

## Contrasting adaptive trait variation in response to drought in two Mediterranean shrubs

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### ABSTRACT

Adaptive evolution and phenotypic plasticity are key mechanisms of climate change responses. However, we still lack a detailed understanding of the strategies different species use to cope with climatic changes such as increased droughts, particularly for species with special edaphic requirements and limited dispersal such as gypsum endemics. In this study, we assessed phenotypic and genotypic selection, phenotypic plasticity and genetic variation in traits potentially related to drought response in two dominant gypsum Mediterranean species, *Helianthemum squamatum* and *Centaurea hyssopifolia*. We established a common garden in which 524 plants from 79 maternal families from both species were grown under two contrasting watering treatments. Our results revealed that selection was stronger under drought than well-watered conditions for both species, but we found contrasting adaptive strategies and genetic variation. In *H. squamatum*, a drought-escape strategy with advanced reproductive phenology and faster growth rates was positively associated with fitness under dry conditions, and most adaptive traits exhibited quantitative genetic variation. In contrast, in *C. hyssopifolia*, selection under dry conditions favored a drought-tolerance strategy with thicker leaves and longer phenologies, but all traits lacked quantitative genetic variation, indicating that their evolutionary potential may be limited. Most traits exhibited significant plasticity in response to drought and genetic variation for trait plasticity in both species, indicating that trait plasticity can evolve independently of the evolution of trait means in these gypsophiles. Our results show that these gypsum endemic species vary in strategies and adaptive potential in response to drought, which contributes to our understanding of potential adaptive responses to climate change in such edaphic specialists.

### 1. Introduction

Climate change is a major threat to plant biodiversity due to the worldwide alteration of temperature and precipitation patterns (Hoffmann and Sgró, 2011; Matesanz and Valladares, 2014). The Mediterranean region is especially vulnerable to climate change due to the expected increase in aridity and environmental heterogeneity, particularly in areas with arid and semiarid conditions such as the Iberian Peninsula (Giorgi and Lionello, 2008; IPCC, 2022). A well-documented response to cope with climate change is migration to less climatically restrictive areas (Jump and Peñuelas, 2005; Parmesan, 2006; and references). However, migration may be limited for species with strict edaphic requirements, fragmented distributions, and/or low dispersal

ability (Blanco-Sánchez et al., 2021; Jump and Peñuelas, 2005; Shaw and Etterson, 2012). In these cases, adaptive responses occurring *in situ* within populations, i.e. evolution by natural selection and adaptive phenotypic plasticity, may be key mechanisms to guarantee their long-term persistence (Chevin and Hoffmann, 2017; Franks et al., 2014; Gomez-Mestre and Jovani, 2013).

Adaptive evolution, a genetically-based shift in the mean phenotype of populations driven by natural selection, is a major force to cope with altering selection pressures, and mounting evidence shows that rapid evolution in response to climate change is occurring in plant populations (Franks et al., 2007; Giménez-Benavides et al., 2007; Hoffmann and Sgró, 2011; Matesanz and Valladares, 2014). In the new conditions imposed by climate change, populations may evolve by natural selection

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if fitness and fitness-related traits (i.e., adaptive traits) exhibit genetic variation within populations (Etterson, 2004; Jump et al., 2009; Lande and Arnold, 1983). However, evolution of adaptive traits may be constrained by a lack of quantitative genetic variation in some cases, or, even in the presence of quantitative genetic variation, by genetic correlations among traits if the direction of the correlation does not match the direction of selection (Arnold, 1992; Etterson and Shaw, 2001; Conner, 2012). Furthermore, since both the adaptive value of traits and the expression of quantitative genetic variation are environment-dependent, testing the potential response to selection in relevant ecological environments that simulate climate change conditions is needed to make reliable predictions of the future evolutionary trajectories of plant populations (Chevin and Hoffmann, 2017; Shaw and Etterson, 2012).

Phenotypic plasticity, the ability of a genotype to express different phenotypes in different environments, is the main response mechanism to buffer rapid environmental changes, and is particularly favored in highly heterogeneous environments (Chevin and Hoffmann, 2017; Hoffmann and Sgró, 2011; Jump and Peñuelas, 2005; Matesanz et al., 2010; Stotz et al., 2021; Van Kleunen and Fischer, 2005). Furthermore, plasticity may also evolve by natural selection, and the evolution of adaptive plastic responses may play a major role in the persistence of plant populations in future environmental conditions (Chevin and Hoffmann, 2017; Jump and Peñuelas, 2005; Matesanz et al., 2010). Although phenotypic plasticity and adaptive evolution are complementary mechanisms that might act simultaneously, the relative importance of both mechanisms to cope with climate change and how adaptive evolutionary responses may differ between co-occurring species is far from resolved (Ghalambor et al., 2007; Merilä, 2015; Nicotra et al., 2010), particularly in Mediterranean semiarid plants (Franks et al., 2014; Matesanz and Valladares, 2014; Parmesan, 2006). Evaluating the contribution of both mechanisms could be especially important for gypsophiles—plants restricted to gypsum soils—since these species live under semiarid and highly heterogeneous conditions, have specific edaphic requirements, and lack long-distance dispersal mechanisms (Blanco-Sánchez et al., 2021; Escudero et al., 2015). To assess the potential response to selection of plant populations and patterns of adaptive plasticity, it is particularly useful to conduct quantitative genetics studies in which individuals of known family structure are grown in common gardens under experimental conditions that simulate contrasting and realistic future environments (de Villemereuil et al., 2016; Franks et al., 2014).

In this study, we assessed the potential response to selection of traits and their plasticities in two dominant gypsophile species, *Helianthemum squamatum* and *Centaurea hyssopifolia*. Since drought is often the primary selection pressure in Mediterranean gypsum habitats (Blondel et al., 2010), we performed an outdoor common garden experiment with two contrasting watering treatments, well-watered and drought, to evaluate phenotypic plasticity, quantitative genetic variation and patterns of phenotypic selection in traits related to drought response. A recent phenotypic selection study in natural conditions revealed the adaptive value of earlier and longer phenologies, less sclerophyllous leaves, and lower water use efficiency associated with a drought-escape strategy for both species (Blanco-Sánchez et al., 2022), but the genetic basis of this strategy, and therefore, its potential to evolve, is to date unknown. Therefore, we predicted that a similar trait syndrome will be adaptive, especially under drought conditions (Blanco-Sánchez et al., 2022). Finally, we hypothesized that both species will show adaptive plasticity to drought and genetic variation for traits and their plasticity, since large populations evolving in highly-variable environments usually express high levels of plasticity and genetic variation for both traits and their plasticity (Chevin and Hoffmann, 2017; Hoffmann and Sgró, 2011; Kelly, 2019; Saltz et al., 2018; Stotz et al., 2021).

## 2. Materials and methods

### 2.1. Study species and seed collection

*Centaurea hyssopifolia* Vahl. (Asteraceae) and *Helianthemum squamatum* (L.) Dum. Cours (Cistaceae) are two of the most dominant gypsophiles of the center of the Iberian Peninsula (Matesanz et al., 2018). Both are small (20–60 cm of height), endemic chamaephytes of Iberian gypsum habitats. *Centaurea hyssopifolia* is restricted to the central Iberian Peninsula, while *H. squamatum* is widely distributed in most Iberian gypsum outcrops (Matesanz et al., 2018). Furthermore, *C. hyssopifolia* shows an earlier reproductive period (from May to July), while the flowering and fruiting phenology of *H. squamatum* lasts from late May to early-mid August (Blanco-Sánchez et al., 2022).

In July 2017, we collected mature seeds from 45 maternal plants per species in a large population in the center of the Iberian Peninsula (Belinchón, Spain; 40° 04' N, 3° 04' W; ~700 m a.s.l.). The site has a typical Mediterranean semiarid climate, with mean annual precipitation and temperature of 419.2 mm and 14.6 °C, respectively, and pronounced summer drought (mean climatic data extracted from the 35-year climatic time series of CHELSA; Karger et al., 2017). In this site, the plant community is established in gypsum hills that harbour populations of thousands of individuals of both study species, forming discrete vegetation patches surrounded by bare soil and biological soil crust. To account for the high environmental variability of gypsum habitats (Blanco-Sánchez et al., 2022), maternal plants were sampled from south and north slopes at three different gypsum hills (22 and 23 maternal plants per species from north and south slopes, respectively). To avoid sampling closely-related individuals, maternal plants were separated by at least three meters from each other.

### 2.2. Common garden experiment

The experiment was performed in the outdoor CULTIVE facilities at URJC (Móstoles, Madrid, Spain). The climatic conditions of this area match those experienced by individuals in natural conditions, providing a realistic experimental environment, with similar climatic conditions and high light intensity typical of Mediterranean gypsum habitats (mean annual precipitation and temperature of 434.4 mm and 14.81 °C, respectively; data extracted from CHELSA time series (Karger et al., 2017); PAR > 1600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  Supp. 1). In August 2017, seeds from each maternal plant were sown in 6 L pots (22 × 20 cm; Alpifer, Valencia, Spain) filled with soil extracted from a nearby gypsum quarry (Yesos Ibéricos-Algiss S.A., Valdemoro, Madrid, Spain). Since both species are mostly outcrossing, individuals from the same maternal plant constituted a maternal family and were considered half-siblings. Before sowing, ten seeds per maternal plant were individually weighed using a Mettler Toledo MX5 microbalance (1  $\mu\text{g}$  precision; Mettler Toledo, Columbus, OH, USA) to obtain a family-level seed mass. To ensure that the substrate did not contain seeds of the study species, control pots were filled and watered for several weeks, showing no germination of either species. Eight pots per maternal family were placed in a greenhouse and maintained in well-watered conditions during germination and seedling establishment. Approximately three months after sowing, in November 2017, pots were moved from the greenhouse to the outdoor cultivation facility. Finally, in January 2018, pots were thinned out to one experimental individual per pot. Due to differences in germination success, the final size of the experiment was 221 plants for *C. hyssopifolia* (35 maternal families, 4–8 half-siblings per family) and 303 plants for *H. squamatum* (44 maternal families, 4–8 half-siblings per family).

To ensure that experimental plants reached the reproductive stage and to minimize potential maternal effects, which are larger in early stages of plant development (Bischoff and Müller-Schärer, 2010), we performed the plasticity experiment and collected phenotypic data in the second growing season. On March 15th, 2019, after ~2 years of growing in common, optimum conditions, 2–4 individuals per maternal

family were randomly assigned to each of two contrasting watering treatments, well-watered and drought. Treatments were implemented by modifying the soil water content (SWC hereafter) of the experimental pots using a drip irrigation system with pressure-compensating emitters (Rain Bird XB05PC; Rain Bird Corporation, CA, USA) and adjusting the number and duration of watering events. In the well-watered treatment, plants were kept at field capacity (~25 % of SWC for our substrate), simulating periods/years when SWC is high for several days, such as during a rainy spring or in wet years. In contrast, SWC in the drought treatment was progressively reduced by decreasing the intensity and frequency of watering events and then maintained at ~50 % of field capacity (12–14 % of SWC), simulating periods later in the season (e.g., early summer) or springs drier than the average. Although similar SWC values to those imposed in our treatments have been registered in natural conditions at different time points in the study population (data not shown), climate change models for the semiarid Mediterranean region predict an increase in the frequency and duration of drought events (IPCC, 2022). Therefore, our drought treatment mimics conditions that will become more common in the near future, being key to assess the response of the study species to climate change. Treatments lasted for ~4 months, ending when plants in the well-watered treatment began to senesce (July 2nd, 2019). To guarantee the successful implementation of both watering treatments, pots were placed under rain exclusion structures that eliminated all natural precipitation and did not substantially affect other environmental conditions (see details of the structures on Supp. 2 and Matesanz et al., 2020a, b). Furthermore, SWC was monitored every 3–5 days in 12 pots per treatment using an HH2 Moisture Meter with an ML3 Sensor (Delta-T devices, Cambridge, UK; see Supp. 3).

### 2.3. Phenotypic and fitness traits measurements

We measured a wide set of functional and fitness traits in all plants (see Supp. 2 for details).

#### 2.3.1. Phenological traits

Reproductive phenology was monitored every three days during the experiment (24 censuses). At each census, we visually assessed the presence of inflorescences with open flowers, fully-developed fruits, and dispersed inflorescences. Using these data, we calculated flowering, fruiting, and dispersal onset and duration.

#### 2.3.2. Leaf morphological traits

At the reproductive peak of each species, we randomly collected five non-senescent, fully-developed leaves per plant. After 12 h of rehydration, the saturated fresh mass of all leaves was weighed using a Mettler Toledo MX5 microbalance. Then, we measured leaf thickness using a dial thickness gauge (Mitutoyo Corporation, Japan). Next, leaves were scanned using an Epson Perfection V370 Photo scanner (Seiko Epson Corporation, Japan), and oven-dried at 60°C for 48 h. Finally, dried leaves were weighed again. From these data, we calculated specific leaf area (SLA), leaf dry matter content (LDMC) and total estimated leaf area (TELA).

#### 2.3.3. Ecophysiological traits

At the time of leaf collection, we also measured the midday maximum photochemical efficiency ( $F_v/F_m$ ), and leaf chlorophyll content. Midday photochemical efficiency was measured from 13:00–16:30 (UTC + 2) during two consecutive sunny days using a Handy PEA+ chlorophyll fluorimeter (Hansatech, UK), adapting leaves to the dark for 30 min before the measurement. Leaf chlorophyll content was measured in three leaves per plant, using a SPAD 502 chlorophyll meter (Konica Minolta, Japan).

#### 2.3.4. Plant size and growth traits

We measured the height of each plant, its maximum diameter, and

the perpendicular diameter to the maximum diameter at the onset and at the end of the watering treatments. We calculated initial and final plant volume of each plant as the volume of a hemispheroid, and relative growth rate (RGR; Supp. 2 for details). Aboveground tissues were harvested and oven-dried, and leaves and stems from each individual were weighed using a Kern ABJ 120-4M analytical balance (1 mg precision; Kern & Sohn GmbH, Germany). From these data, we calculated aboveground biomass, as the sum of leaf and stem biomass, and the leaf:stem ratio.

#### 2.3.5. Reproductive fitness traits

We haphazardly collected three mature inflorescences per plant before seed dispersal, storing them individually in paper bags. Then, inflorescences were thoroughly dissected, obtaining the mean number of viable seeds per inflorescence. To assess the mean seed mass, five viable seeds per plant were randomly selected and individually weighed. Finally, before the end of the experiment, we counted the number of viable inflorescences of all plants. From these data, we calculated two integrated plant-level fitness variables: i) total seed number, as the product of the number of inflorescences and the number of seeds per inflorescence, and ii) total seed mass, as the product of total seed number and the mean seed mass.

### 2.4. Statistical analyses

#### 2.4.1. Phenotypic and genotypic selection analyses

To identify traits under selection in each species and treatment, we calculated phenotypic (Lande and Arnold, 1983) and genotypic (Rauscher, 1992) selection differentials and gradients. Selection differentials ( $S'$ ), i.e., the covariance between relative fitness and a particular standardized trait, assess the total relationship between traits and fitness (total selection; including direct selection and indirect selection caused by correlations with other traits). Selection gradients ( $\beta'$ ), i.e., the vector of partial regression coefficients of relative fitness on standardized traits, assess direct selection on the traits, removing the effect of correlations with other traits. First, trait values were standardized as  $\frac{X-\mu}{\sigma}$ , where  $X$  is the trait value of an individual, and  $\mu$  and  $\sigma$  are the mean and the standard deviation, respectively, of the trait in each watering treatment. Then, reproductive fitness was relativized in each species and watering treatment as individual fitness divided by the mean value of fitness for a given treatment. To estimate genotypic selection differentials and gradients, we calculated the mean of each functional and fitness trait (standardized and relativized, respectively) for each maternal family in each treatment. To assess directional selection, we calculated linear selection differentials and gradients. To evaluate stabilizing and disruptive selection, we estimated quadratic selection differentials for each species and treatment (see Supp. 2). To avoid potential multicollinearity in our selection analyses, we computed both variance inflation factors (VIFs) and pairwise phenotypic correlations for each trait in both species and watering treatments (see Supp. 4). As recommended, we excluded from our models predictors with VIF > 10 (Dormann et al., 2013). Therefore, due to their high correlation with other traits (>0.7) and high VIFs, TELA was not included in the models of either species, fruiting duration in the models of *H. squamatum*, and dispersion onset and duration in the models of *C. hyssopifolia*.

Phenotypic and genotypic selection analyses were performed using linear mixed models and generalized linear mixed models (LMMs and GLMMs) with functions *lmer* and *glmer* (package *lme4*; Bates et al., 2015). We included relative reproductive fitness as the dependent variable, and the standardized trait (in selection differentials) or traits (in selection gradients) as independent variables. To account for potential factors that may affect the relationship between traits and fitness, initial plant volume of each individual and the mean seed mass from each maternal family were also included as covariates, and the identity of each maternal family was included as a random factor (in phenotypic

selection analyses). Selection analyses excluding the covariates and the random factor provided very similar results (Supp. 5), but the explained variance of the latter models was slightly lower. Since reproductive fitness did not follow a gaussian distribution in either treatment in *C. hyssopifolia* and in the drought treatment in *H. squamatum* (the distribution was positively skewed), models were performed using family = “Gamma” and link = “log” in these cases, and family = “gaussian” with link = “identity” in the models performed for *H. squamatum* in the well-watered treatment. Finally, to account for multiple testing, results from selection analyses were corrected using false discovery rate (FDR) in each species and treatment (Benjamini and Hochberg, 1995) using function *p.adjust*. Because the total seed mass fitness variable includes total seed number, and models using both fitness variables resulted in very similar selection patterns, we only show selection analyses using total seed mass in the main text (see Supp. 6 and 7 for results using total seed number).

Finally, to assess if genetic trade-offs between traits may constrain adaptive evolution, we assessed the genetic variance-covariance matrices (G-matrices) for all traits for each species and treatment (Supp. 8), which quantified the additive genetic variance of traits (diagonal) and the genetic covariance among traits, using function *MCMCglmm* (package *MCMCglmm*; Hadfield et al., 2019) with scaled trait values and gaussian priors. Bayesian models were fitted including all the studied traits as dependent variables and maternal family as a random factor. Each model was run for one million iterations, with a burn-in period of 100000 iterations and a thinning interval of 1000.

#### 2.4.2. Quantitative genetic variation

To quantify the degree to which phenotypic differences among individuals within each watering treatment were genetically-based, i.e., the presence of quantitative genetic variation in both species, we performed for each trait and treatment a mixed model including individual trait values as dependent variables, seed mass of each maternal family as covariate and the identity of maternal family as a random factor. We compared this model to the same model excluding the random term maternal family using a likelihood ratio test (Zuur et al., 2009), with function *lrtest* (package *lmer*; Zeileis and Hothorn, 2002). A significant effect from a Chi-square test comparing these models ( $P < 0.05$ ) indicated genetic differences among maternal families. Results were again corrected by FDR. Families with fewer than three replicate half-siblings in a given treatment were dropped from these analyses.

**Plastic responses to drought, genetic variation and selection for plasticity**— To test the effect of the watering treatments on phenotypic expression (phenotypic plasticity), we fitted linear mixed models for each species with the individual trait values as the dependent variable, treatment as a fixed factor, and maternal family and the family-by-treatment interaction as random factors. The significance of the fixed factor was assessed using function *Anova* (package *car*; Fox et al., 2012), with type III sum of squares and the Kenward–Roger approach, and the proportion of variance explained by each model ( $R^2$ ) was calculated using function *summary* (R Core Team, 2018). A significant effect of treatment indicated phenotypic variation between watering conditions (phenotypic plasticity). Then, to quantify the specific response to drought of each maternal family, assess the presence of genetic variation for plasticity for each trait, and estimate the selection differentials and gradients for plasticity, we calculated the relative phenotypic distances between individuals from the same maternal family in different treatments, which allows calculating an index of plasticity (RDPI index) for each maternal family and comparing them statistically (Valladares et al., 2006). To evaluate the presence of genetic variation for plasticity among maternal families, we performed two different models for the RDPIs of each trait with maternal seed mass as a covariate, including and excluding the random term maternal family, and compared them using likelihood ratio tests. Results were corrected by FDR. Significant differences between models ( $P < 0.05$ ) indicated that maternal families responded differently to drought, i.e., the presence of genetic variation

for plasticity. Finally, to assess if plastic responses were under selection, we estimated genotypic selection differentials and gradients (see above) including standardized RDPIs values for each trait (or traits) as fixed factors, maternal seed mass as a covariate, and two different relativized fitness variables as dependent variables (Caruso et al., 2006). We first assessed if plasticity was adaptive in the sense that the most plastic genotypes have the highest average fitness across environments (Van Kleunen and Fischer, 2005). To test this, selection analyses for plasticity were performed using mean fitness across treatments. Second, to evaluate whether plasticity could enhance fitness under stressful conditions, models were fitted using relativized fitness under the drought treatment.

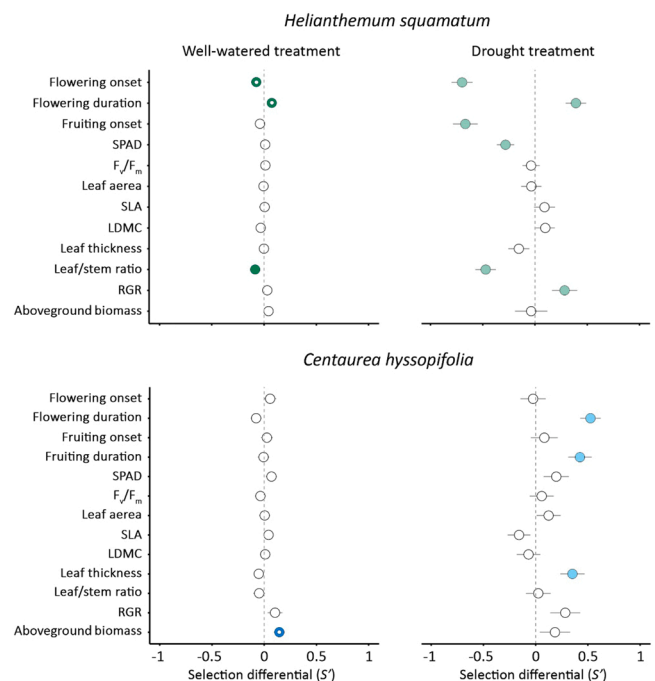
All analyses were performed in R v4.0.5 (R Core Team, 2018).

### 3. Results

#### 3.1. Selection patterns across watering conditions

In both species, we found a higher number of traits under linear selection in the drought treatment than in the well-watered treatment. The magnitude of selection, based on the larger selection differentials and gradients, was also higher under drought (Fig. 1; Supp. 9). We did not find evidence of stabilizing or disruptive selection for any trait in either treatment or species. Although a few functional traits showed significant  $C'$  and  $\gamma'$  coefficients (Supp. 10), there were not clear maxima or minima of fitness associated with intermediate phenotypic values.

*Helianthemum squamatum* and *Centaurea hyssopifolia* differed in both the number and the identity of the traits under selection. For *H. squamatum* in drought conditions, there were significant selection differentials for phenological traits, with individuals with earlier flowering and fruiting phenology and with longer flowering periods having higher fitness (Fig. 1; Supp. 9). Also, lower leaf chlorophyll content



**Fig. 1.** Linear phenotypic selection differentials ( $S'$ ) and their standard error for *H. squamatum* and *C. hyssopifolia* in both watering conditions using total seed mass as fitness variable. Significance levels after FDR corrections: colored circle =  $P < 0.05$ ; small white dot inside colored circle =  $0.05 < P < 0.1$ ; white = n.s. ( $P > 0.05$ ) selection differentials. Significant and marginally significant selection differentials in the drought treatment are shown in light green and light blue for *H. squamatum* and *C. hyssopifolia*, respectively, and in dark green and dark blue for *H. squamatum* and *C. hyssopifolia* under well-watered conditions, respectively.



(SPAD), lower leaf:stem ratio, and higher relative growth rate (RGR) were associated with greater reproductive fitness under drought (Fig. 1; Supp. 9). Under drought conditions, flowering and fruiting onset, individual leaf chlorophyll content, and leaf:stem ratio were under negative direct selection, while flowering duration was under positive direct selection (Supp. 9). In well-watered conditions, leaf:stem ratio was under negative total and direct selection (Fig. 1; Supp. 9). In this species, genotypic selection analysis showed very similar results compared to those obtained using individual trait values, especially under drought conditions. In this treatment, families with advanced flowering and fruiting phenology, longer flowering periods, lower leaf:stem ratio and higher RGR showed higher fitness (Supp. 9). Conversely, there was no significant genotypic selection in well-watered conditions (Supp. 9).

In *C. hyssopifolia* under drought, we found that longer phenologies were associated with greater fitness, with significant selection differentials for flowering and fruiting duration and a significant selection gradient for flowering duration (Fig. 1; Supp. 9). In well-watered conditions, selection differentials showed that only greater aboveground biomass was marginally associated with fitness (Fig. 1; Supp. 9). We did not find any trait under direct selection in *C. hyssopifolia* in the well-watered treatment (Supp. 9). Genotypic selection analyses showed no trait under total or direct selection in either the well-watered or the drought treatment (Supp. 9).

### 3.2. Quantitative genetic variation and genetic correlations

In *H. squamatum*, we found significant quantitative genetic variation for several traits in both watering conditions, some of which were under selection (Table 1). Specifically, in drought conditions, traits under selection that exhibited genetic variation were flowering and fruiting onset, leaf:stem ratio, and total seed number. In the well-watered treatment, flowering onset, and leaf:stem ratio showed genetic variation in *H. squamatum* (Table 1). Furthermore, G-matrices did not show any genetic correlation of the opposite sign to the direction of selection in this species (Supp. 8).

In contrast to *H. squamatum*, there was no significant genetic variation for any trait under selection in either treatment in *C. hyssopifolia* (Table 1).

### 3.3. Plastic responses, genetic variation and selection for plasticity

We found significant plasticity (differences in phenotypic expression between treatments) for most traits in both species (Fig. 2; Supp. 11). Likelihood-ratio tests performed with RDPI values showed genetic variation for the plasticity of all traits except for fruiting onset in *C. hyssopifolia* (Table 1; Fig. 3).

Individuals of *H. squamatum* significantly advanced their flowering and fruiting onset (~1.5 and 4.2 days, respectively) in response to drought, and there was a significant reduction in the duration of flowering (~5.7 days) under drought conditions (Fig. 2). In response to drought, plants also showed significant changes in leaf morphology, producing smaller (~18.2 % decrease in leaf area) and thicker leaves (~10.2 % increase in leaf thickness), and increasing their leaf:stem ratio by ~9.5 % (Fig. 2). Furthermore, in well-watered conditions, plants showed higher values of leaf chlorophyll content and photochemical efficiency (~9.25 % increase for both traits). In addition, drought constrained plant growth, resulting in a 10-fold reduction in RGR and 1.25 times reduction in aboveground biomass (Fig. 2). Finally, water stress significantly reduced individual fitness. Plants in the well-watered treatment showed greater total seed number and total seed mass (12.8 and 10.4 times greater) than in the drought treatment (Fig. 2).

In *C. hyssopifolia*, the onset of fruiting was significantly advanced in response to drought (~2 days), and plants showed a significant reduction in flowering and fruiting duration (18.7 and 13.4 days, respectively; Fig. 2). Morphological traits were also significantly affected by drought. In the drought treatment, individuals produced smaller and thinner

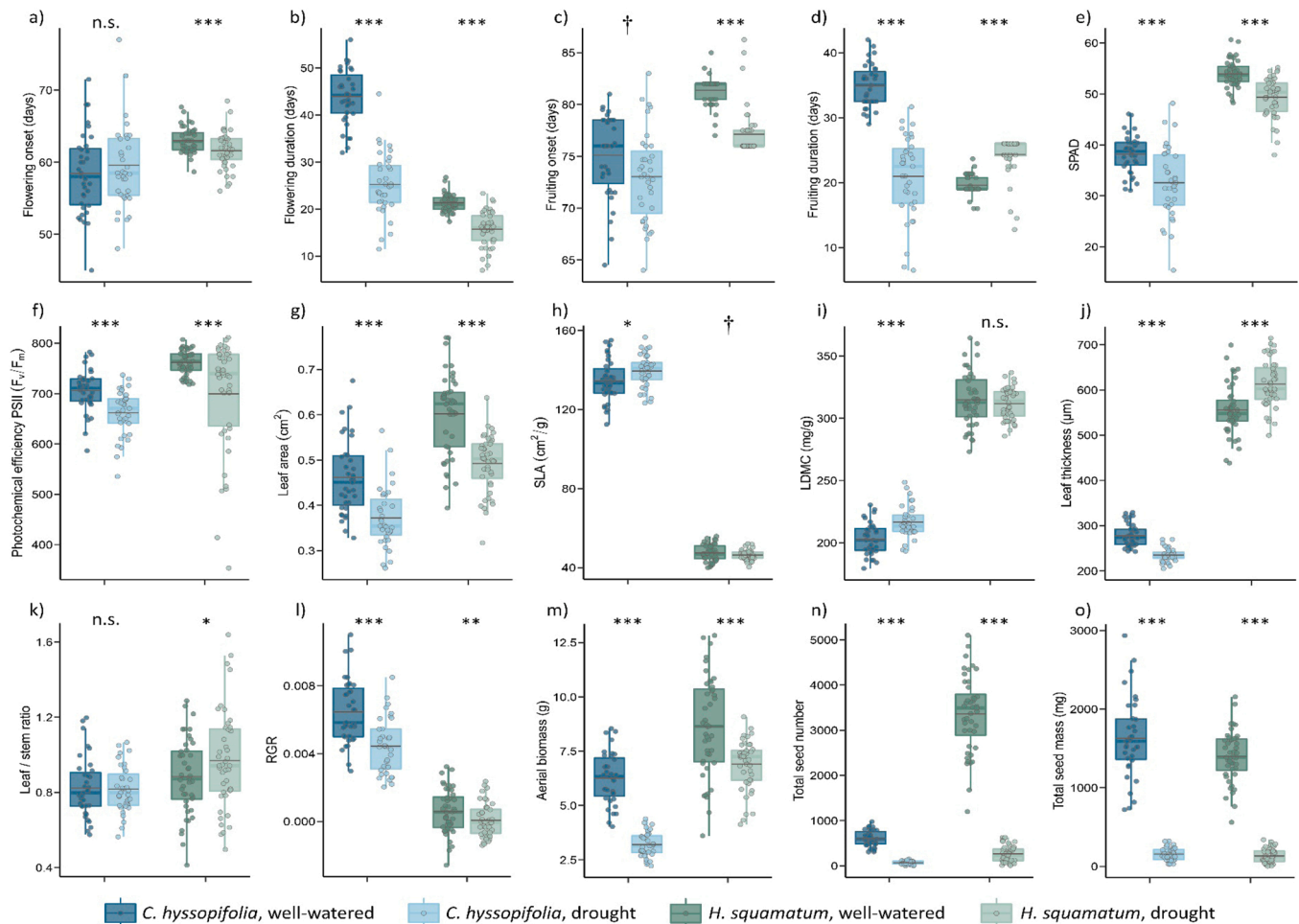
**Table 1**

Results of Likelihood Ratio tests assessing the presence of genetic variation for functional traits (within treatments) and their plasticity (across treatments, using RDPIs values for each trait) for both study species.  $\chi^2$  statistics and *P*-values after FDR correction are shown. Traits with significant genetic variation within treatments, and significant genetic variation for plasticity are shown in bold. Traits under selection (as shown by linear selection differentials and gradients) are underlined. Traits in both bold type and underlined were traits under selection with genetic variation (i.e., with significant differences among maternal families).

<i>H. squamatum</i>	Genetic variation for functional traits				Genetic variation for plasticity	
	Well-watered treatment		Drought treatment		Across treatments (RDPIs)	
	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>
Flowering onset	<b>8.899</b>	<b>0.010</b>	<b>32.480</b>	<b>&lt;0.001</b>	72.228	<0.001
Flowering duration	<u>1.863</u>	<u>0.301</u>	<u>3.302</u>	<u>0.097</u>	95.878	<0.001
Fruiting onset	0.358	0.592	<b>19.533</b>	<b>&lt;0.001</b>	55.253	<0.001
SPAD	1.352	0.343	<u>0.000</u>	<u>1.000</u>	26.366	<0.001
F <sub>v</sub> /F <sub>m</sub>	1.432	0.343	<b>5.638</b>	<b>0.031</b>	126.013	<0.001
Leaf area	3.636	0.132	<b>9.387</b>	<b>0.008</b>	62.165	<0.001
SLA	<b>11.785</b>	<b>0.003</b>	<b>7.426</b>	<b>0.018</b>	36.104	<0.001
LDMC	<b>13.303</b>	<b>0.002</b>	1.318	0.293	33.394	<0.001
Leaf thickness	1.039	0.392	<b>5.642</b>	<b>0.031</b>	6.850	0.009
Leaf:stem ratio	<b>8.550</b>	<b>0.010</b>	<b>35.379</b>	<b>&lt;0.001</b>	65.447	<0.001
RGR	2.705	0.200	<u>2.109</u>	<u>0.186</u>	14.062	<0.001
Aboveground biomass	<b>15.206</b>	<b>0.001</b>	0.564	0.488	33.096	<0.001
Total seed number	0.895	0.402	<b>6.373</b>	<b>0.027</b>	110.818	<0.001
Total seed mass	0.215	0.643	4.439	0.055	113.400	<0.001
<i>C. hyssopifolia</i>	Well-watered treatment		Drought treatment		Across treatments (RDPIs)	
	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>
Flowering onset	2.874	0.211	-1.14E-13	1.000	<b>17.419</b>	<0.001
Flowering duration	0.846	0.580	<u>1.14E-13</u>	<u>1.000</u>	6.696	0.010
Fruiting onset	<b>8.008</b>	<b>0.034</b>	3.41E-13	1.000	0.790	0.374
Fruiting duration	<b>6.252</b>	<b>0.047</b>	<u>0.000</u>	<u>1.000</u>	<b>33.902</b>	<0.001
SPAD	2.729	0.211	6.287	0.182	<b>69.832</b>	<0.001
F <sub>v</sub> /F <sub>m</sub>	0.636	0.580	0.114	1.000	<b>33.895</b>	<0.001
Leaf area	<b>7.335</b>	<b>0.034</b>	2.286	0.792	<b>23.219</b>	<0.001
SLA	1.762	0.346	1.14E-13	1.000	<b>31.621</b>	<0.001
LDMC	0.200	0.755	1.14E-13	1.000	<b>40.664</b>	<0.001
Leaf thickness	4.786	0.086	<u>0.000</u>	<u>1.000</u>	<b>40.900</b>	<0.001
Leaf:stem ratio	<b>14.061</b>	<b>0.003</b>	0.080	1.000	27.444	<0.001
RGR	0.704	0.580	1.990	0.792	<b>42.923</b>	<0.001
Aboveground biomass	<u>5.68E-14</u>	<u>1.000</u>	0.732	1.000	<b>40.109</b>	<0.001
Total seed number	0.488	0.606	2.27E-13	1.000	19.565	<0.001
Total seed mass	0.135	0.764	0.735	1.000	<b>22.134</b>	<0.001

leaves (decrease of ~19.3 % and 15.5 %, respectively; Fig. 2). Furthermore, we also observed reduced leaf chlorophyll content and photochemical efficiency under water stress (~13.6 % and 6.2 %, respectively; Fig. 2). Finally, a reduction in size and fitness was also observed under drought conditions. In the well-watered treatment, plants had more aboveground biomass, total seed number and total seed mass (~2, 9 and 10 times higher, respectively), compared to the drought treatment.

Finally, selection analysis performed on the plasticity indices showed that plasticity was under selection only in *H. squamatum*. Specifically, selection analyses performed using relativized mean fitness across



**Fig. 2.** Phenotypic variation across watering treatments and study species. a) flowering onset; b) flowering duration; c) fruiting onset; d) fruiting duration; e) leaf chlorophyll content (SPAD); f) photochemical efficiency of PSII ( $F_v/F_m$ ); g) leaf area; h) SLA; i) LDMC; j) leaf thickness; k) leaf/stem ratio; l) RGR; m) Aboveground biomass; n) total seed number; o) total seed mass, for each species in each watering treatment. Boxplots show median, first and third quartiles, and mean is represented using a gray line. Upper whiskers show 1.5 times the interquartile range (or the maximum value in case it is lower), while lower whiskers show 1.5 times the interquartile range (or the minimum value if it is higher). Dots represent mean trait values of each maternal family. Phenotypic differences between treatments (i.e., phenotypic plasticity) are shown. Significance levels: n.s. = not significant; †  $0.05 < P < 0.1$ ; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

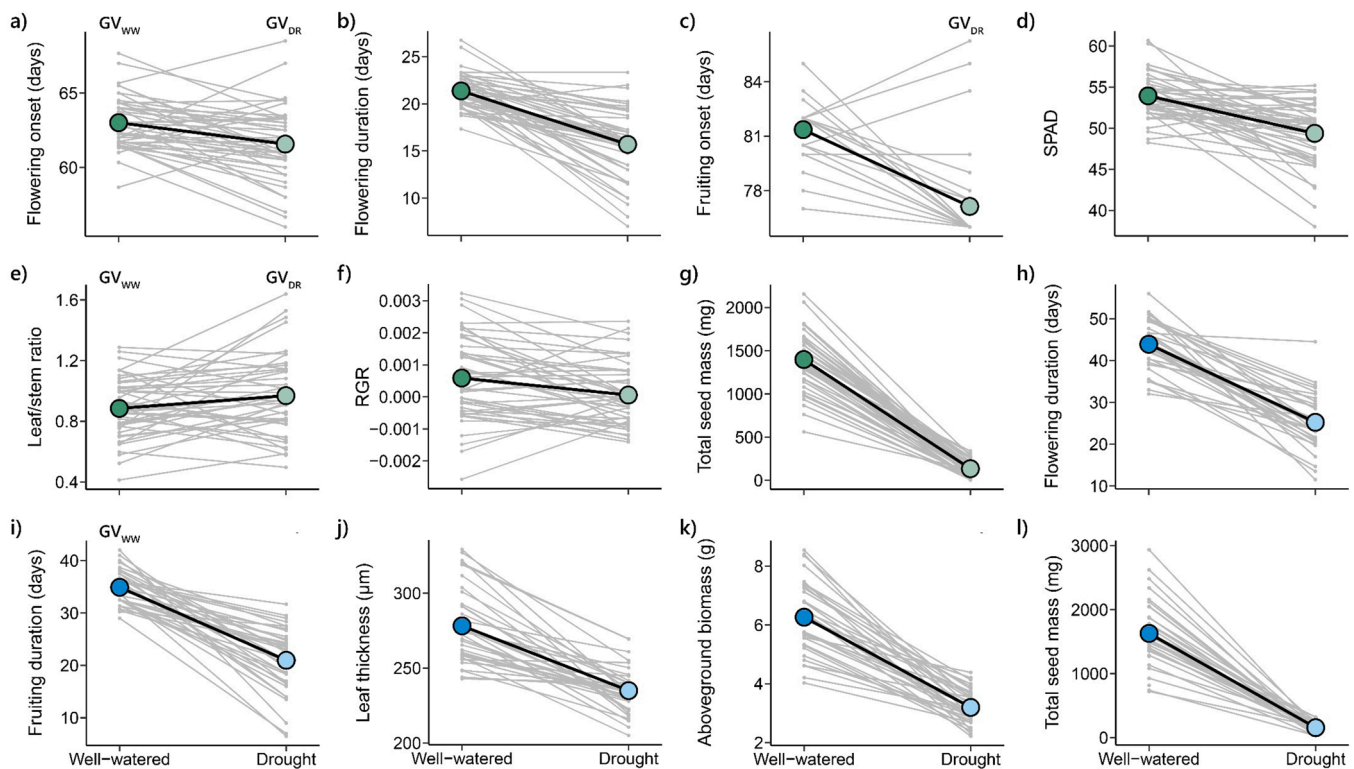
environments showed that the plasticity of flowering duration was negatively related to fitness ( $S' = -0.122$ ,  $P = 0.007$ ), indicating that maternal families with a lower reduction in flowering duration under drought conditions (i.e., flatter reaction norms, lower plasticity) showed higher fitness. In the models performed using relativized fitness under drought, we also found that higher plasticity in leaf chlorophyll content (SPAD) and SLA indicating that also families with a higher reduction in leaf chlorophyll content and SLA in response to drought were associated with higher fitness in *H. squamatum* (Supp. 12).

#### 4. Discussion

Our results showed strong selection and plasticity in response to drought in a large population of Mediterranean gypsophiles, but we found substantial differences between species in adaptive traits and in evolutionary potential as shown by quantitative genetic variation. In *H. squamatum*, selection favored earlier and longer reproductive phenology, higher RGR and lower leaf chlorophyll content, coupled with significant genetic variation for several traits and fitness, indicating that adaptive evolution may occur in this species in response to continuing climate change. In contrast, drought tolerance traits such as thicker leaves were favored by selection in *C. hyssopifolia*, but the lack of genetic variation suggested that these traits may be constrained in their

evolution in response to further drought selection. Furthermore, plastic responses to drought and genetic variation for plasticity were found in both species, suggesting that plasticity may play a key role in buffering the climatic conditions imposed by climate change. Overall, our results showed differences in the potential evolutionary responses of two dominant gypsophile species, which may affect their persistence in a climate change context.

Selection patterns within watering treatments highlighted the importance of drought as a key selective pressure for Mediterranean plant species, agreeing with previous studies (Blanco-Sánchez et al., 2022; Ramírez-Valiente et al., 2021). In *H. squamatum*, earlier and longer reproductive phenology, higher RGR, and lower leaf chlorophyll content were significantly associated with individual fitness under drought conditions, a syndrome consistent with a drought-escape strategy (Franks, 2011; Volaire, 2018; Welles and Funk, 2021), which was also found to be adaptive in natural conditions (Blanco-Sánchez et al., 2022). Several studies have reported the adaptive value of an advanced phenology in Mediterranean taxa to escape from drought (Blanco-Sánchez et al., 2022; Franks, 2011). Furthermore, earlier phenologies could be favored by the adaptive value of higher RGR. Especially under drought conditions, the onset of reproduction depends on resource acquisition rate, which is often correlated with individual growth rate (Segrestin et al., 2020; Welles and Funk, 2021), since more



**Fig. 3.** Phenotypic means (large dots), average reaction norms (black lines) and maternal families reaction norms (gray lines) for the traits under selection and total seed mass fitness variable in both species: a) flowering onset in *H. squamatum*; b) flowering duration in *H. squamatum* c) fruiting onset in *H. squamatum*; d) SPAD in *H. squamatum*; e) leaf/stem ratio in *H. squamatum*; f) RGR in *H. squamatum*; g) total seed mass in *H. squamatum*; h) flowering duration in *C. hyssopifolia*; i) fruiting duration in *C. hyssopifolia*; j) leaf thickness in *C. hyssopifolia*; k) aboveground biomass in *C. hyssopifolia*; l) total seed mass in *C. hyssopifolia*. Dark green and light green dots indicate phenotypic means for *H. squamatum* under well-watered and drought treatments, respectively. Dark blue and light blue dots indicate phenotypic means for *C. hyssopifolia* under well-watered and drought treatments, respectively. The presence of significant quantitative genetic variation within well-watered and drought conditions is shown with GV<sub>WW</sub> and GV<sub>DR</sub>, respectively. All traits showed plastic responses to drought and significant genetic variation for plasticity between families (i.e., non-parallel reaction norms).

acquisitive individuals may complete their lifecycles earlier and escape the most stressful conditions. In addition, plants often show lower chlorophyll content under drought and/or high irradiance conditions (Dai et al., 2009; Letts et al., 2012; Matesanz et al., 2020b). This reduction may be adaptive in stressful habitats such as Mediterranean gypsum ecosystems, since it prevents damage in the photosynthetic system caused by photoinhibition (Dai et al., 2009; Letts et al., 2012). Importantly, we found genetic variation for several adaptive traits and for reproductive fitness in drought conditions in *H. squamatum*, and patterns of trait covariance suggest that genetic correlations will not likely constrain trait evolution. Such heritable variation indicates the potential of this species to evolve higher reproductive output under drought conditions, associated with the evolution of an acquisitive resource strategy, which may be crucial given the predicted increased aridity for the Mediterranean region.

In contrast to *H. squamatum*, selection favored individuals with thicker leaves and longer flowering and fruiting periods under dry conditions in *C. hyssopifolia*, but both traits and fitness lacked genetic variation. More sclerophyllous leaves often have smaller cells with thicker walls, and are usually associated with conservative resource-use and drought-tolerance strategies that minimize water loss (Blumenthal et al., 2020; Ramírez-Valiente et al., 2020; Solé-Medina et al., 2022). Accordingly, selection studies have previously reported the adaptive value of thicker leaves to tolerate drought (Etterson, 2004; Ramírez-Valiente et al., 2014, 2011). Indeed, sclerophyllous leaves are usually associated with longer periods of photosynthetic activity during the growing season (Ramírez-Valiente et al., 2011; and references therein), favoring longer reproductive phenologies (Blumenthal et al., 2020; Ocheltree et al., 2020). However, the observed selection on increased

leaf thickness under dry conditions in our experiment differed from the results obtained in previous studies under natural conditions (Blanco-Sánchez et al., 2022). Volaire (2018) argued that shifts between drought-related strategies may be a consequence of different levels of water availability. Differences in adaptive traits between studies might be also related to differences in the onset of drought. In natural conditions, gypsophiles encounter severe water stress mostly in the later stages of the season (Blanco-Sánchez et al., 2022; Escudero et al., 2015), while our common garden simulated the increment of aridity caused by climate change and individuals experienced drought conditions during the entire growing season.

Nevertheless, *C. hyssopifolia* lacked heritable variation for adaptive traits and fitness in the studied population, potentially constraining their evolution. The absence of within-population quantitative genetic variation in this species was not likely caused by past selection that could have eroded genetic variation of adaptive traits. In such a scenario, low phenotypic variation would be expected (Blows and Hoffmann, 2005; Matesanz et al., 2010), but this was not the case in this species. Quantitative genetic variation can also be reduced in small and isolated populations by stochastic processes such as genetic drift (Shaw and Etterson, 2012), but our studied population harbored hundreds of individuals. Although we cannot pinpoint the exact reason behind the lack of quantitative genetic variation, the contrasting levels of genetic variation between species have important implications for their future evolutionary responses, since adaptive evolution requires within-population quantitative genetic variation (Blows and Hoffmann, 2005; Jump et al., 2009; Shaw and Etterson, 2012). Our results highlight the fact that two dominant species co-occurring in semiarid Mediterranean habitats and that are subject to similar selection pressures may



substantially differ in their potential to respond to selection at the population level, which will likely alter the dynamics of the plant community over time. Nevertheless, because quantitative genetic variation can vary across populations, i.e., populations of the same species may differ in their evolutionary potential (Matesanz et al., 2014; Matesanz and Valladares, 2014; Ramírez-Valiente et al., 2011), further studies with other populations of *C. hyssopifolia* would be needed to assess the evolutionary potential of this species in a climate change scenario.

In contrast to the differences in adaptive strategies under drought between the two study species, both showed significant plasticity to drought and genetic variation for plasticity in most functional traits (Fig. 3). Some of these plastic responses were consistent with adaptive responses to drought based on previous evidence. For instance, individuals of both species reduced their leaf area under drought conditions, which minimizes evapotranspiration under water stress (Matesanz et al., 2020b; Matesanz and Valladares, 2014). However, selection analyses indicated that plasticity was not under selection in either species. Furthermore, in some instances, plastic responses were in the opposite direction to the direction of selection found within the drought treatment (cf. Figs. 1 and 2). Determining the adaptive value of plasticity is not straightforward, and statistical approaches that quantify the contribution of phenotypic change to fitness may fail because environmental conditions affect both phenotypic expression and fitness, making it impossible to isolate the effect on fitness of the phenotypic change across environments (Auld et al., 2010; Sultan, 2004, 2000). Our experiment shows the limitations of evaluating adaptive plastic responses based on selection patterns assessed both across and within environmental conditions, highlighting the need for a novel and robust approach to statistically assess the adaptive value of plasticity.

Although the precise drivers promoting high levels of plasticity and variation in norms of reaction (i.e., genetic variation for plasticity) are yet not fully understood, several factors have been discussed (reviewed in Saltz et al., 2018; see also Kelly, 2019), highlighting the role of fluctuating selection pressures and environmental heterogeneity. Gypsum habitats have high coarse- and fine-grained spatiotemporal environmental variation (Blanco-Sánchez et al., 2022; Escudero et al., 2015; Matesanz et al., 2020a), which may have favored the expression of phenotypic plasticity in gypsophile species (Matesanz et al., 2010, 2020a; Sultan and Spencer, 2002; Via et al., 1995). Indeed, the fine-grained heterogeneity of gypsum habitats may select for different norms of reaction within populations (Sih, 2004; Via et al., 1995), maintaining genotypes (or families) expressing differential plasticity if heterogeneous environmental conditions impose variable selective pressures that favor genotypes with contrasting plastic responses (i.e., highly plastic genotypes in highly-variable microsites, and those with lower plasticity in more stable microsites). In contrast, constant directional selection as a consequence of harsh and predictable environments may reduce genetic variation for plasticity (Blows and Hoffmann, 2005; Matesanz et al., 2010), resulting in similar response patterns across families. Therefore, it is likely that a particular plastic response has not evolved by natural selection during the evolutionary history of these species, with environmental heterogeneity having a critical role in promoting the presence of genetic variation for plasticity and maintaining it over time.

The presence of genetic variation for plasticity at the intrapopulation level has important evolutionary implications for these species. First, it could be advantageous for populations inhabiting gypsum ecosystems because it allows a wide variety of phenotypic responses in such stressful heterogeneous habitats. This diversity may be maintained if families expressing different response patterns are equally fit (i.e., if spatiotemporal heterogeneous conditions favored different phenotypic responses). Indeed, high levels of genetic variation for plasticity are often correlated with higher resistance of populations against environmental-driven changes such as those caused by climate change (Kelly, 2019; Matesanz et al., 2010), since genetic variation is the substrate for natural

selection (Fisher, 1930; Matesanz and Valladares, 2014). Second, our results showed that, in both species, quantitative genetic variation of a particular trait and its plasticity might remarkably differ, and therefore both trait means and plasticities may evolve independently (see also Pigliucci, 2005; Weijtschedé et al., 2006). Surprisingly, in contrast to previous results both within and among populations (Scheiner, 1993; Lázaro-Nogal et al., 2015; Matesanz et al., 2017; Matesanz and Valladares, 2014), in some instances the evolutionary potential of plasticity in the study species was higher than the evolutionary potential of traits, especially in *C. hyssopifolia*. These results suggested that, particularly in *C. hyssopifolia*, the evolution of adaptive norms of reaction may play a more important role than the evolution of trait means in the adaptation to the changing environmental conditions driven by climate change.

Overall, our results indicate that traits and trait plasticities have the potential to evolve in gypsum endemics, with the evolutionary direction and evolutionary potential varying among species, traits, and environmental conditions. Thus, phenotypic plasticity and adaptive evolution can interact to shape adaptive responses in these habitat specialists, with implications for species responses to climatic changes more broadly.

### CRedit authorship contribution statement

**Mario Blanco-Sánchez:** Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. **Steven J. Franks:** Methodology, Formal analysis, Writing – review & editing, Visualization. **Marina Ramos-Muñoz:** Investigation, Resources, Data curation, Writing – review & editing. **Beatriz Pías:** Investigation, Resources, Data curation, Writing – review & editing. **José Alberto Ramírez-Valiente:** Methodology, Formal analysis, Writing – review & editing, Visualization. **Adrián Escudero:** Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Silvia Matesanz:** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

Phenotypic data are available on Figshare (<https://doi.org/10.6084/m9.figshare.c.6415805.v1>).

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2023.105253](https://doi.org/10.1016/j.envexpbot.2023.105253).



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