Adaptation to contrasting habitats underlies distinct plastic responses to environmental variation in two closely related *Senecio* species

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Abstract

Phenotypic plasticity can maintain population fitness in novel or changing environments if it allows the phenotype to track the new trait optimum. Understanding how adaptation to contrasting environments determines plastic responses can identify how plasticity evolves, and its potential to be adaptive in response to environmental change. We sampled 79 genotypes from populations of two closely related but ecologically divergent ragwort species (*Senecio*, Asteraceae), and transplanted multiple clones of each genotype into four field sites along an elevational gradient representing each species' native range, the edge of their range, and conditions outside their native range. At each transplant site, we quantified differences in survival, growth, leaf morphology, chlorophyll fluorescence and gene expression for both species. Overall, the two species differed in their sensitivity to the elevational gradient. As evidence of plasticity, leaf morphology changed across the elevational gradient, with changes occurring in opposite directions for the two species. Differential gene expression across the four field sites also revealed that the genetic pathways underlying plastic responses were highly distinct in the two species. Despite the two species having diverged recently, adaptation to contrasting habitats has resulted in the evolution of distinct sensitivities to environmental variation, underlain by distinct forms of plasticity.

Keywords: adaptation, differential gene expression, environmental sensitivity, evolutionary history, genotype-by-environment interactions, phenotypic plasticity, physiological plasticity, specialisation

Introduction

The resilience of natural populations and communities to environmental change relies on their ability to adjust their phenotype to track changes in the environment (Chevin et al. 2010). This can occur via adaptive evolutionary responses across generations (Bell and Gonzalez 2009), and/or by plasticity within a generation where a given genotype generates different phenotypes depending on the environment to which it is exposed (Via et al. 1995; Ghalambor et al. 2007; Charmantier et al. 2008). Both mechanisms (plasticity and evolutionary change) are part of the response of populations to environmental change (Baythavong and Stanton 2010; Chevin et al. 2013). Where genetic variation in plastic responses is high, selection on plastic responses could help to increase the resilience of natural populations in the face of environmental change (Nussey et al. 2005). If we are to understand the potential for plasticity to help maintain the resilience of natural populations in response to environmental change, we need to first understand how adaptation shapes plasticity. We also need to quantify the phenotypic change induced by plasticity when genotypes from natural populations experience novel conditions, and whether there is genetic variation for such plasticity.

The effect of adaptation on the nature and magnitude of plastic responses will depend on how plasticity and selection interact, and the predictability of the environment (de Jong 2005). Phylogeny (Pigliucci et al. 1999; Kellermann et al. 2018), ecology (Kulkarni et al. 2011) and highly predictable seasonality (Oostra et al. 2018) have been shown to determine plastic responses. However, we do not know to what extent shared evolutionary history constrains plastic responses in different species, or whether plasticity is determined by specialisation to a given environment. It is predicted that more ecologically specialised species should be less phenotypically plastic and struggle to respond to environmental change (Lortie and Aarssen 1996; Debat and David 2001; Dal Santo et al. 2018). For example, plants adapted to higher elevations can show reduced plasticity due to specialisation to their particular environment (Schmid et al. 2017). By quantifying how plasticity varies among closely related but ecological divergent species, we can better understand how adaptation to contrasting habitats shapes plasticity, with important consequences for understanding how species can respond to novel environmental conditions in the future.

Genetic variation in plasticity can promote the rapid evolution of plasticity through selection on genotypes that vary in their level of plasticity (Lande 2009; Chevin and Lande 2011). When genotypes vary in their response to the environment, they exhibit genotype-by-environment interactions (G×E) that underlie plastic responses (Pigliucci 2005; Josephs 2018). Plasticity is expected to be maintained within specific parameters when the environment is predictable, leading to adaptive plasticity within the environmental limits experienced during adaptation (Bradshaw 1965; Schlichting 1986; Baythavong and Stanton 2010). Whether such plasticity will continue to be adaptive when exposed to novel conditions, such as those imposed by

climate change, remains an empirical issue (Ghalambor et al. 2007). Strong stabilising selection created by predictable environments is expected to lead to specific plastic responses and reduce genetic variation for plasticity (Oostra et al. 2018). By contrast, populations adapted to a wider range of habitats that are more spatially and temporally variable are predicted to maintain genetic variation in plastic responses, increasing the potential for selection on plasticity (Chevin et al. 2010). Detecting and characterising patterns of G×E for a range of naturally occurring genotypes can help us understand whether evolutionary responses can occur even if plasticity is constrained in certain directions (Via 1993; Chevin and Hoffmann 2017).

The genetic architecture underlying variation in plasticity is largely unknown (Fusco and Minelli 2010). Plastic responses at the gene expression level are most likely controlled either by epiallelic control of the genes themselves or allelic variation in the regulators of the genes (Rockman and Kruglyak 2006). If allelic (sequence changes) or epiallelic (e.g. DNA methylation, chromatin remodelling, post-transcriptional modifications) variation underlying the traits become fixed during adaptation, constraints to plasticity may arise (Gibson and Wagner 2000; Shaw et al. 2014; Oostra et al. 2018). For example, at the level of a given genotype, homogeneous or predictable environments should lead to stable epiallelic controls that are resistant to resetting, which can induce a loss of plasticity within a generation (Herman et al. 2014). At a population level, predictable environments will reduce genetic variation in plasticity through purifying selection acting either on the genetic regulators (e.g. transcription factors) or long-term epiallelic changes, such as transgenerational DNA methylation (Colicchio et al. 2015; Oostra et al. 2018). Canalisation and specialisation can therefore reduce plasticity in the traits involved in adaptation to any given environment.

In this study, we quantify variation in environmental sensitivity in two closely related species of ragwort that are adapted to contrasting habitats located at different elevations on Mt. Etna, Sicily. *Senecio chrysanthemifolius* (hereafter, *S.ch*) is an annual/short-lived perennial with dissected leaves that occupies disturbed habitats (e.g., abandoned land and roadsides), as well as gardens, vineyards and fruit orchards on the foothills of Mt. Etna c. 400-1,000m a.s.l (above sea level) (**Fig. 1a**), and more broadly, Sicily. By contrast, *S. aethnensis* (hereafter, *S.ae*) has a perennial life history and entire leaves and is endemic to lava flows c. 2,000-2,600m a.s.l on Mt. Etna that are covered by snow each winter (**Fig. 1b**). These two species diverged relatively recently (Chapman et al. 2013), with an estimate of c.150,000 years before present (Osborne et al. 2013) that corresponds to the approximate time of the uplift of Mt. Etna, which created the new high altitude environment to which *S.ae* is adapted (Chapman et al. 2013). The recent shared ancestry of the two species is reflected by very low genetic divergence, despite large differences in habitat, phenotype and life history (Chapman et al. 2016).

A) *S. chrysanthemifolius* **B)** *S. aethnensis*

Fig. 1 *Senecio chrysanthemifolius* occupies disturbed habitats below c.1,000m a.s.l, and has thin, dissected leaves. *Senecio aethnensis* inhabits lava flows and has thicker, smooth-margined leaves with a thick waxy cuticle.

Given *S.ae* exists in small populations endemic to high elevations on Mt. Etna, while *S.ch* is found in a variety of lower-elevation habitats across Sicily, we predicted that *S. ae* would show higher specialisation in its plasticity, associated with: (a) lower tolerances to conditions outside its home range, and (b) lower levels of plasticity that prevent *S.ae* responding positively to environmental variation. To test this, we sampled c.40 genotypes of each species from several natural populations. We then reciprocally transplanted multiple cuttings of each genotype to four transplant sites (6-15 cuttings/individual/transplant sites) across an elevational range that included the home range of each species (500m for *S.ch* and 2,000m for *S.ae*), and two intermediate elevations (1,000m and 1,500m). We found some support for our predictions, with both species suffering at elevational extremes outside their range, but this was associated with similar levels of plasticity in both species. However, the direction of plasticity differed greatly, with species-specific patterns observed at the phenotypic, physiological and gene expression levels.

Materials and methods

Sampling natural populations

We sampled fruits (achenes), hereafter referred to as 'seeds' as they are functionally equivalent, and took cuttings from individuals in natural populations of both species after plants started flowering (May-June 2017 for *S.ch*, and July 2017 for *S.ae*). The difference in timing was because *S.ae* develops more slowly and flowers later than *S.ch* due to it occupying a high-elevation habitat. We only sampled from plants that were large enough to take material for more than 30 cuttings. For *S.ch*, we sampled from 88 individuals at five sites, each a geographically separated patch of individuals representing potentially discrete populations (Table S1). For *S.ae*, we sampled from 87 individuals at four different elevations (2,600m, 2,500m, 2,400m and 2,300m a.s.l [above sea level]) on both the North and South slopes of Mt. Etna (Table S1). Where possible, we avoided sampling plants less than 10m apart to minimise the risk of sampling close relatives, but this was difficult for *S.ch* because patches of individuals were very small (<30 individuals in a 100m radius).

Physiological differences between species

To identify physiological differences between species under common garden conditions, we grew plants from seeds in a growth cabinet with controlled conditions: 350µmol m^{-2} s^{-1} light intensity (photosynthetic photon flux density), 25/20°C±3°C day/night temperature, 65-70% humidity, 14/10h photoperiod and 400µmol mol^{-1} ambient CO_2 concentration. Seeds were germinated using mechanical scarification, and seedlings transplanted into 70 mm square pots with standard potting mix. From eight maternal families of *S.ch* we grew 24 individuals, and from 10 maternal families of *S.ae* we grew 30 individuals. Seedlings were grown for two months and physiological measurements taken. With a Dualex+® instrument (ForceA, France), we measured leaf pigment content for concentrations of chlorophyll, anthocyanins, flavanols and estimated the nitrogen balance index. Using an LCpro (ADC BioScientific, UK), we measured photosynthetic gas exchange. Intrinsic water use efficiency (iWUE) was calculated as a ratio between photosynthesis and stomatal conductance. To measure chlorophyll fluorescence, we used an IMAGING-PAM M-series chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). With the output of the fluorometer, we quantified two mechanisms of physiological light defence. We quantified the unregulated dissipation of heat as the quantum yield of non-regulated energy dissipation, calculated as $Y(NO) = \frac{1}{NPQ+1+qL(\frac{F_m}{F_o}-1)}$, where qL is the parameter representing photochemical quenching, and $NPQ = \frac{F_m - F_m'}{F_m'}$, where F_m and F_m' represent the maximal fluorescence in a dark and light adapted state, respectively (Kramer et al. 2004). To quantify the regulated dissipation of heat, we calculated the quantum yield of regulated energy dissipation as $Y(NPQ) = 1 - Y(II) - Y(NO)$ (Kramer et al. 2004).

Field transplant experiment

In the glasshouse, cuttings (i.e., clones) from all individuals sampled from natural populations (hereafter, genotypes) were cut into 5cm stem segments, each possessing 2-3 leaf nodes. Each smaller cutting was then dipped in rooting plant growth regulator for softwood cuttings (Germon® Bew., Der. NAA 0.5%, L. Gobbi, Italy) and placed in a compressed mix of coconut coir and perlite (1:1) in one cell of an 84-cell tray. All cuttings from each genotype were kept together in one half of a tray, with tray positions randomised regularly to randomise environmental or positional effects. Trays were kept moist and checked regularly for cuttings that successfully produced roots (roots extending out of the bottom of tray). For each genotype, rooted cuttings were randomised into experimental blocks and transplanted at four field sites. From the initial genotypes, we transplanted 37 *S.ch* genotypes and 42 *S.ae* genotypes that produced enough cuttings with roots.

Field transplant sites were located at four elevations (500m, 1,000m, 1,500m and 2,000m a.s.l) along a transect on the south-eastern side of Mt. Etna. The 500m site was located in a garden among fruit trees and grape vines, the 1,000m site on an abandoned vineyard among oak trees (*Quercus ilex*), the 1,500m site among an apple and pear orchard, and the 2,000m site surrounded by pine trees on a lava flow from 1983. Soil is characterised as a silty sand at elevations between 500m and 1,500m, but changes to volcanic sand at 2,000m. At each transplant environment we deployed four data loggers (Tinytag Plus, Gemini Data Loggers, UK) at each site, which measured temperature hourly. We also took three soil samples for each transplant site, which were analysed for 36 variables that included nutrients, salts and ions (Nucleo Chimico Mediterraneo laboratories, Catania, Italy). To analyse the soil data, we used Multi-Dimensional Scaling (MDS) to calculate the scaled distance between replicate soil samples taken at all transplant sites.

Genotypes were replicated at each transplant site by transplanting multiple cuttings from each genotype into three identical experimental blocks. The position of cuttings was randomised with respect to genotype, and transplanted into 20×7 grids, with cuttings separated from each other by 40cm (*S.ch* block n=109; site n = 327; total N = 1,308; *S.ae* block n = 130; site n = 390; total N = 1,560). Depending on the number of cuttings that successfully produced roots, we transplanted 6-15 cuttings per genotype at each transplant site (exact numbers presented in Table S1). Cuttings of *S.ch* were transplanted in June-July 2017, whereas cuttings of *S.ae* were transplanted (into separate experimental blocks) at the start of August 2017. The difference in timing was because we were restricted to sampling from natural populations of *S.ae* much later than *S.ch*. Following the transplant, cuttings were watered daily for three weeks to encourage establishment. To prevent death during high temperatures in July-August (consistently greater than 35ºC), we watered cuttings daily during this period, which allowed assessments of phenotypic responses to what were still stressful conditions.

Characterising morphology

We recorded mortality approximately every two weeks and measured phenotypic traits of all plants at a single time point (November 2017). To characterise leaf morphology, we sampled and pressed 3-5 young but fully expanded leaves from each cutting (five and four months after transplant for *S.ch* and *S.ae*, respectively). Leaves were scanned and morphology quantified using the program Lamina (Bylesjo et al. 2008), which generates estimates of leaf area, perimeter, the number of indentations, and the average width and depth of each indentation. To estimate the density of indentations along the leaf margin, we standardised the number of indentations by the perimeter. To capture leaf complexity we calculated perimeter^2/area^2, where lower numbers indicate fewer indentations, i.e. more entire leaves.

Chlorophyll fluorescence

To quantify photosynthetic capacity for both species across the elevational gradient, at each transplant site we measured chlorophyll *a* fluorescence for four leaves on each of three clones from five genotypes of each species. We took measurements at two transplant sites each day within one week in October 2017. For a temporal replicate, we measured the same clones at the same site on a second day. To take measurements, we put leaf clips on four leaves of each plant and dark-adapted the plants for 30 minutes by covering them with large black plastic containers. We then took fluorescence induction curve measurements for 2 seconds at 3,500µmol s^{-1}m^{-2} photosynthetic photon flux density from each leaf (clip) using a Handy PEA instrument (Hansatech Instruments Ltd., UK). The raw experimental data was processed by Handy PEA software and then by HandyBarley software. Using the JIP test (Tsimilli-Michael and Strasser 2013), we calculated the total performance of photosystem I and II as: $PI_{total} = \frac{F_V}{F_M} \times \frac{V_J}{M_0} \times \frac{F_V}{F_0} \times \frac{1-V_J}{V_J} \times \frac{1-V_I}{V_I-V_J}$, where F_0 is the minimal fluorescence intensity, F_M the maximal fluorescence, F_V the maximal variable fluorescence ($F_V = F_M - F_0$), M_0 the approximated initial slope of fluorescence change (normalised on F_V), and V_J and V_I the relative variable fluorescence levels recorded at 2ms and 30ms, respectively (also normalised on F_V).

Statistical analyses of plasticity

To quantify morphological plasticity across the four transplant sites we first estimated the mean for all leaf traits for a given cutting at a given transplant site. We standardised the morphological data to a mean of zero and standard deviation of one, and then used a Principal Components Analysis (PCA) with five leaf morphology traits. The first two principal components described 88.7% of the total variation. We used these two PC axes for analyses of plasticity in leaf morphology described below.

To compare differences in growth, survival, leaf morphology and chlorophyll fluorescence across transplant sites and for both species, we used linear mixed models in R v.3.6.1 (R Core Team 2019) within the package

'*lme4*' (Bates et al. 2015),

$$y_{ijklm} = T_i + S_j + T_i \times S_j + T_i \times G_{k(j)} + B_{l(i)} + e_{m(ijkl)} \ . \qquad (1)$$

Separate implementations of equation 1 were used for different variables of interest, each of which was included as the univariate response variable (y_{ijklm}). Changes in the response variable across transplant sites were modelled by the *j*th species (S_j) in the *i*th transplant site (T_i) and their interaction ($T_i \times S_j$), which were all included as fixed effects. Random effects included the interaction between transplant site and genotype $T_i \times G_{k(j)}$ (individuals sampled in the natural populations), and experimental block within each environment ($B_{l(i)}$). The residual error variance was captured by $e_{m(ijkl)}$.

Equation 1 was implemented as a generalised linear mixed model with a binomial error distribution for survival (after summer 2017 and after winter 2018). The remaining phenotypic traits were normally distributed (plant height, leaf area, specific leaf area, principal components of leaf morphology and chlorophyll fluorescence), for which we used a linear mixed model with a Gaussian error distribution. For each implementation we tested the significance of the interaction between transplant site and species using likelihood ratio tests. To test whether differences in morphology between transplant sites were significant for each species, we used the R package '*emmeans*' (Lenth 2019) to conduct pairwise t-tests adjusted for multiple comparisons.

To test for significant differences in G×E within each species, we applied equation 1 separately for each species. We tested the significance of transplant site (the only fixed effect) using the Kenwood-Roger test in the R package '*pbkrtest*' (Højsgaard 2017). To separate the effect of genotype from G×E, we included genotype and the genotype×elevation interaction as separate random effects. We tested the significance of all random effects using likelihood ratio tests. To identify whether G×E was created by changes in the magnitude of among-genotype variance across elevation, or by differences in reaction norms (i.e., a change in rank of genotypes across elevation), we used the parameters estimated from equation 1 on PC1 and PC2 to calculate

$$\sigma^2_{G\times E} = \frac{\sum_{i=1}^{h}\sum_{j=1}^{h}\left[2\sigma_i\sigma_j(1-r_{ij})+(\sigma_i-\sigma_j)^2\right]}{h(h-1)}, \qquad (2)$$

where σ represents square root of the variance among genotypes for the *i*th and *j*th transplant sites. The number of sites is represented by *h*, for which we only compared the elevational extremes (500m and 2,000m). The first half of the equation represents G×E as differences in reaction norms, with r_{ij} representing the genotypic correlation between the *i*th and *j*th habitats. The second half of the equation represents G×E as

changes in the magnitude of among genotype variance (Cockerham 1963; Johnson 2007; Friedman et al. 2019).

Changes in gene expression: Sampling of plant tissue and RNA extraction

To quantify gene expression, we sampled young leaves from all cuttings at a single specific time-point following the initial transplant. More specifically, after transplanted cuttings showed sufficient growth (12-15 new, fully expanded leaves also associated with the emergence of branches), we collected 2-3 young leaves from all plants (July 2017 for *S.ch*; October 2017 for *S.ae*), which we stored in RNAlater at -20ºC. Three genotypes of each species were selected at random, and three clones sampled at each transplant site (36 samples per species). We extracted RNA from each sample using QIAgen RNeasy kits with β-mercaptoethanol added to the extraction buffer. Library preparation and RNA sequencing was performed at the Oxford Genomics Centre on an Illumina Hiseq4000 platform, producing 75bp paired-end reads.

Transcriptome assembly

The quality of raw reads was assessed in FastQC v0.11.4 and low quality bases and adaptors trimmed using TrimGalore v0.6. (Phred quality cut-off score = 20). For each species, trimmed reads from all samples were combined and a reference transcriptome was *de novo* assembled using Trinity v2.8.4 (Haas et al. 2013). Each transcriptome was filtered using the EvidentialGene (http://arthropods.eugenes.org/EvidentialGene/) and contaminants were removed using the MCSC Decontamination pipeline (Lafond-Lapalme et al. 2017), with the filter set to Viridiplantae. Orthologous transcripts between the two species were identified using Orthofinder v2.3.3 with default parameters (Emms and Kelly 2019). The two transcriptomes were filtered to contain only orthologous transcripts between the two species. The final orthologous transcriptomes each contained 23,622 transcripts with an N50 of 1509 and 1515 for *S.ae* and *S.ch*, respectively. Annotation of the transcriptomes resulted in 7579 unique GO terms for 14701 transcripts (mean of 7.2 GO terms/transcript).

Differential expression

Trimmed reads were mapped to each species reference transcriptome using Salmon v0.13.1 (index kMer length = 29, --gcBias) (Patro et al. 2017). Transcript abundance estimates were imported into R using the txImport pipeline. Read counts were normalised by transcript size, library size and filtered based on counts > 5 across half of all samples. Differential expression was estimated in DESeq2 (Love et al. 2014) according to

$$Counts \sim Genotype + Transplant\ Site + Genotype \times Transplant\ Site\ . \qquad (3)$$

Each treatment was compared with the home transplant site of each species (2000m for *S.ae* and 500m for *S.ch*), with differentially expressed genes determined based on an adjusted p-value < 0.01 (Benjamini and Hochberg 1995) and a log fold change > 2 for overexpression or < -2 for underexpression. Log fold changes

were shrunk using the '*apeglm*' method and were used to rank genes based on high overexpression and underexpression.

To compare the magnitude of transcriptional responses between transplant sites, genotypes and their interactions, we estimated an average log2-fold change of all genes as a response to each contrast of each factor (Love et al. 2014). Significant differences in this response for each transcript were compared using a two-sided Wilcoxon signed-rank test.

Annotation and functional enrichment

Each transcriptome was annotated following the Trinotate pipeline (Bryant et al. 2017). Nucleotide sequences were used to perform a blastx search against the Uniprot database, selecting in each case the single best hit (< 1e-5). Predicted amino-acid sequences were generated using TransDecoder v5.5 (https://transdecoder.github.io) and protein sequences were blasted against the Uniprot database. Protein sequences were also used to search the Pfam database for conserved protein motifs using HMMER (http://hmmer.org). Finally, protein sequences were used to search for signalling proteins using Signalp (Armenteros et al. 2019).

To test for significant representation of functional categories among differentially expressed genes, Gene Ontology enrichment analyses were performed using topGO v2.3.6 (Alexa and Rahnenfuhrer 2019). Enrichment was determined using genes that were significantly differentially expressed (adjusted p values < 0.01) between the native transplant site and the furthest transplant site and Kolmogarov-Smirnoff (KS) test using the 'weight' algorithm.

Results

Physiological differences between species under laboratory conditions

Under common garden conditions *S.ch* and *S.ae* showed large differences in plant physiology, reflecting strong habitat differences. The maximum quantum yield of photosystem II (F_v/F_m), a specific indicator of photoinhibition, did not differ between two species (data not shown). However, compared to *S.ch*, *S.ae* exhibited significantly greater values for the quantum yield of non-regulated energy dissipation [Y(NO)] (**Fig. 2a**; t=2.351, P=0.0217), which indicates that both photochemical energy conversion and protective regulatory mechanisms are less efficient in *S.ae*. Higher values of Y(NO), and similar values of Y(NPQ) for the two species (data not shown), reflect suboptimal capacity of photoprotective reactions, which could lead to greater photodamage for *S.ae*. *S.ch* showed evidence of higher intrinsic water use efficiency than *S.ae* (**Fig. 2b**; t=3.875, P=0.0002), suggesting that *S.ch* leaves possess traits allowing better adaptation to

drought than *S.ae*. We found evidence that the two species differed in the concentration of leaf pigments in the cuticle of their leaves. *S.ae* showed greater leaf cuticle concentrations of chlorophyll (**Fig. 2c**; t=2.085, P=0.0388) and flavanols (**Fig. 2d**; t=4.399, P<0.0001).

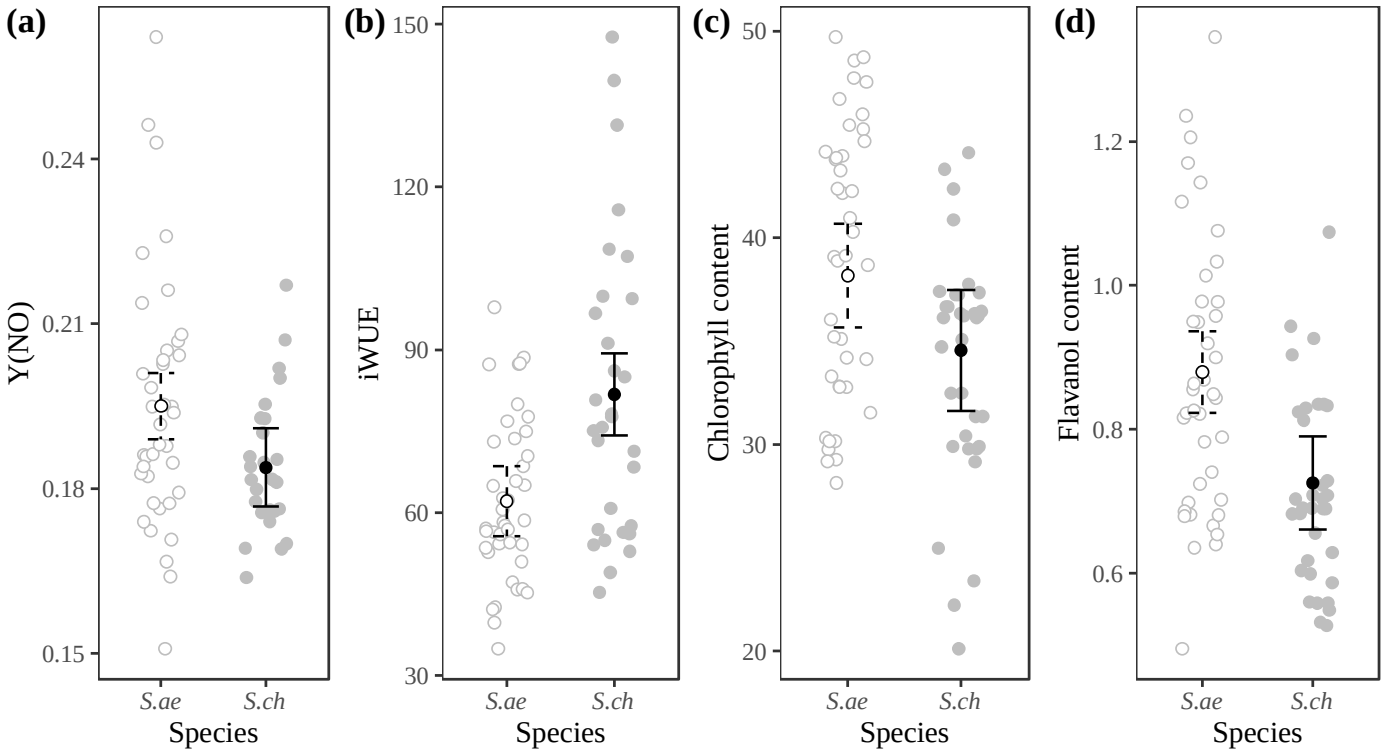

Fig. 2 Physiological differences between species grown from seeds under common garden conditions in the laboratory. Filled circles and solid lines represent *S.ch*, while unfilled circles and dashed lines represent *S.ae*. Gray circles represent individual plants measured and credible intervals represent the 95% confidence intervals. (**a)** *S.ae* exhibited greater values of Y(NO), suggesting it will be more prone to photodamage. (**b)** *S.ch* showed higher intrinsic water use efficiency, while *S.ae* showed higher leaf chlorophyll content **(c)** and a higher flavanol content **(d)**.

Transplant survival, growth and flowering

Temperature data loggers at the transplant sites revealed contrasting climatic conditions associated with elevation variation, with extreme heat (regularly exceeding 40ºC) at 500m and 1,000m during summer, and extreme cold (regularly below 0ºC) at 1,500m and 2,000m during winter (**Fig. 3a**). Soil profiles separated the four transplant sites in a linear fashion along the first axis (MDS1), which represented a gradual change in soil type and reduction in nutrients (amount of organic material, total nitrogen, cation exchange capacity and exchangeable ions) at higher elevations (**Fig. 3b**). The second axis (MDS2) described differences between the 1,000m site and the other sites, associated with greater concentrations of various salts (soluble nitrates, calcium and magnesium).

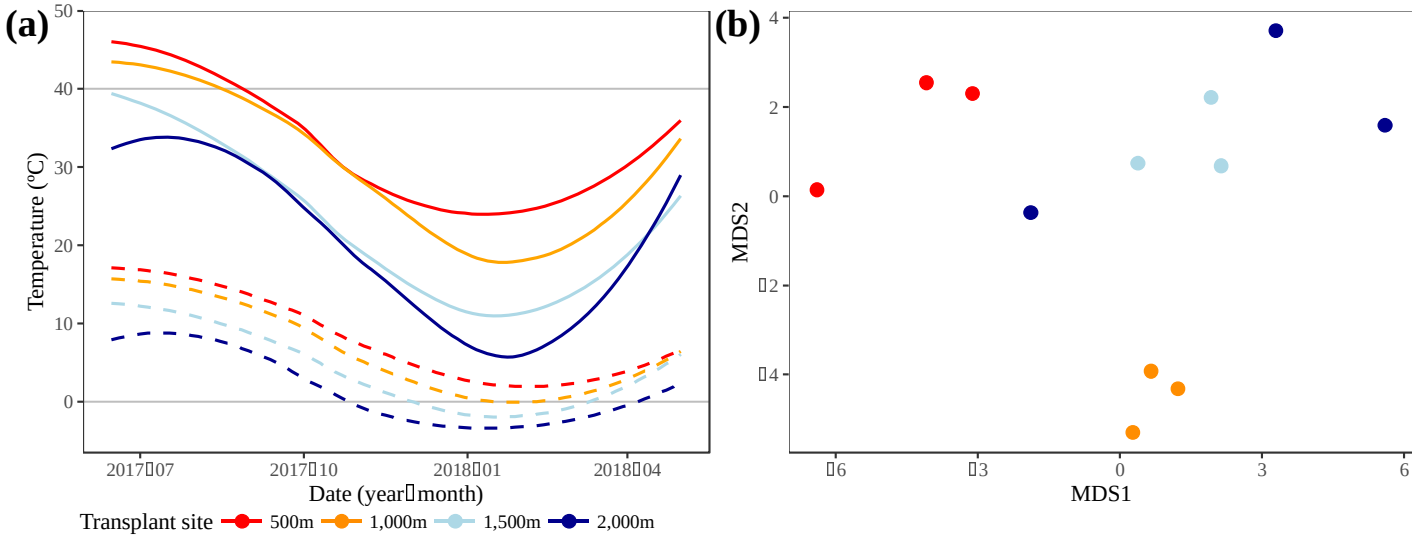

Fig. 3 Differences in environment for the four transplant sites at four elevations. **(a)** Average daily maximum (solid lines) and minimum (dashed lines) for three data loggers at each site, for the duration of the transplant. Gray shading represents the standard error for estimating the coefficients. Higher elevations remained below 40ºC in the summer and dropped well below zero in the winter. **(b)** Differences in soil composition for 35 soil variables captured by a multidimensional scaling analysis.

Transplanted cuttings of *S.ae* showed high mortality at low elevations over summer, but survived well at high elevations over winter (**Fig. 4a**). The *S.ae* plants that did survive at low elevations grew vigorously (**Fig. 4b**). By contrast, *S.ch* survived well at all elevations over summer, but suffered high mortality over winter at 2,000m (**Fig. 4a**). *S.ch* grew well at all elevations and showed a greater difference in growth across elevation than *S.ae* (**Fig. 4b**). To test whether the survival rates in this 2017 experiment were consistent across years, we conducted a similar transplant in 2018 by transplanting both species at the same time (total N = 984 cuttings) in spring (April) and providing less supplementary water. In the 2018 transplant experiment we found very similar patterns of survival. After summer, only 6% and 3% of *S.ae* plants remained at 500m and 1,000m, respectively, as compared to 79% and 39% for *S.ch* (**Table S2**), which suggested that the 2017 experiment represented typical patterns of mortality.

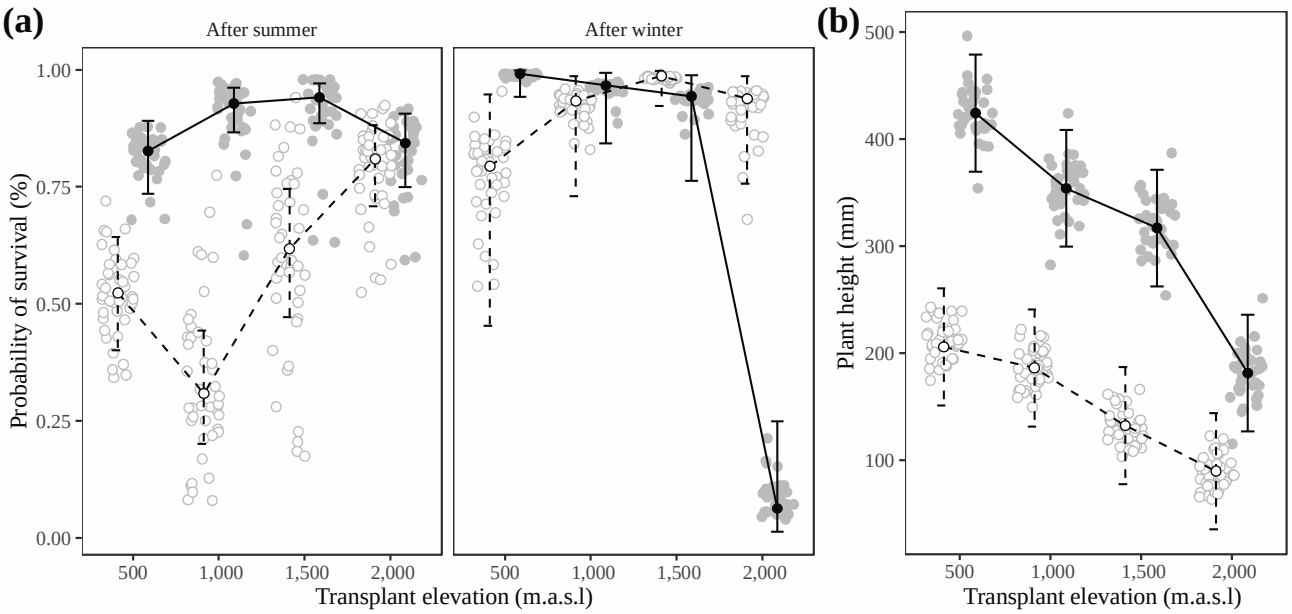

Fig. 4 Variation in survival, growth and flowering success of both species across all transplant sites. Filled circles and solid lines represent *S.ch*, while unfilled circles and dashed lines represent *S.ae*. Grey points represent the mean of all cuttings for each genotype sampled in the natural populations. Credible intervals represent 95% confidence intervals. (**a**) Survival after summer was high for *S.ch*, but low for *S.ae* away from its home site. Both species survived well after winter, except for *S.ch* at high elevation. (**b**) Plants grew larger at lower elevations, and *S.ch* grew taller overall.

Morphological and physiological plasticity

Despite low survival of *S.ae* at lower elevations, enough clones remained to measure almost all genotypes at each transplant site (only 2-3 *S.ae* genotypes missing at 500m, 1,000m and 1,500m). Plasticity in leaf morphology was estimated by quantifying changes in PC1 and PC2 across elevations. The first two principal components described 74.8% and 13.9% of total variation, respectively (**Fig. 5**).

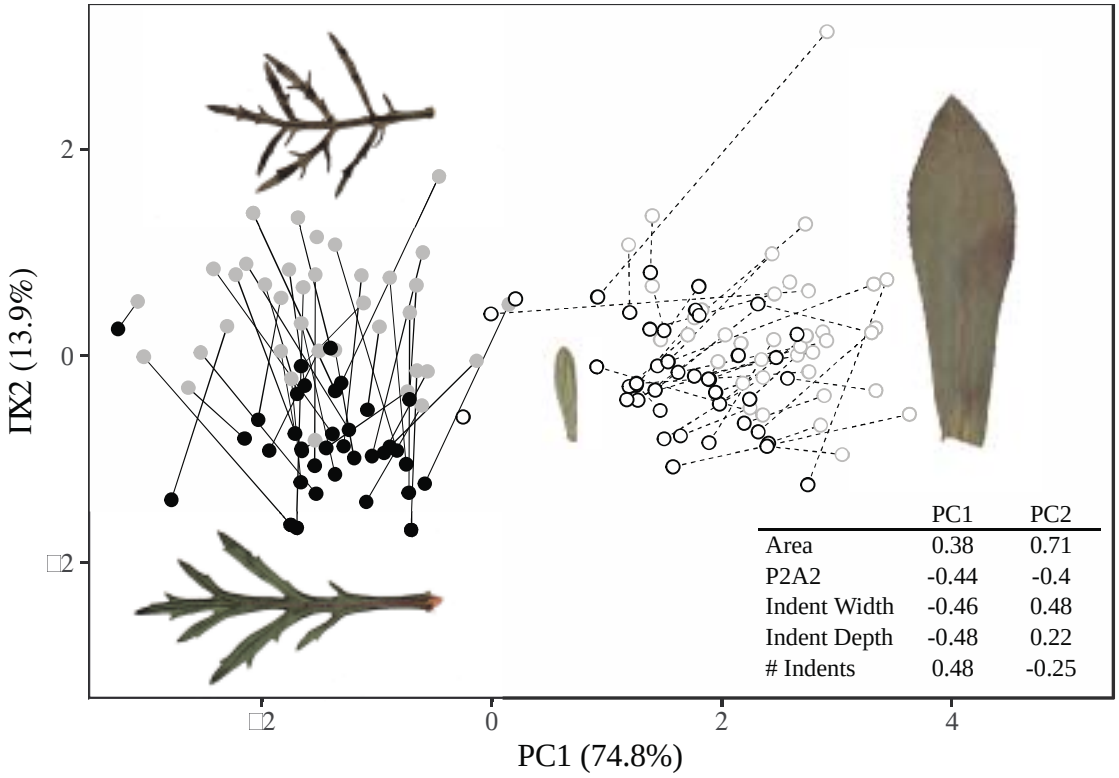

Fig. 5 Principal component analysis for leaf morphology of both species measured at the four transplant elevations, but with only the extreme (500m and 2,000m) elevations presented for simplicity. Filled circles and solid lines represent all genotypes of *S.ch*, and unfilled circles and dashed lines represent the *S.ae* genotypes. Gray circles represent morphology at 500m, with black circles representing morphology at 2,000m. Table inset shows the trait loadings for both PC axes. *S.ae* changes morphology between 500m and 2,000m for PC1, which also represents species differences. By contrast, *S.ch* changes morphology between 500m and 2,000m for PC2. Inset leaf images represent the extreme differences across elevation for PC1 (*S.ae*), and for PC2 (*S.ch*).

Both species showed morphological plasticity as changes in leaf morphology across the four transplant sites, but patterns differed between the two species. The first principal component described differences between species as well as phenotypic differences associated with elevation for *S.ae* (**Fig. 6a**; PC1 species×elevation χ^2 (3) = 28.83, P<0.0001). The second principal component described differences between the four transplant sites for *S.ch*, but not for *S.ae* (PC2 species×elevation χ^2 (3) = 7.33, P=0.0621). Only the highest and lowest elevation transplant sites were significantly different in leaf size for *S.ch*, whereas *S.ae* showed significant and consistent differences in leaf size across the range of transplant sites (**Fig. 6b**; species×elevation χ^2 (3) = 9.01, P=0.0290).

To test whether species differed in their leaf investment across elevation, we calculated specific leaf area (SLA; leaf area per unit leaf weight) and found a steep reduction in SLA as elevation increased for *S.ae*, but a shallower reduction for *S.ch* (**Fig. 6c**; species×elevation χ^2 (3) = 22.54, P<0.0001), suggesting *S.ae* changed leaf investment at lower elevations by producing lighter leaves for a given leaf size.

To estimate traits associated with physiological responses of these species, we measured chlorophyll fluorescence and calculated the total performance index (PI_{total}; total photosynthetic activity), which reflects the energy conservation capacity of the photosynthetic machinery. We found that *S.ch* showed no change in PI_{total} across elevation, while *S.ae* showed a steady decline, suggesting reduced photosynthetic activity of *S.ae* at lower elevations (**Fig. 6d**; species×elevation χ^2 (3) = 24.59, P<0.0001).

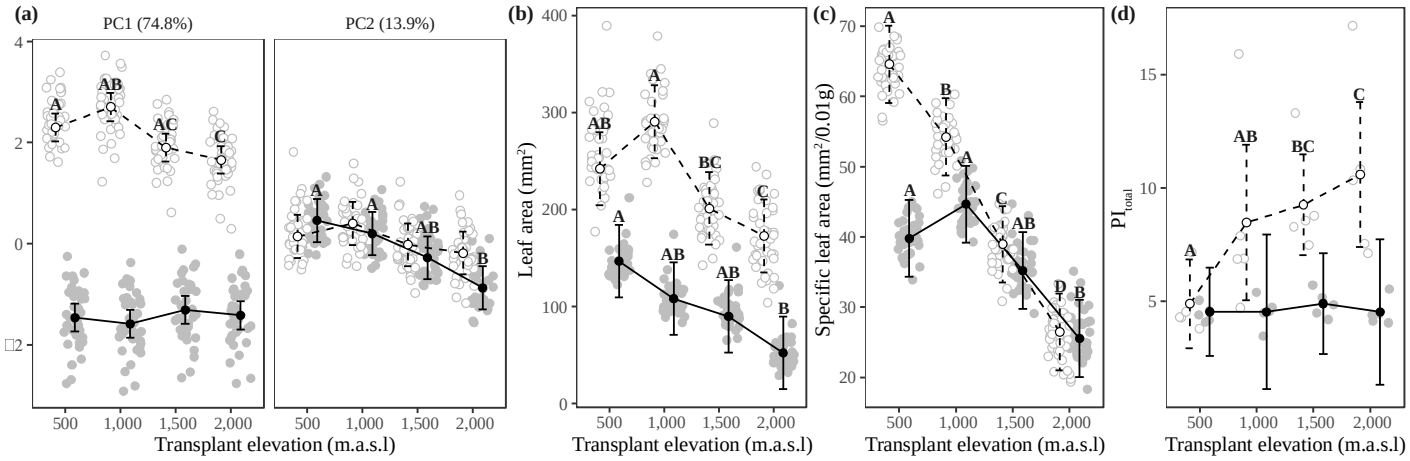

Fig. 6 Variation in leaf morphology, leaf size and leaf weight across transplant sites. Filled circles and solid lines represent *S.ch*, while unfilled circles and dashed lines represent *S.ae*. Points with credible intervals (95% confidence intervals) represent the mean for each species, with lines connecting the transplant sites. Letters denote significant differences between transplant sites tested using pairwise tests conducted within each species. Grey points represent the mean of all cuttings for each genotype, within species. (**a**) *S.ae* showed significant changes in multivariate leaf morphology across transplant sites for PC1, whereas *S.ch* showed significant changes only for PC2. (**b**) *S.ae* had larger leaves than *S.ch* at all sites and showed slightly increased plasticity in leaf size. (**c**) Leaves for both species increased in SLA towards lower elevations, and *S.ae* exhibited a greater increase than *S.ch*. (**d**) *S.ae* showed lower total photoysynthetic performance (PI_{total}) at lower elevations, while *S.ch* did not change.

Together, these results indicate statistically strong and contrasting patterns of morphological plasticity in these two species. *S.ae* exhibited reduced leaf investment with elevation, which was associated with reduced photosynthetic activity at lower elevations. By contrast, *S.ch* showed smaller reductions in leaf area and smaller changes in leaf investment across elevation, as well as lower and more consistent physiological activity.

To test whether genotypes responded differently across transplant sites within species, we tested for significant G×E underlying morphological plasticity. We found that both species exhibited significant differences among genotypes (**Table 1**), while significant G×E was only absent for PC2 in *S.ae*. Generally, this meant that genotypes varied greatly in their response to the four transplant sites, reflected by the high percentage of G×E attributed to changes in variance rather than differences among reaction norms (**Table 1**; **Fig. S1**).

Table 1: Testing for G×E in leaf morphology using mixed effects models. F-ratio is shown for the fixed-effect of elevation, and χ^2 statistics presented from the likelihood ratio tests for the remaining random effects of genotype, genotype×elevation and block.

						% of G×E change in:	
Species	PC	Elevation	Genotype	Genotype×Elevation	Block	Reaction norm	Variance
S.ae	PC1	**$F_{3,9.0}$ = 9.28; P=0.0041**	**χ^2(1) = 196.84; P<0.0001**	**χ^2(10) = 36.53; P<0.001**	**χ^2(1) = 46.68; P<0.001**	6.89	93.11
	PC2	$F_{3,8.5}$ = 2.84; P=0.1016	**χ^2(1) = 224.14; P<0.0001**	χ^2(10) = 14.14; P=0.1667	**χ^2(1) = 56.22; P<0.001**	4.79	95.21
S.ch	PC1	**$F_{3,10.8}$ = 4.18; P=0.0339**	**χ^2(1) = 672.14; P<0.0001**	**χ^2(10) = 62.049; P<0.001**	**χ^2(1) = 5.41; P=0.02**	10.08	89.92
	PC2	**$F_{3,8.4}$ = 6.67; P=0.0132**	**χ^2(1) = 304.91; P<0.0001**	**χ^2(10) = 49.35; P<0.001**	**χ^2(1) = 246.91; P<0.001**	7.6	92.4

Differential gene expression between transplant sites, genotypes and species

Patterns of gene expression within each species reflected differences between transplants sites and between genotypes (**Fig. S2**). In *S.ch*, the total number of differentially expressed genes between transplant sites increased with increasing elevation. By contrast, *S.ae* exhibited more differentially expressed genes as elevation decreased, with the greatest number of differentially expressed genes at 1,000m (**Fig. S3**). Overall, the number of differentially expressed genes was greater for *S.ae* (**Fig. S3**), which was reflected by stronger differences among the sampled genotypes, and greater numbers of differentially expressed genes showing G×E interactions (**Fig. 7a**). In *S.ae*, the average transcriptional change across all contrasts (between all genotypes or between all transplant sites) was 8% greater as a result of genotypic differences than the environmental response and 9% greater than the G×E interaction ($p < 0.001$, Z = -10.89; **Fig. 7b**). In *S.ch* we observed a contrasting pattern, with the average expression change resulting from G×E 6% and 8.5% greater than genotypic and environmental responses respectively ($p < 0.001$, Z = 25.04; **Fig. 7b**). These results suggest that a greater proportion of the transcriptome in *S.ae* showed transcriptional plasticity between genotypes (G×E) than for *S.ch*.

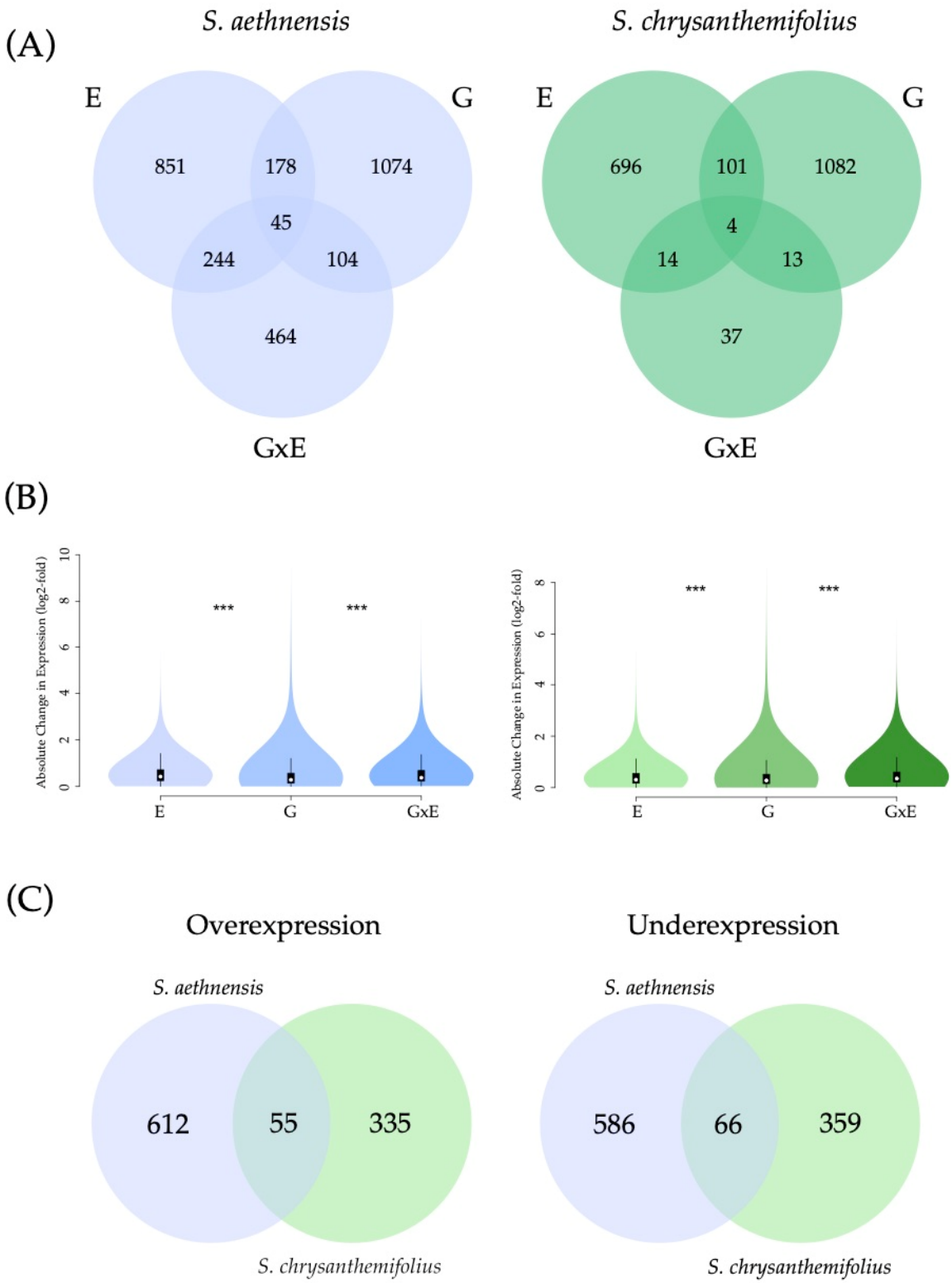

Fig. 7 Contrasting patterns of gene expression between species. (**a**) Total numbers of differentially expressed genes (-2 < lfc > 2) between genotypes (G), transplant sites (E) and interactions between genotype and environment (G×E). (**b**) Average expression changes in response to G, E and G×E. Asterisks denote significant differences (Wilcoxon signed-rank test, $p < 0.001$). (**c**) Overlapping overexpressed and underexpressed genes between the home and furthest transplant site in each species.

A comparison of the genes that were differentially expressed between the home site and the most novel

environment (i.e., elevational extremes) for each species indicated very little overlap between the two species, with just 5.5% and 6.5% of overexpressed and underexpressed genes shared between species (**Fig. 7c**). This suggests independent genetic pathways underlie plastic responses to elevation in these two species. We plotted the expression profile of the ten genes in each species with the largest change in overexpression and underexpression between 2,000m and 500m. In each case we observed a contrasting pattern between the two species, with strong overexpression or underexpression in one species but a relatively unchanged expression profile in the other species (**Fig. 8**).

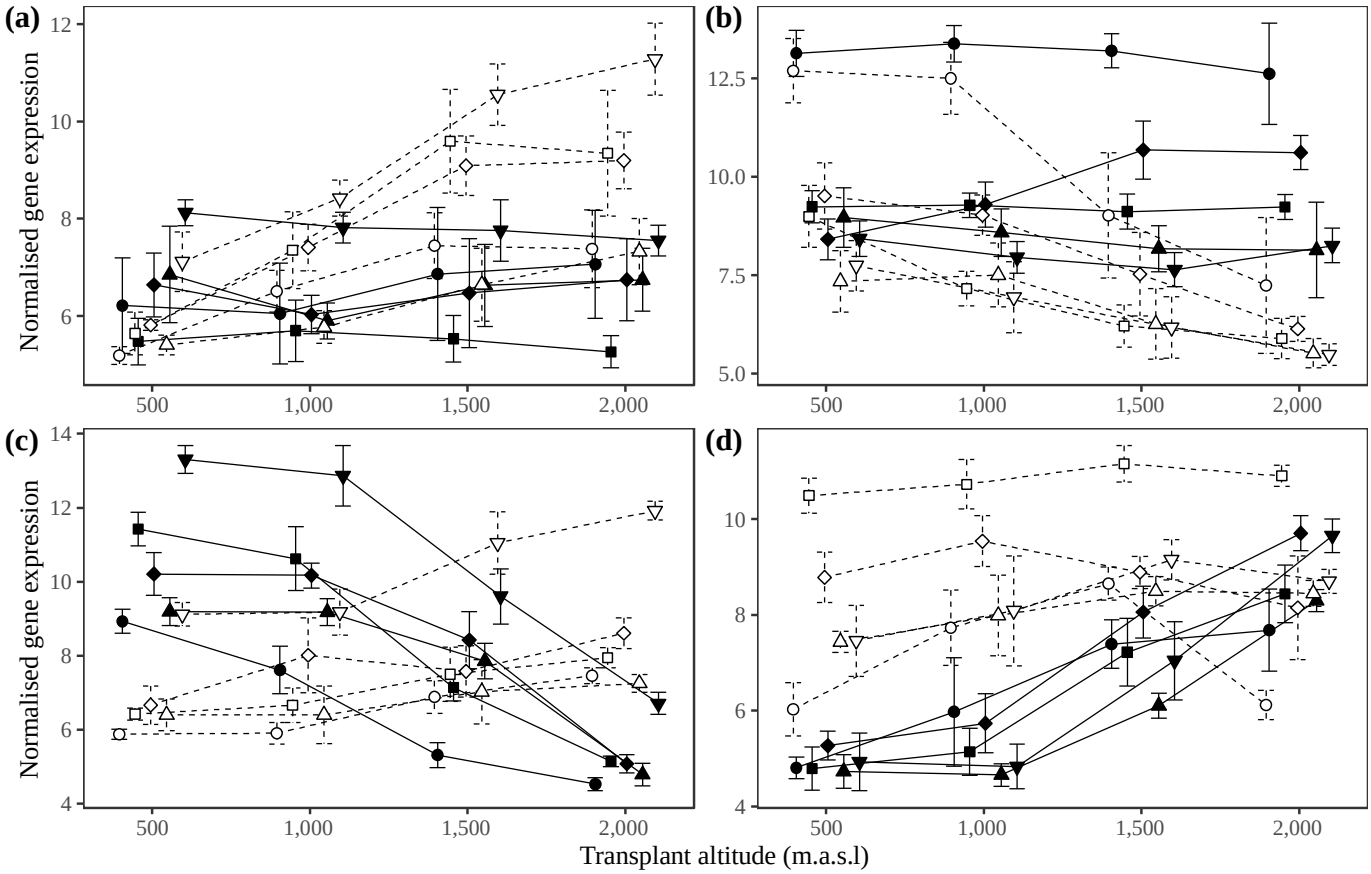

Fig. 8 Normalised expression profiles across all transplant sites for *S.ch* (solid lines and circles) compared to *S.ae* (dashed lines and unfilled circles). This includes the five genes (represented by different shapes) that are most strongly underexpressed in *S.ae* **(a)**, overexpressed in *S.ae* **(b)**, and the five genes most strongly underexpressed in *S.ch* **(c)** and overexpressed in *S.ch* **(d)**. Strong overexpression or underexpression in one species was reflected by little to no change in gene expression in the same gene for the other species.

Functional enrichment analyses of differentially expressed genes between the transplant sites representing their home versus novel environmental conditions, revealed 38 significant GO terms in *S.ch* and 30 in *S.ae*. Comparing the significant terms between species revealed that only four functional categories of genes were shared (GO:0006412 translation, GO:0009637 response to blue light, GO:0009768 photosynthesis and GO:0000028 ribosomal small subunit assembly; **Tables S3** and **S4**). These data also suggest that the

phenotypic response to elevational change in each species involves mostly different genetic pathways. In *S.ae*, GO terms indicated potential physiological changes to the leaf cuticle, including fatty acid biosynthesis, wax biosynthesis and cutin biosynthesis (**Table S3**). In *S.ch*, GO terms specifically involved responses to changing light conditions, including response to blue light, protein-chromophore linkage, light-harvesting in Photosystem I and response to high light intensity (**Table S4**).

Discussion

In this study, we used extensive field transplants of cuttings (clones) from 79 genotypes to quantify the survival, growth, and responses at the phenotypic and gene expression levels for two closely related but ecologically divergent species to an elevation gradient. We tested how these two species differed in plasticity, and characterised genotypic variation in plasticity within and outside each species' elevational range. We predicted that due to strong stabilising selection and a small geographic range, the more ecologically restricted species, *S. aethnensis* (*S.ae*), would perform more poorly (than *S.ch*) away from its home site, which would be reflected by reduced plastic responses overall and reduced genetic variation in plasticity. Conversely, we predicted that because *S. chrystanthemifolius* (*S.ch*) occupies a range of habitats with higher spatial and temporal environmental variation, *S.ch* would show greater plasticity and more genetic variation in plasticity, which would help this species to maintain performance across an elevational gradient.

In support of our predictions, we found that *S.ae* showed reduced survival (**Fig. 4a**) and photosynthetic activity (**Fig. 6d**) away from its home site after summer, while *S.ch* showed low but consistent photosynthetic activity across the elevational range, and only reduced performance after winter at the site furthest from its home range. Against our predictions, both species showed similar levels of plasticity in morphology across the elevational gradient, but morphological plasticity occurred in different phenotypic directions for the two species (**Fig. 5** and **Fig. 6a**). This suggests that following adaptation morphological plasticity might be restricted to certain areas of phenotypic space, which is different for populations adapting to contrasting habitats. Similarly, gene expression responses to the elevational gradient involved highly distinct genetic pathways for the two species (**Figs. 7-8**; for *S.ch*, genes underlying the sensing and response to light; for *S.ae*, the composition of the leaf cuticle). Surprisingly, in both species we identified genetic variation in plasticity as G×E underlying plastic responses (**Table 1**), but a stronger pattern of G×E in gene expression for *S.ae* compared to *S.ch* (**Fig. 7**). These results indicate that adaptation to their contrasting habitats has determined the gene expression and morphological pathways each species is able to access when exposed to

environmental variation.

Cuttings used for the field transplants in this study were from adult individuals in the wild, which suggests that because they successfully bypassed selection that removed other individuals from the population, they likely represent genotypes that are well adapted to their local environments. However, the plastic responses shown by cuttings from these genotypes, as well as representing only a subset of the genotypes available from seed produced in the natural populations, are likely to reflect many developmental decisions that have already been made by genotypes in earlier life that shape their responses to environmental variation (Morey and Reznick 2000; Weinig and Delph 2001). To better understand how populations of short-lived species can respond to environmental change, it will be important to compare patterns of plasticity for segregating genetic variation versus established genotypes or seeds produced in the wild, which will determine how plasticity emerges in natural populations, and how they may respond to environmental change.

Adaptation influences plastic responses

Our data suggest that *S.ae* and *S.ch* have specialised to contrasting habitats, which has created differences between the species in the level and direction of plastic responses to conditions outside their natural habitats, even though these species are very closely related (Taylor and Aarssen 1988; Emery et al. 1994; Ho and Zhang 2018). Where a previous study indicated that under controlled conditions there were minimal differences in the transcriptome of each species (Chapman et al. 2013), our field studies revealed that even within common gardens, each species has evolved a distinct transcriptional regime. These contrasting results are likely due to greater environmental heterogeneity under field conditions.

It has been demonstrated that variation in transcriptomic plasticity between species, i.e. greater changes in gene expression of key genes and networks, correlates with the ability to maintain fitness by matching the phenotype to environmental changes (Wellband and Heath 2017). While the extent of transcriptomic plasticity was similar between species, large changes in the expression of particular genes in one species was often contrasted by small or non-existent changes in the other species, suggesting stress outside their natural range results in a species-specific plastic response and inability to converge on similar expression profiles. Such contrasting responses for the two species suggest that adaptation to their local environment, rather than phylogenetic history, has a greater bearing on transcriptomic plasticity. Transcriptomic studies in other plant species have also reported that the local environment is a major determinant of transcriptomic changes when transplanted (Akman et al. 2016).

We identified hundreds of loci in each species showing a plastic response, and there was clear evidence for changes in a small number of functional suites of genes that were highly distinct between the two species. *S.ch* showed the greatest change in genes relating to photosynthesis, light response and circadian

rhythm, which are likely a response to the changing temperature and light intensity with increasing altitude (Beis and Patakas 2012). By contrast, the greatest changes observed in *S.ae* were associated with the plant cuticle, including the biosynthesis of cutin, waxes and fatty acids. Changes in the cuticle could reflect a response to various biotic and abiotic stressors at the lower altitude, such as pathogens and water loss (Serrano et al. 2014). This was reflected by consistent photosynthetic activity across all elevations in *S.ch*, suggesting a broad ecological range where plasticity could maintain growth and survival. By contrast, transplanting *S.ae* away from its home elevation resulted in high mortality, and rapid reductions in leaf allocation and photosynthetic activity. This supports our predictions that the species with the smallest ecological range will show higher specialisation, which would reduce its tolerance to environmental variation. Theory predicts that plants growing in harsh environments will show reduced plasticity because the cost of a mismatch with the environment is greater (Alpert and Simms 2002), and there is compelling evidence in other high-elevation plant species that specialisation does reduce plasticity in flowering time (Schmid et al. 2017) and morphology (Emery et al. 1994). A narrow sensitivity to environmental variation suggests that adaptation to the high elevation environment has resulted in a reduced ability for *S.ae* to respond positively to high temperatures, which may influence its persistence in response to climate change.

Understanding how plasticity evolves is important for understanding how species can respond to environmental variation (Bradshaw 1965; Baythavong and Stanton 2010). In our results, such distinct sensitivities of closely related species to the same environmental variation are important for predicting ecosystem-level responses to environmental change because they will likely lead to different responses. However, different taxa can evolve different plastic responses, even when they inhabit similar environments (Puijalon and Bornette 2004), suggesting that to better understand ecosystem-level responses to environmental change we need to first identify how plastic responses to environmental variation arise in different species, and at what spatial/temporal scale. Studies comparing plasticity among multiple species are not common, but suggest that the direction of plastic responses differs among species, and that such differences are likely important for species persisting in their particular habitat (Marshall et al. 1985; Huang et al. 2009). Fine-scale plasticity may be required for adapting to environments that impose strong stabilising selection (e.g., the alpine environment), which will likely hinder their ability to track large environmental variation (Emery et al. 1994; Baythavong 2011). Future studies should focus on understanding how plasticity arises, and identify whether such plasticity will be adaptive in response to future environmental change.

Genotype-by-environment interactions underlying plastic responses

In both *Senecio* species we found significant patterns of G×E interactions underlying plastic responses to the elevational gradient, which suggests a rapid evolutionary response of plasticity to shifts in environmental

variation is possible. At the level of gene expression, prevalent G×E interactions in *S.ae* suggests greater genetic variance in the response to the environment, which potentially reflects genotype-specific responses to microenvironmental variation within their home range. However, with only three genotypes per species, stronger G×E in gene expression for *S.ae* could be an artefact of sampling. Consistent with other studies (e.g., Friedman et al. 2019), G×E patterns in leaf morphology were largely created by changes in scale (amount of variance among genotypes) across the elevational gradient, rather than by genotypic-specific (i.e., crossing of reaction norms) responses to the environment. If the G×E interaction underlying plastic changes in phenotype and gene expression is maladaptive (i.e., the more plastic genotypes have lower fitness), we may be overestimating the potential for natural populations to respond positively to environmental change (Acasuso-Rivero et al. 2019). Future work should more closely link patterns of G×E with fitness variation to understand whether there is genetic variation for adaptive plasticity, even if the overall response of the species is a net reduction in performance. If such genetic variation exists, selection for adaptively plastic genotypes could lead to the rapid evolution of novel forms of adaptive plasticity (Sultan 2004; Wadgymar et al. 2018). Whether such evolution of adaptive plasticity requires a crossing of reaction norms, or whether selection can act upon differences in scale needs to be tested. Therefore, associating genotypes that vary in their level of plasticity with fitness across environmental variation could test whether more specialised genotypes determine the level of plasticity, and the conditions under which plasticity is adaptive.

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Authors' contributions

JB, SH, GW, SCozzolino, AC and DF designed the study. GW, AC and SCatara conducted the glasshouse and fieldwork. JC and BN extracted RNA and handled the transcriptome data. MP measured chlorophyll fluorescence, and VV grew plants and measured physiological differences between the two species. GW and JC analysed the data and wrote the manuscript with important contributions from all authors. All authors gave final approval for publication.

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