked in the field by a plastic response to shading, which triggered investment into more efficient light capture. Similarly, divergent patterns in functional trait diversity were observed, with trait diversity detected from field observations primarily reflecting phenotypic plasticity to small-scale environmental heterogeneity, while heritable trait diversity measured under controlled conditions was related to the population density of flowering individuals. Our findings underscore the complex effects of land use abandonment on plant genetic resources, which have important implications for grassland restoration and conservation. Experimental evidence clearly shows that higher genotypic diversity and trait variability are important for ecosystem functioning and stability (Cook-Patton et al., 2011; Prieto et al., 2015; Reusch et al., 2005). Our study demonstrates that the assessment of functional trait composition and diversity in plant populations should not solely rely on field-based trait observations but should be measured in common garden conditions to remove the masking effects of phenotypic plasticity. Future research and ecosystem

monitoring should ideally combine the assessment of plant functional traits reflecting plant resource acquisition and conservation with novel sequencing techniques for quantifying adaptive genetic variation (Heuertz et al., 2023; Kardos et al., 2021) to better understand the links between the phenotypic traits relevant for wider ecosystem functions and the genomic markers used in population biology.

AUTHOR CONTRIBUTIONS

Anastasia Tõnisson: Conceptualisation, planning, fieldwork, genotype propagation, data collection, statistical analysis, writing—original draft, writing—review and editing. **Jenalle Eck:** Data collection, writing—review and editing. **Yuying Jing:** Fieldwork, data collection, writing—review and editing. **Piia Kängsep:** Data collection, writing—review and editing. **Lauri Laaspere:** Data collection, writing—review and editing. **Jianlu Wu:** Data collection, writing—review and editing. **Laura Puura:** Fieldwork, data collection, writing—review and editing. **Mari Torsus:** Data collection, writing—review and editing. **Kadri Koorem:** Conceptualisation, planning, fieldwork, data collection, writing—review and editing. **Mari Moora:** Conceptualisation, planning, writing—review and editing. **Siim-Kaarel Sepp:** fieldwork, data collection, writing—review and editing. **John Davison:** Fieldwork, statistical analysis, writing—original draft, writing—review and editing. **Marina Semchenko:** Conceptualisation, planning, funding acquisition, fieldwork, genotype propagation, data collection, statistical analysis, writing—original draft, writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

Marina Semchenko is an Associate Editor of the *Journal of Ecology*, but took no part in the peer review and decision-making processes for this paper.

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.70238>.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.wstqjq30w> (Tõnisson et al., 2025).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Site information.

Table S2. Results of linear mixed models testing the effect of land use (abandoned versus grazed) on site characteristics.

Table S3. Results of linear mixed model testing the effect of land use on plant functional traits under field and common garden conditions.

Table S4. Results of linear mixed models testing the effects of site productivity and PCA axes 1 and 2, which respectively reflect major axes of variation in site conditions, on plant functional traits under field conditions and after 8 months of propagation under common growth conditions.

Table S5. Comparison of linear mixed-effects models predicting trait volumes under field and common conditions.

Table S6. Results of linear mixed models testing the effects of land use, site productivity, and PCA axes 1 and 2, which respectively

reflect major axes of variation in site conditions, on multivariate trait volumes under field conditions and after 8 months of propagation under common growth conditions.

Table S7. Relationships between different site characteristics and multivariate trait volumes measured in the field and after 8 months of propagation under common growth conditions.

Figure S1. Differences in root traits between populations originating from abandoned and grazed sites in the field (a–c) and after 8 months of propagation under common conditions (d–f).

Figure S2. Plant functional trait data for each population.

Figure S3. Relationships between plant functional traits measured in field conditions and the first principal component of site characteristics (a–d), which is strongly related to land use, and site productivity (e–f).

Figure S4. Relationships between plant functional traits measured

under common growth conditions and the first two principal component axes of site characteristics and productivity of the sites where populations originated from.

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