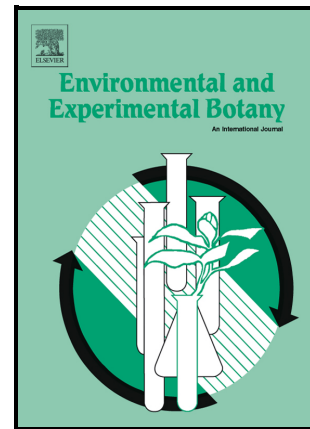


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## **Drivers of phenotypic variation and plasticity to drought in populations of a Mediterranean shrub along an environmental gradient**

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### **Abstract**

Assessing the factors driving intraspecific phenotypic variation is crucial to understand the evolutionary trajectories of plant populations and predict their vulnerability to climate change. Environmental gradients often lead to phenotypic divergence in functional traits and their

plasticity across populations. We studied the entire environmental range of the Mediterranean gypsum endemic shrub *Helianthemum squamatum* to evaluate the factors underlying quantitative population differentiation and phenotypic plasticity to drought, using a common garden with 16 populations that covered the main geographic and the entire climatic range of the species. Sampling followed a hierarchical approach to assess trait genetic variation within and among four distinct geographical regions. We found high but similar plastic responses across populations, which were consistent with adaptive plasticity to drought, including advanced phenology, more sclerophyllous leaves, higher water use efficiency and larger seeds in dry conditions. Despite these generally similar plastic responses, we found significant population differentiation in quantitative traits, part of which was structured at the regional scale. Such differentiation was not associated with environmental variation, including differences in climate and soil conditions. This suggests that non-adaptive processes might have had a role on genetic differentiation in *H. squamatum*, likely due to the island-like configuration of gypsum habitats and the lack of effective seed dispersal of the study species. Our results emphasize the role of phenotypic plasticity in adaptive drought response and the importance of considering both adaptive and non-adaptive processes shaping intraspecific phenotypic variation, which is crucial for predicting plant population vulnerability to climate change.

**Keywords:**

intraspecific trait variation, gypsophiles, natural selection, phenotypic plasticity, population differentiation, non-adaptive evolution.

## **1. Introduction**

Plant species exhibit significant intraspecific phenotypic variation due to both adaptive and non-adaptive processes. Adaptive variation can come from genetic differentiation driven by natural selection and differences in phenotypic plasticity (Franks et al., 2014; Nicotra et al.,

2010; Valladares et al., 2014). Phenotypic plasticity is not only a relevant source of adaptive phenotypic variation to cope with environmental changes (i.e., plastic rescue), but may also buy time for adaptive evolution to occur (Chevin and Hoffmann, 2017; Fox et al., 2019; Miner et al., 2005). However, plasticity may shield genetic variation from selection when it favors the expression of optimum, adaptive phenotypes across different environmental conditions (Collins et al., 2007; Valladares et al., 2007; Fox et al., 2019). Within populations, genotypes may vary both in their mean phenotypes and in their responses to the environment, indicating that adaptive traits and their plasticity may evolve by natural selection (Chevin et al., 2013; Crispo, 2008; Pigliucci, 2005). When populations encounter contrasting environmental conditions that impose differential selective pressures, natural selection may favor distinct phenotypes and plastic responses across populations, leading to patterns of clinal phenotypic variation and divergent plasticity (Brouillette et al., 2014; Kawakami et al., 2011; Kingsolver and Diamond, 2011; Sork, 2016). Accordingly, significant associations between phenotypic differences –measured in a common environment– or differences in plasticity across populations and relevant environmental gradients offer insights into the selective pressures driving adaptive differentiation (Blanco-Sánchez et al., 2024 and references therein).

Non-adaptive evolutionary processes, such as gene flow patterns and genetic drift, can also significantly affect phenotypic variation within and among populations (Sork, 2016). Gene flow tends to homogenize genetic variation across populations, potentially counteracting natural selection (Garant et al., 2007; Sork, 2016), but it can also introduce novel phenotypic variation that can be subsequently selected (Frankham, 2015; Whiteley et al., 2015). Historical colonization events may increase spatial genetic structure and create clinal phenotypic variation due to isolation by distance and founder effects (Keller and Taylor, 2008). The spatial configuration of populations can exacerbate these non-adaptive processes by promoting uneven colonization and gene flow (Bailey et al., 2006; Blanco-Sánchez et al., 2021). In the

Mediterranean basin, gypsum soils exhibit an island-like spatial arrangement due to their discontinuous distribution (Blanco-Sánchez et al., 2021; Escavy et al., 2012; Escudero et al., 2015), which, along with limited dispersal mechanisms of plants restricted to gypsum soils (gypsophiles; Meyer, 1986), might influence genetic and phenotypic differentiation among their populations (Blanco-Sánchez et al., 2021).

Furthermore, gypsophile populations have been subjected to different selective pressures during their evolutionary history, mainly associated with the stressful environmental conditions of gypsum habitats (Blanco-Sánchez et al., 2024). As in other Mediterranean and semiarid ecosystems, water availability constitutes the most critical selective pressure (Blondel et al., 2010). Indeed, previous evidence shows that temperature and precipitation gradients have driven intraspecific adaptive differentiation and high phenotypic plasticity across populations of a dominant gypsophile (Blanco-Sánchez et al., 2024). Another potentially important aspect is whether variation in the chemical composition of gypsum soils has also promoted phenotypic evolution in gypsophile populations. Despite the need to understand the drivers behind intraspecific phenotypic variation to robustly predict the vulnerability of plant populations to climate change, we still lack consistent evidence about how environmental conditions and the spatial configuration of gypsum habitats have shaped population differentiation for most gypsophiles.

Our study assessed patterns of intraspecific phenotypic variation in the Mediterranean dominant gypsophile *Helianthemum squamatum* at two geographical scales: among regions corresponding to distinct gypsum outcrops and associated gypsophile vegetation (Mota et al., 2011), and among populations with different climatic and soil conditions within these regions. We evaluated the potential role of natural selection associated with variations in climatic and soil composition conditions in population differentiation and patterns of plasticity to drought in this species, and discussed the potential influence of non-adaptive processes related to

evolutionary and colonization history. We measured a set of ecophysiological and fitness traits using an outdoor common garden experiment with two contrasting and ecologically meaningful watering treatments. We addressed the following questions: 1. Is there phenotypic differentiation among populations and regions, both in trait means and their plasticity? 2. If so, is this variation related to climatic or edaphic variation across populations? We hypothesized that divergent selection along climatic gradients has shaped intraspecific phenotypic differentiation in *H. squamatum*, in parallel with other gypsum endemics (Blanco-Sanchez et al., 2024). Alternatively, the spatial arrangement of gypsum habitats may have significantly affected colonization events and gene flow patterns among populations, resulting in non-adaptive phenotypic differentiation observed at the regional scale.

## 2. Materials and methods

### 2.1. Study species and population sampling

*Helianthemum squamatum* (L.) Dum. Cours. (Cistaceae) is a representative gypsophile shrub in the Iberian Peninsula gypsum habitats (Mota et al., 2003; Palacio et al., 2007). The species is found in the eastern half of the Iberian Peninsula (Fig. 1a), with an additional population in northern Africa (Escudero et al., 1999; López González, 1993). This dwarf chamaephyte (10 – 40 cm) is diploid, mainly outcrosser with partial self-compatibility, and has a short lifespan up to 10 years (Aragón and Escudero, 2008; Olano et al., 2011). The flowering period in *H. squamatum* occurs from May to August, corresponding with the summer drought (Aragón and Escudero, 2008). Seeds of *H. squamatum* lack long-distance dispersal mechanisms, showing a mucilage coating that favors seed adhesion to the soil (Aragón and Escudero, 2008; Escudero et al., 1999; Matesanz et al., 2018).

In mid-July 2020, at the peak of seed maturation, we collected seeds from individual plants in 16 different populations of *H. squamatum* in the Iberian Peninsula (Fig. 1a; Table S1), covering the main geographic and the entire climatic range of the species. Populations were sampled following a hierarchical approach, with four populations nested in each of four different regions. These four regions roughly matched large river basins in the Iberian Peninsula (Ebro, Tajo, Júcar-Segura, and Guadalquivir basins; Fig. 1a, Table S1), which correspond to distinct gypsum outcrops and gypsophile vegetation habitats (Mota et al., 2011). Gypsum outcrops in the Mediterranean basin often show an island-like spatial configuration due to their immersion in other soil types, and such natural fragmentation has been aggravated by human activities related to agricultural intensification (Blanco-Sánchez et al., 2021; Escavy et al., 2012; Escudero et al., 2015; Mota et al., 2011). In each region, selected populations encompassed the climatic variation within the region, and showed substantial climatic variation among them (i.e., populations within a given region were not climatic replicates). To characterize the climatic conditions of the sampled populations in each region, mean annual temperature and annual precipitation data were extracted from 50 random points (separated at least 50 meters from each other) within a 5 km buffer around each population, using WorldClim bioclimatic layers (see below). From these data, linear mixed models were performed using climatic variables as the dependent variable, region as a fixed factor and population nested in region as a random factor. These models did not show significant differences among regions in annual mean temperature or annual precipitation ( $P > 0.05$ ). Thus, our sampling protocol allowed us to assess the role of adaptive evolutionary processes related to environmental differences among populations on phenotypic differentiation and discuss other potential non-adaptive sources of variation. In this design, a pattern of phenotypic differentiation where individuals from populations with similar environmental conditions express more similar phenotypes would provide evidence that trait variation has been shaped by adaptation to such

conditions. In contrast, if individuals from populations within a particular region are phenotypically more similar than to those from other regions, intraspecific trait variation would likely be shaped by non-adaptive processes resulting from colonization patterns, differential gene flow and other stochastic events.

In each population, we collected mature inflorescences from 12 maternal plants that were at least 2m apart from each other, to avoid sampling closely related individuals. Inflorescences were bagged separately using paper bags, and seeds were cleaned in the laboratory. Climatic data of sampled populations was extracted from WorldClim bioclimatic layers (Fick and Hijmans, 2017) and soil-water balance layers developed by Trabucco and Zomer (2010), using a 2 km buffer around each population to account for small-scale climatic variability with ArcMap 10.5 (ArcGIS Desktop, ESRI, CA, USA). Overall, sampled populations spanned a gradient of temperature and precipitation, from 12.4 to 16.5 °C of annual mean temperature and from 291 to 488 mm of annual precipitation (WorldClim, Table S1). To characterize soil chemical and nutrient composition, we randomly collected three soil cores (0-20 cm depth) in each population. We quantified total S (a proxy of gypsum content), total C and its organic and inorganic fractions, and total N using an elemental analyzer (TruSpec CHNS, LECO, MI, USA). The percentage of soil gypsum content was calculated by multiplying total S by the molecular mass of gypsum (172.17) and dividing this value by the molecular mass of S (32.07). Furthermore, organic matter content and available Olsen P were estimated by chromic acid digestion and standardized protocols with NaHCO<sub>3</sub> (Olsen, 1954), respectively. Soil analyses were performed at IPE-CSIC in Zaragoza, Spain.

## ***2.2. Common garden experiment and watering treatments***



Before sowing, we randomly selected nine field-collected maternal plants, and we weighed ten seeds per maternal plant in a Mettler Toledo MX5 microbalance (1  $\mu\text{g}$  precision; Mettler Toledo, Columbus, OH, USA). From these values, we calculated the mean of the ten individually weighed seeds to obtain a family-level seed mass. Since the pollination of *H. squamatum* is mostly outcrossing, individuals originated from seeds collected in the same maternal plant were considered half-siblings and constituted a maternal family. In early August 2020, we sowed field-collected seeds in 0.5 L pots with gypsum soil extracted from a nearby gypsum quarry (Yesos Ibéricos-Algiss S.A., Valdemoro, Madrid, Spain). Because seeds of *H. squamatum* show physical dormancy (Pérez-García et al., 1995), seeds were mechanically scarified using 500 grit sandpaper before sowing to maximize seed germination. Pots were maintained at field capacity (see below) in the greenhouse of the CULTIVE laboratory at URJC (Móstoles, Madrid, Spain) for ~2 months. Furthermore, to guarantee that the used gypsum soil did not contain seeds of the study species in the seed bank, control pots were also filled and maintained at field capacity without sowing. Control pots showed no germination of *H. squamatum*.

In early October 2020, plants were individually transplanted into 6 L plastic pots (22 cm in height; 20 cm maximum diameter and 17.5 cm minimum diameter; Alpifer, Valencia, Spain) filled with the same gypsum soil, and plants were moved from the greenhouse to the outdoor cultivation facilities where the common garden experiment was performed. The phenotypic characterization of experimental individuals was performed during their second growing season, to maximize that all individuals were in reproductive phase. In early March 2022, after ~2 years of growth under common, well-watered conditions, 2-3 half-siblings per maternal family and population were randomly assigned to two ecologically-meaningful experimental treatments that reflected natural variation in water availability: well-watered and drought (Fig. 1b and c). In the well-watered treatment, plants were kept at field capacity for our gypsum substrate (~24% of soil water content, SWC hereafter; Fig. S1), simulating water availability in

populations with milder climatic conditions and/or during periods with higher soil moisture (e.g., in early spring). In contrast, SWC in the drought treatment was gradually reduced and then maintained at ~50% of field capacity (~12% of SWC; Fig. S1), which simulated water availability at populations from sites with harsher conditions and/or during periods with lower soil moisture (e.g., in early summer). The final size of the experiment was 764 plants (4 regions  $\times$  4 populations/region  $\times$  9 maternal families/population  $\times$  2 watering treatments  $\times$  2-3 half-siblings/treatment).

To implement the watering treatments, we used a drip-irrigation system with Rain Bird XB05PC pressure-compensating emitters (Rain Bird Corporation, CA, USA) and modified the duration and number of watering events to reach the SWC of our watering treatments. During the experiment, SWC was measured thrice a week in 20 random pots per treatment using an HH2 Moisture Meter with an ML3 Sensor (Delta-T devices, Cambridge, UK; Fig. S1). Because natural precipitation could interfere with the watering treatments, pots were moved under rain-exclusion structures (Fig. 1b). These structures excluded all-natural precipitation without substantially affecting environmental conditions (see environmental conditions recorded under rain-exclusion structures in Fig. S2; and details of rain-exclusion structures in Matesanz et al., 2020a). Watering treatments finished when plants in the well-watered treatment began to senesce, lasting approx. 4 months (from mid-March to early July).

### ***2.3. Collection of functional traits***

We measured ecophysiological and reproductive fitness traits in experimental individuals (Table 1, see Methods S1 for detailed protocols on trait collection). During the experiment, we monitored the onset of flowering (first fully-opened flower) in all plants 4-5 times per week. Physiological and leaf morphological traits were characterized at the reproductive peak of experimental individuals (early June 2022). Specifically, we measured leaf area (LA), specific

leaf area (SLA), leaf dry matter content (LDMC), leaf density (LD) and midday photochemical efficiency in dark-adapted leaves ( $F_v/F_m$ ). From the leaves collected to characterize leaf morphological traits, we also determined leaf C and N content, and C and N stable isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) as proxies of integrated water use efficiency (WUE) and N assimilation, respectively (Ariz et al., 2015; Robinson et al., 2000). While the relationship between  $\delta^{13}\text{C}$  and WUE is not universally linear,  $\delta^{13}\text{C}$  remains a reliable indirect measure of WUE, often showing a linear correlation in studies conducted under stable environmental conditions, water limitation, and focused on lower taxonomic levels (e.g. populations of the same species) (Farquhar et al., 1989; Mininni et al., 2022), as in our study. Furthermore, we counted the inflorescences of each plant (NI) and estimated the individual seed mass per plant (ISM). From the latter traits, we determined two integrated variables of reproductive fitness: total seed number (TSN) and total seed mass (TSM). At the onset and the end of the watering treatments, we also measured the height, maximum diameter, and orthogonal diameter to the maximum diameter in all plants. With these measurements, we calculated volume-based relative growth rate (RGR\_vol). Finally, at the end of the experiment we collected all reproductive biomass (RB) and aerial biomass (AB) of all plants and weighed them in an analytical balance.

#### ***2.4. Statistical analyses***

All analyses were performed using R v 4.3.1 (R Core Team, 2023).

##### *2.4.1. Genetic differentiation and patterns of phenotypic plasticity*

To evaluate the presence of genetically-based phenotypic differentiation among regions and the effect of watering treatments on phenotypic traits, we used linear mixed models with restricted maximum likelihood (REML) with individual trait values as the dependent variables. Region, watering treatment, and their interaction were included as fixed factors, family-level seed mass as a fixed covariate, and maternal family and population nested within region as random factors.

Furthermore, to identify the independent effect of regional differences not related to differences in environmental conditions among populations, we also included as covariates the first three axes of an environmental Principal Component Analysis (PCA) performed to summarize the environmental conditions of populations (see below). A significant effect of region in these models would evidence genetically-based phenotypic differences between regions likely due to historical, non-adaptive processes. A significant effect of treatment indicates phenotypic differences across treatments, and a significant region by treatment interaction ( $R \times T$ ) indicates that the effect of drought differs among regions. Models were performed using the *lmer* function (lme4 package; Bates et al., 2015). The significance of fixed factors was assessed using the *Anova* function (package car; Fox and Weisberg, 2011), with type III sum of squares and the Kenward-Roger approximation to determine the residual degrees of freedom. To avoid type I errors related with multiple testing, significance levels for each term of the model were corrected by false discovery rate (FDR; Benjamini and Hochberg, 1995) using the *p.adjust* function. Differences between regions within each watering treatment were assessed via Tukey post hoc tests on marginal means, using the *emmeans* function (package emmeans; Lenth 2019). Furthermore, marginal  $R^2$ , which represents the variance explained by fixed factors, and conditional  $R^2$ , which represents variance explained by both fixed and random factors, were calculated using the *r.squaredGLMM* function (package MuMIn; Barton 2019). When necessary, data were transformed (SLA, LD, RB, AB, NI and TSM were square-root- and FO was log-transformed) in models to meet assumptions of normality and homoscedasticity.

To evaluate the presence of phenotypic differences among populations not explained by regional differentiation, for each trait, we compared the full mixed model described above with a reduced model dropping the factor population by using the *anova* function (package stats) and REML fit. A significant effect in the test comparing both models indicates significant phenotypic differentiation among populations not ascribable to differences among regions.

Because we found significant population differentiation in most traits (i.e., population explained a significant proportion of the phenotypic variance; see Results), we further evaluated the presence of phenotypic differences between populations, the effect of treatments on phenotypic traits, and whether plastic responses differed between populations (i.e., non-parallel norms of reaction between populations;  $P \times T$  interaction) using linear mixed models with restricted maximum likelihood (REML) for each phenotypic trait. These models were performed including population, treatment, and their interaction as fixed factors, family-level seed mass as a fixed covariate, and maternal family as a random factor. A significant effect of population indicates genetically-based phenotypic differentiation among populations; a significant effect of treatment indicates significant phenotypic differences across treatments (phenotypic plasticity); and a significant population-by-treatment interaction indicates differences in plasticity among populations ( $P \times T$ ). Finally, for each trait, we assessed the percentage of phenotypic variance explained by fixed and random factors in the models performed at the regional and the population level. Variance partitioning analyses were performed using the *calcVarPart* function (package *variancePartition*; Hoffman and Schadt, 2016).

#### 2.4.2. Trait–environment and plasticity–environment associations

To determine whether climate and soil conditions were relevant selective pressures driving phenotypic population differentiation and differential plasticity across populations, we assessed the association between i) population mean phenotypes and ii) their plasticity in the common garden experiment, and environmental conditions at the population sites. A significant relationship between environmental conditions and i) the phenotype of populations and ii) their plasticity would indicate the presence of adaptive differentiation and differential plasticity, respectively, driven by a specific environmental factor (Vitasse et al., 2013; Bansal et al., 2015; Solé-Medina et al., 2022, Blanco-Sanchez et al., 2024).

These analyses were conducted by univariate and multivariate Pearson correlations between population trait means in both well-watered and drought conditions, trait plasticity, and local environmental conditions of populations, i.e., climatic variables and soil chemical composition (see Methods S2 for details on specific analyses). In short, environmental conditions of populations were summarized through a PCA (environmental PCA). Four key environmental variables (i.e., bio1: annual mean temperature; bio12: annual precipitation; bio15: precipitation seasonality and the percentage of gypsum) were selected based on their loadings across the first three axes of the environmental PCA. Then, trait means of populations in each watering treatment were determined using linear mixed models, and population trait variation in each treatment was summarized through two PCAs (phenotypic PCAs). For traits showing significant  $P \times T$  interaction, two plasticity indices were calculated to quantify differences in plasticity among populations (RDPIs and phenotypic distances; Valladares et al., 2006), and a multivariate norm of reaction was calculated for each population based on a vector of multivariate phenotypic change by using a PCA (see Blanco-Sánchez et al., 2024 and Solé-Medina et al., 2022 for a similar approach). Pairwise Pearson correlations were performed to evaluate both univariate and multivariate trait-environment and plasticity-environment associations (see Methods S2).

### **3. Results**

#### ***3.1. Phenotypic differentiation among regions***

We observed significant and marginally significant differentiation between regions, particularly in performance and reproductive fitness traits (effect of Region in Table 1 and Fig. 2). Specifically, populations from the Júcar-Segura region showed, on average, lower number of inflorescences, total seed mass and total seed number (Fig. 2), and lower aerial biomass and RGR (Table 1; Fig. 2). Such differentiation was also found in a leaf morphology trait, with

populations from the Ebro region showing significantly higher leaf area (Table 1). When significant, the term Region explained between 1 and 12.6 % of the phenotypic variance (Fig. S3).

For several traits, we also found that phenotypic differences between regions were more pronounced within a particular treatment (significant Region  $\times$  Treatment interaction; Table 1 and Fig. 2), which were mainly due to the stronger differentiation of populations from Júcar-Segura in a given watering treatment. For instance, under well-watered conditions, populations from the Júcar-Segura showed significantly lower total seed number, total seed mass and RGR compared to populations from all other regions. Furthermore, populations from the Júcar-Segura showed significantly higher individual seed mass than populations from the Ebro and Tajo basin and higher  $\delta^{13}\text{C}$  (indicating higher water use efficiency; WUE) compared to populations from the Ebro and Guadalquivir basins. These differences were comparatively lower in the drought treatment (Fig. 2). When the term Region  $\times$  Treatment was significant, it explained between 1.6 and 6.3 % of the variance (Fig. S3).

### ***3.2. Phenotypic differentiation among populations and plasticity to drought***

We also found significant differences between populations that were not ascribable to regional differences in all traits except for aerial biomass, leaf area, and  $\delta^{15}\text{N}$  (Population effect in Table 1 and S2). In other words, populations showed genetically based phenotypic differentiation for most traits, and the Population term explained a substantial amount of variance (Fig. 3).

Phenotypic expression of all functional traits was significantly affected by the watering treatments (Table S2). Indeed, the largest source of variation for most traits was Treatment (13-60%; Fig. 3). In drought conditions, the midday photochemical efficiency ( $F_v/F_m$ ), aerial biomass and RGR were significantly lower compared to the well-watered treatment (a reduction of 31, 20 and 93% respectively, Table S2 and S3). On average, individual plants showed an

earlier flowering onset in response to drought (an advance of ~3 days, Fig. 4a, Table S2). Experimental individuals also showed significant differences across treatments in traits related to leaf morphology, with lower SLA and higher LDMC and LD in the drought treatment (i.e., a reduction of ~14 % and increase of ~16 and 18% in response to drought, respectively; Fig. 4). Furthermore, drought conditions affected experimental individuals in leaf chemical composition traits (Fig. 4; Table S2). Specifically, experimental individuals shown a ~6% higher  $\delta^{13}\text{C}$  and leaf C content, and a ~10 and 20% lower  $\delta^{15}\text{N}$  and leaf N content, respectively, in the drought treatment. Finally, total reproductive biomass, number of inflorescences, total seed number and total seed mass were 2-fold higher in the well-watered treatment compared to drought conditions (Fig. 4; Table S2 and S3).

Phenotypic responses to water availability treatments were generally similar among populations (i.e., no significant population-by-treatment interaction,  $P \times T$ ), as also shown by the variance explained by the interaction Population  $\times$  Treatment, which was almost negligible for most ecophysiological traits (Fig. 3). However, we found significant differences in the plastic responses of populations in reproductive fitness traits (Table S2, Fig. 4). In agreement to patterns of regional differentiation, populations from the Júcar-Segura region showed lower performance and reproductive fitness in the well-watered treatment compared to populations from the other regions (Fig. 4).

The effect of maternal seed size was not significant for most functional traits (except for individual seed mass, Table S2). Conditional  $R^2$  was higher than marginal  $R^2$  in the models of all traits, indicating substantial variation across families within populations.

### ***3.3. Trait–environment and plasticity–environment associations***

The first, second and third axes of the environmental PCA that summarized environmental conditions of populations explained 34.3, 27.26, and 11.13% of the variance, respectively. The



first axis was negatively associated with mean temperature and positively associated with mean precipitation. The second axis was negatively associated with precipitation seasonality (populations with higher values have higher precipitation stability along the year; Fig. S4a). The third axis was negatively associated with soil total N, C, P, and organic matter, and positively associated with soil gypsum content (Fig. S4b).

The phenotypic PCAs for both treatments showed similar variance explained and trait loadings (Fig. S5). PC1 of both phenotypic PCAs explained 38.68 and 39.12% of the variance (well-watered and drought, respectively) and were positively associated mainly with fitness traits, RGR and aerial biomass. PC2 explained 24.11 and 26.23% of the variance (well-watered and drought, respectively) and showed positive loadings for flowering onset, leaf area, leaf density, LDMC and  $F_v/F_m$ , and negative loadings for SLA.

Neither univariate nor multivariate analyses showed a significant association between the environmental (climate and soil composition) conditions at populations sites and i) the phenotypes of populations in both watering treatments or ii) the plasticity of populations for any trait (see Tables S4, S5 and S6).

#### **4. Discussion**

Our research sheds light on the factors underlying intraspecific phenotypic variation and phenotypic plasticity patterns in the dominant gypsophile *Helianthemum squamatum*. Populations responded plastically to drought through changes in morphology, phenology and reproductive fitness, some of which may reflect adaptive plasticity. Plastic responses were generally similar across populations, with mostly absent or weak differentiation in plasticity patterns. Despite the largely common response to drought, we found substantial genetically based phenotypic differences between regions, particularly in reproductive fitness traits, and among populations. However, the strong population differentiation observed was associated

neither with climatic differences nor with differences in soil composition among populations. These results point out to a minor role of climate and soil conditions as drivers of adaptation, suggesting that other abiotic or biotic conditions or non-adaptive evolutionary processes may have influenced population differentiation in *H. squamatum*. Overall, our results indicate that plasticity may be a key mechanism to cope with environmental variation, and provide insights into future population vulnerability to climate change.

#### *4.1. Common patterns of adaptive plasticity to drought across populations*

Drought significantly affected the phenotypic expression of individuals in all functional traits. Some of these responses, including lower growth rates, plant size and photochemical efficiency in drought conditions, likely reflected the strong resource limitation imposed by drought (Matesanz et al., 2020a; Van Kleunen and Fischer, 2005). However, we also found adaptive responses in morphological, phenological and reproductive traits. Specifically, under drought conditions, plants produced more sclerophyllous leaves, with reduced leaf area and SLA, and higher leaf density and LDMC, which has been documented as an adaptation to water limitation in Mediterranean plants (Capon et al., 2009; Lambrecht et al., 2017). Also, experimental individuals showed higher WUE (estimated by  $\delta^{13}\text{C}$  isotope composition) under drought (Fig. 4). Sclerophyllous leaves with higher WUE are typically linked to a more conservative use of resources to reduce water loss and maximize plant survival instead of reproduction (Blanco-Sánchez et al., 2023; Blumenthal et al., 2020; Solé-Medina et al., 2022). However, populations advanced flowering phenology in response to drought, which is frequently associated with higher acquisition and resource-use rates and has been identified as a key trait in characterizing a drought escape strategy (Kooyers, 2015; Pérez-Ramos et al., 2013). Similar to our findings, several studies have described the adaptive value of an advanced phenology in Mediterranean species, as it ensures reproduction before the driest periods by escaping from drought (Blanco-Sánchez et al., 2022; Franks, 2011; Welles and Funk, 2021).

Overall, our results show that plastic responses to drought induced phenotypes associated with both more conservative and acquisitive resource-use strategies. Recent studies with Mediterranean and semiarid species (including gypsophiles) have shown that drought-related acquisitive and conservative strategies can occur simultaneously in patterns of adaptive population differentiation and that drought can trigger plastic responses associated with both strategies (Blanco-Sánchez et al., 2023, 2024; Brouillette et al., 2014; Kooyers et al., 2015; Matesanz et al., 2020a), suggesting the prevalence of this mixed strategy to cope with high levels of abiotic stress.

We also found adaptive plastic responses to drought in reproductive fitness traits. On average, populations produced larger seeds under drought conditions, indicated by the significant effect of treatment and the not significant population-by-treatment interaction in this trait (Fig. 4f; Table S2). Larger seeds may promote germination and increase the likelihood of seedling establishment and survival in water-limited environments (Metz et al., 2010). Previous studies have found the adaptive value of larger seeds in response to drought in different Mediterranean (Matesanz et al., 2020b; Metz et al., 2010) and gypsophile species (Blanco-Sánchez et al., 2024; Ramos-Muñoz et al., 2024). Indeed, in *H. squamatum*, adaptive plasticity in this trait was identified not only in response to immediate drought but also across generations, as a transgenerational effect leading to larger seeds in offspring of drought-stressed maternal plants (Ramos-Muñoz et al., 2024). These results suggest that larger seeds are adaptive to cope with drought and highlight the crucial role of phenotypic plasticity to thrive in the restrictive and heterogeneous environmental conditions of gypsum ecosystems.

Plastic responses to drought were generally similar across populations, as shown by the very small proportion of variance explained by the interaction population-by-treatment for most traits (Fig. 3). These results suggest that, over the evolutionary history of *H. squamatum*, similar plasticity might have been favored across populations, leading to a convergence in their

responses to environmental stress (i.e., homogenizing selection on plasticity; Pigliucci and Kolodynska, 2002). Previous studies have reported similar patterns of plasticity across populations in the dominant gypsophile *Lepidium subulatum*, not only within specific regions (Matesanz et al., 2020a), but also along its entire distribution range (Blanco-Sánchez et al., 2024). Since differences in environmental heterogeneity across populations has been shown to be a major driver of population differentiation in plasticity (Blanco-Sánchez et al., 2023; Kelly, 2019; Lázaro-Nogal et al., 2015), these results may reflect the high spatiotemporal variation that occur in all gypsum habitats, regardless of differences in mean climatic conditions. Nevertheless, plasticity in WUE (estimated through  $\delta^{13}\text{C}$ -Carbon isotope composition; Fig. 2 and Table S2), a key trait in semiarid environments, significantly varied across populations from different regions. Populations with more adaptive WUE responses save water under drought conditions but maximize water use when it is not limited, i.e., they express high WUE only under drought (Nicotra and Davidson, 2010). In contrast, populations from the Júcar-Segura region showed a lower magnitude of plasticity for this trait and the opposite pattern, with a generally higher WUE in well-watered conditions and a lower WUE increase in response to drought. Furthermore, despite the lack of a population-by-treatment interaction in individual seed mass, regional models showed a lower seed mass increase in the populations from the Júcar-Segura region in response to drought (cf. Figs. 2 and 4), which also suggests lower adaptive plasticity in these populations. These differences in plasticity may partly explain the lower reproductive output of populations from the Júcar-Segura region.

#### *4.2. Drivers of regional and population phenotypic differentiation*

Despite the generally similar plastic responses found across populations, we detected significant population differentiation for all traits (Figs. 3 and 4; Tables 1 and S2), part of which was structured at the regional scale (Figs. 2 and S3; Table 1). However, neither regional nor population differentiation were associated with differences in climate and/or soil composition.

Although analyses for individual climatic variables showed not significant differences among regions, the PCA showed a subtle clustering of the Júcar-Segura populations along the axes (see Fig. S4). Therefore, we included the three first axes of the environmental PCA in the models that evaluated phenotypic differentiation among regions. Even after accounting for environmental differences (which did not have a significant effect on phenotypic differentiation; Table 1), these models detected regional differentiation in several ecophysiological, performance and fitness traits including leaf area, aerial biomass, total seed number and total seed mass (Table 1 and Fig. 2). Furthermore, we did not find significant univariate or multivariate associations between the expressed phenotypes in our common garden and the environmental conditions of populations (Table S4 and S6), indicating a lack of clinal variation. Our results contrasted with our hypotheses and previous evidence on the key role of climatic differences among populations as a driver of adaptive differentiation in other Mediterranean and gypsophile species (Blanco-Sánchez et al., 2024; Solé-Medina et al., 2022; Ramírez-Valiente et al., 2022).

Ecophysiological adaptations of *H. squamatum* may partly explain these results. This species can use different water sources during the growth and reproductive season, changing from free water during spring to water contained in gypsum crystals during summer (De La Puente et al., 2022; Palacio et al., 2014), which would consequently alleviate water scarcity and diminish the action of selection exerted by drought. *Helianthemum squamatum* also has shown a lower reduction in photochemical efficiency under drought in natural conditions compared to other species (Aragón et al., 2008, 2009). These features, combined with steep changes in leaf angle during the day in this species (described as an adjustment to prevent heat and photosynthetic damage, e.g., Aragón et al., 2008; Pearcy et al., 2005), suggests the ability of this species to withstand high levels of abiotic stress.

Alternatively, even though our study evaluated the main abiotic selective pressures in Mediterranean gypsum ecosystems, other unmeasured abiotic and biotic factors, and importantly, combinations of environmental factors, may underlie the detected patterns of population differentiation. Under natural conditions, gypsophile populations experience a complex mosaic of environmental variation that may interact with the effects of drought, both abiotic (e.g. warming) and biotic factors (e.g. antagonistic interactions like competition or herbivory). These complex interactions may lead to non-clinal combinations of selective pressures, making it challenging the detection of patterns of adaptive intraspecific variation. Future common garden studies should incorporate realistic combinations of environmental factors reflecting natural variation to improve our assessment of adaptive phenotypic differentiation.

Finally, the lack of adaptive population differentiation and the presence of regional phenotypic structure in *H. squamatum* might reflect the influence of non-adaptive neutral evolutionary processes rather than past natural selection associated with environmental gradients. Previous studies have shown a stronger spatial genetic structure at the landscape level and higher  $F_{ST}$  (population differentiation assessed via molecular markers) compared to other gypsophile species, indicating more limited gene flow in the study species (Blanco-Sánchez et al., 2021; Matesanz et al., 2018). Limited dispersal ability is also evident locally, as *H. squamatum* often forms dense patches of conspecific individuals, a pattern likely favored by the presence of an anchoring mucilage on their seeds (Escudero et al., 1999). This limited gene flow and dispersal ability, along with high anthropogenic and natural fragmentation of gypsum ecosystems, may explain the observed patterns of regional and population phenotypic differentiation. These results would suggest that historical colonization events and uneven gene flow patterns have likely influenced intraspecific population differentiation in *H. squamatum*.

(Bailey et al., 2006; Keller and Taylor, 2008) and agree with other studies highlighting the role of neutral processes on intraspecific variation (López-Goldar et al., 2019; Voltas et al., 2024).

Non-adaptive evolutionary processes in *H. squamatum* may also underlie the fitness differences observed between populations of different regions. Specifically, populations from the Júcar-Segura region had lower reproductive fitness in our common garden, i.e., lower total seed number and total seed mass in both experimental conditions. These populations have smaller sizes (personal observation), increasing the likelihood of experiencing bottlenecks, founder effects, genetic drift and inbreeding. These factors can lead to genetic load, which would partly explain the lower fitness of the populations from this region (Blanquart et al., 2013; Franks et al., 2014; Shaw and Etterson, 2012; Whitlock and Davis, 2011). Furthermore, these processes may lead to lower intrapopulation genetic diversity, consequently hampering further adaptive evolution in response to climate change (Franks et al., 2014). Importantly, these results reflect the vulnerability of the populations from the Júcar-Segura region in a climate change context.

## 5. Conclusions

Our results revealed high but generally similar patterns of adaptive plasticity among populations of *H. squamatum* in response to drought, suggesting the key role of phenotypic plasticity to thrive in the stressful semiarid conditions of gypsum habitats. We found significant genetically-based phenotypic differentiation among populations, which, contrary to our expectations, was not associated with climatic or edaphic gradients. These results contrast with previous findings in other gypsophile species, suggesting that co-occurrent dominant gypsophiles with similar ages of divergence and subjected to the same selective pressures may have contrasting evolutionary trajectories and consequently, patterns of adaptive differentiation. Our results highlight the need to understand how the interplay between adaptive evolution,

phenotypic plasticity and non-adaptive processes determine the patterns of intraspecific phenotypic variation, and consequently how plant populations will respond in a climate change context.

### **CRedit authorship contribution statement**

Marina Ramos-Muñoz: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. María Clara Castellanos: Investigation, Supervision, Writing – review & editing. Mario Blanco-Sánchez: Investigation, Data curation, Writing – review & editing. Beatriz Pías: Investigation, Resources, Data curation, Supervision, Writing – review & editing. José Alberto Ramírez-Valiente: Investigation, Writing – review & editing. Raquel Benavides: Investigation, Writing – review & editing. Adrián Escudero: Investigation, Resources, Supervision, Writing – review & editing. 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.

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### **Data availability statement**

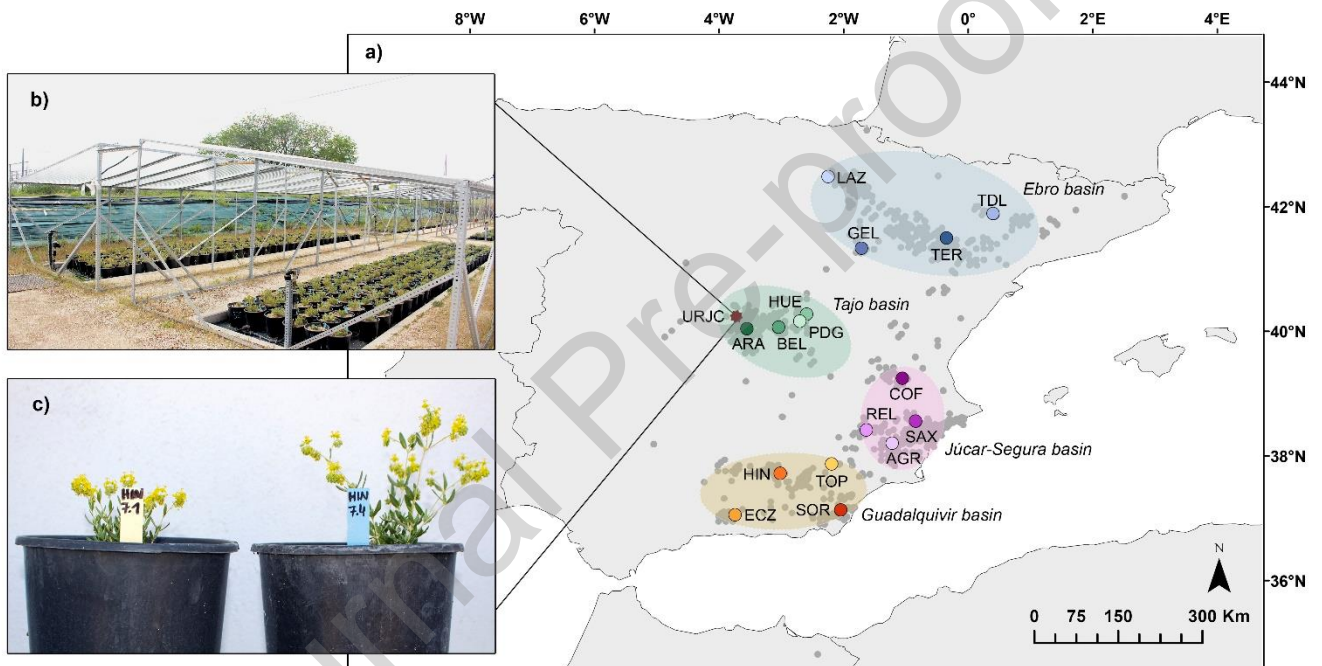
Phenotypic data will be uploaded to a public repository upon manuscript acceptance.



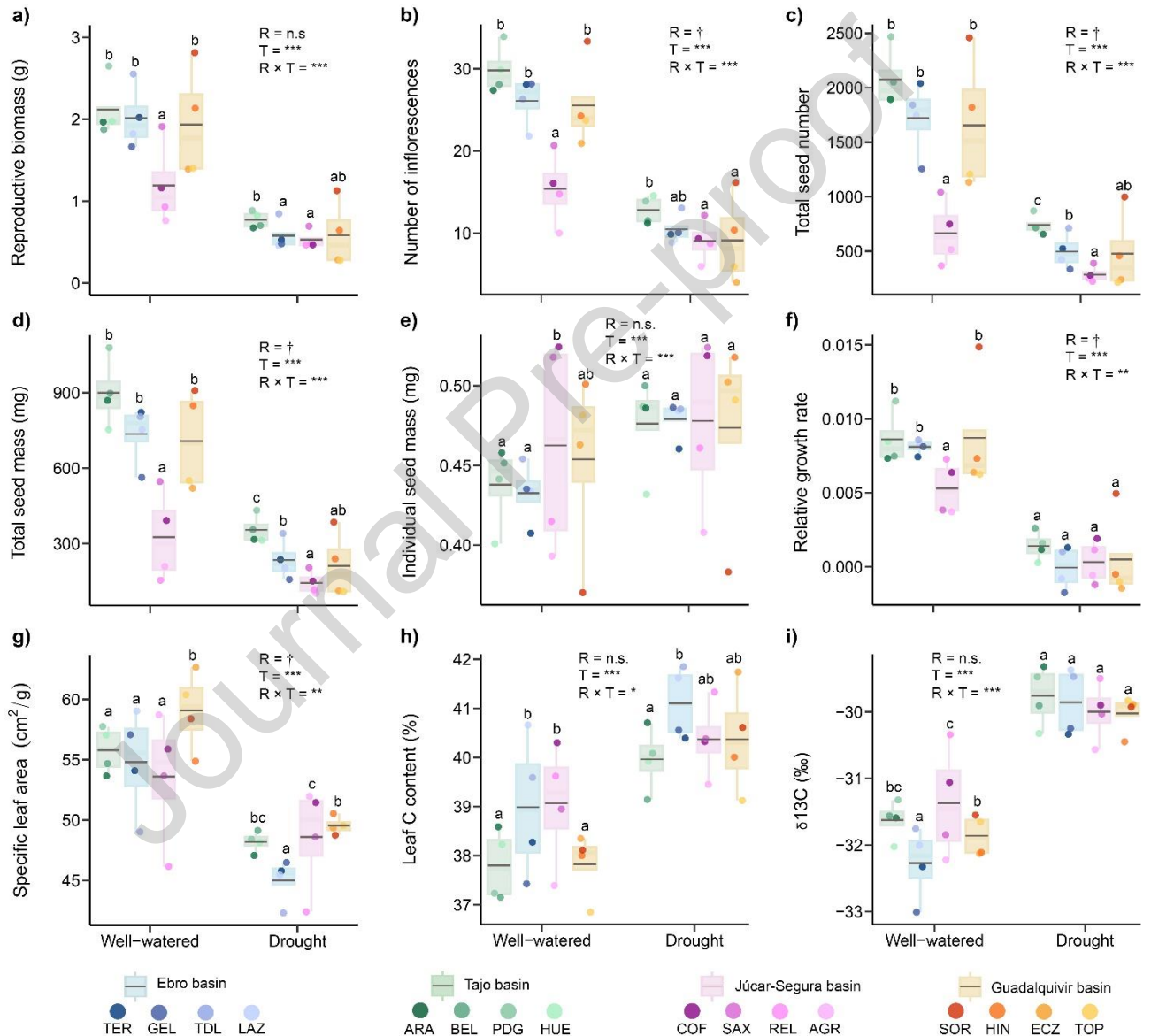
**Table 1.** Results of linear mixed models testing the effects of region, treatment, their interaction on functional traits. The three first axes of environmental PCA were included as fixed covariates to differentiate the effect of neutral evolutionary processes and adaptive processes associated with environmental differences on phenotypic differentiation. Family and population (nested in region) were included as random factors, and the significance of the random term Population is also included in the table. F-statistic ( $\chi^2$  for the random factor) is shown for each term. Significant terms are shown in bold ( $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ; † indicates a marginally significant effect (in italics),  $0.05 < P < 0.1$ ).  $R^2_m$  = marginal  $R^2$ ;  $R^2_c$  = conditional  $R^2$ .  $df = 1$  for all fixed terms. See text for details on statistical analysis.

	Region	Treatment	Region × Treatment	Maternal seed size	PC1	PC2	PC3	Population	$R^2_m$	$R^2_c$
	<i>F</i>	<i>F</i>	<i>F</i>	<i>F</i>	<i>F</i>	<i>F</i>	<i>F</i>	$\chi^2$		
<b>Ecophysiological traits</b>										
Reproductive biomass (RB)	2.49 5	<b>663.2</b> ** 33 *	<b>8.01</b> ** 7 *	0.32 9	0.0 66	1.2 46	<i>0.1</i> 88	<b>29.4</b> ** 65 *	0.4 59	0.6 28
Number of inflorescences (NI)	<b>6.81</b> 2 †	<b>564.3</b> ** 85 *	<b>10.2</b> ** 49 *	0.08 8	0.6 88	1.2 21	1.6 38	<b>8.60</b> ** 1 *	0.4 57	0.5 81
Total seed mass (TSM)	<b>4.02</b> 6 †	<b>486.3</b> ** 21 *	<b>7.01</b> ** 25 *	0.78 7	0.0 84	1.1 67	0.1 35	<b>19.9</b> ** 54 *	0.4 35	0.6 06
Total seed number (TSN)	<b>5.52</b> 7 †	<b>545.8</b> ** 53 *	<b>9.09</b> ** 2 *	3.74 6	0.0 01	1.8 62	0.0 56	<b>16.8</b> ** 6 *	0.4 71	0.6 34
Individual seed mass (ISM)	2.33 7	<b>57.81</b> ** 4 *	<b>4.35</b> ** 9 *	<b>52.3</b> ** 46 *	5.8 19	0.9 43	1.2 96	<b>15.2</b> ** 22 *	0.3 39	0.5 13
Relative growth rate (RGR)	<b>4.78</b> 8 †	<b>491.97</b> ** 4 *	<b>5.36</b> ** 2 **	0.01 1	0.5 42	5.1 47	0.9 78	<b>15.2</b> ** 22 *	0.4 16	0.5 29
Aerial biomass (AB)	<b>5.26</b> †	<b>70.627</b> ** *	1.14 7	0.01 5	0.1 8	0.0 85	4.4 68	2.04 4	0.2 14	0.3 32
Leaf area (LA)	<b>10.4</b> 86 *	<b>214.75</b> ** 4 *	0.11	2.22 7	8.3 08	0.4 11	1.1 3	0.08 1	0.3 14	0.4 07
Specific leaf area (SLA)	0.79 6	<b>366.31</b> ** 2 *	<b>4.96</b> ** 5 **	1.44 4	0.1 21	0.5 72	1.9 81	<b>20.4</b> ** 68 *	0.3 23	0.5 63
Leaf density (LD)	1.09 7	<b>241.62</b> ** 5 *	0.83 1	1.96 5	2.6 25	1.2 38	4.5 64	<b>24.5</b> ** 78 *	0.2 78	0.3 69
Leaf dry matter content (LDCM)	1.14 3	<b>546.85</b> ** 2 *	0.65 7	2.36 2	2.3 92	0.9 54	4.3 44	<b>33.0</b> ** 01 *	0.4 09	0.5 79
F <sub>v</sub> /F <sub>m</sub> (Fv/Fm)	0.95 2	<b>216.27</b> ** 2 *	0.58 6	1.86 1	0.0 03	2.0 10	0.5 28	<b>4.85</b> * 8	0.2 45	0.3 11
Flowering onset (FO)	0.25 3	<b>144.09</b> ** 3 *	1.95 9	0.08 3	1.5 60	0.0 74	0.6 38	<b>22.1</b> ** 40 *	0.1 43	0.5 23
Leaf C content (C)	0.89 7	<b>65.618</b> ** *	<b>3.01</b> * 9 *	1.39 3	1.2 41	0.5 49	3.8 60	<b>12.8</b> ** 36 *	0.3 13	0.5 13
Leaf N content (N)	0.85 7	<b>2576.0</b> ** 41 *	1.08 5	0.24 4	0.5 92	0.3 30	2.5 06	<b>11.2</b> ** 96 *	0.3 26	0.4 40
δ <sup>13</sup> C (d13C)	1.52 0	<b>595.83</b> ** 0 *	<b>7.67</b> ** 1 *	0.05 2	2.6 04	0.5 56	1.9 73	<b>7.90</b> ** 1 *	0.4 87	0.6 49
δ <sup>15</sup> N (d15N)	1.13 4	<b>41.601</b> * *	0.32 2	0.87 0	0.0 21	0.2 87	0.6 30	0.30 4	0.3 26	0.4 40

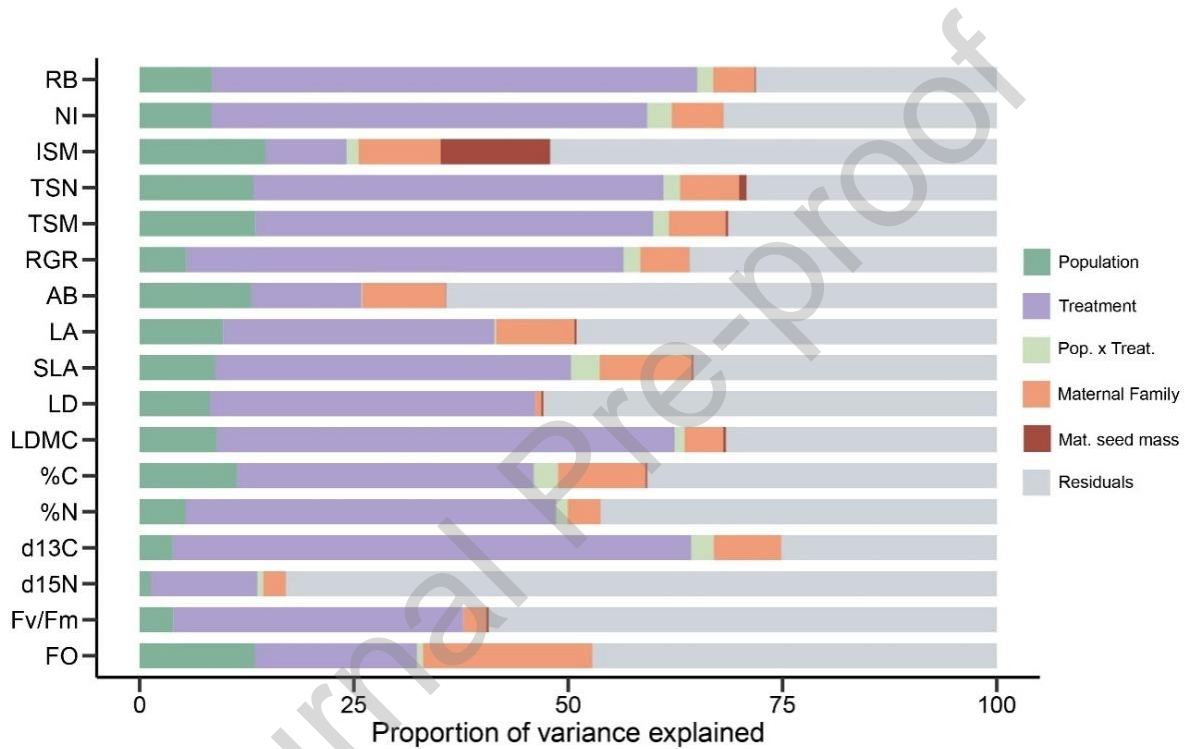
**Figure 1.** a) Location of sampled populations and worldwide distribution of *Helianthemum squamatum* (grey-colored dots; data extracted from GBIF.org (23 June 2020) GBIF Occurrence Download <https://doi.org/10.15468/dl.twuds9>). Sampling of selected populations followed a hierarchical approach, with populations (represented with differently-colored dots) nested within regions (ovals). Regions correspond to different river basins in the Iberian Peninsula and distinct gypsum outcrops (see text). Note that climatic conditions did not significantly differ among regions, with substantial climatic variation found within regions. Coordinates, main climatic variables, and codes of population can be found in Table S1; b) Common garden set up, with experimental individuals growing below rain exclusion structures used to avoid all-natural precipitation; c) Effect of the watering treatments on phenotypic expression. Half-siblings from the same maternal family and population grown in well-watered (right) and drought (left) treatments are shown.



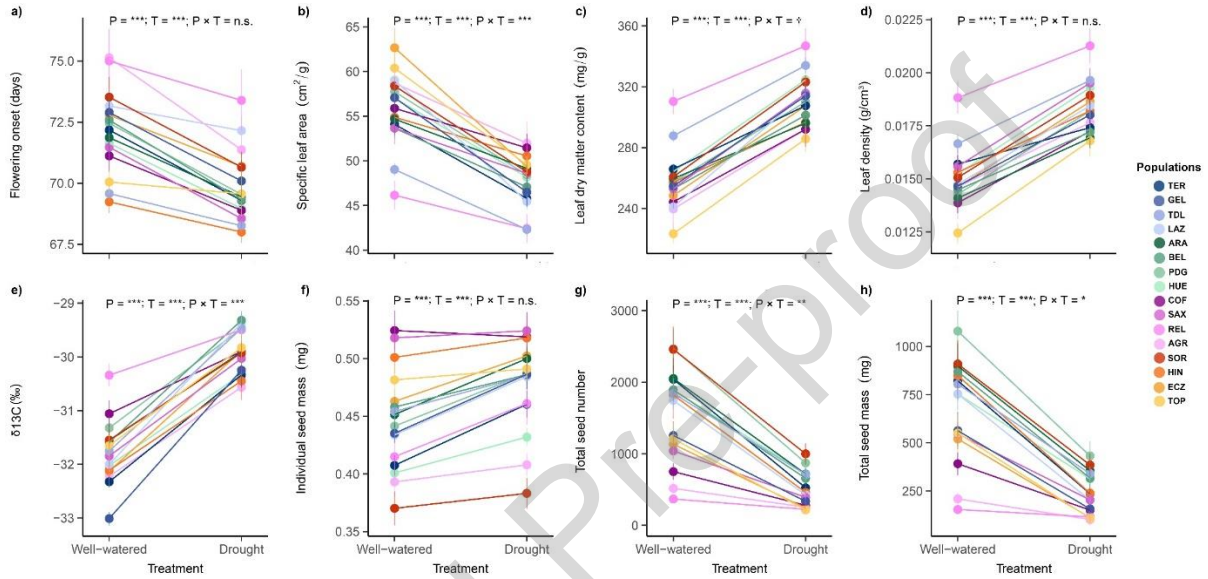
**Figure 2:** Phenotypic variation across sampled regions and watering treatments: a) Reproductive biomass; b) Number of inflorescences; c) Total seed number; d) Total seed mass; e) Individual seed mass; f) Relative growth rate; g) Specific leaf area; and i)  $\delta^{13}\text{C}$  (leaf Carbon stable isotope ratio). Each boxplot shows median, first and third quartiles and mean phenotype (black line) for each region in each watering treatment. Upper and lower whiskers show 1.5 times the interquartile range. Dots represent mean phenotype of different populations. Significance of region (R), treatment (T) and their interaction ( $R \times T$ ) are shown for each trait. Different letters indicating significant ( $P < 0.05$ ) differences among regions using post hoc tests. Significance levels: n.s. = not significant; †  $0.05 < P < 0.1$ ; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .



**Figure 3.** Variance partitioning for each trait of fixed and random factors from the linear mixed-effects models employed to evaluate the presence of population differentiation (Population term), phenotypic plasticity (Treatment term) and differential plasticity among populations (Population  $\times$  Treatment term). Stacked bars show the percentage of variation explained by each explanatory variable (in different colors). FO: Flowering onset (days);  $F_v/F_m$ : midday photochemical efficiency;  $\delta^{15}\text{N}$ : leaf Nitrogen isotope ratio (‰);  $\delta^{13}\text{C}$ : leaf Carbon isotope ratio (‰); %N: leaf Nitrogen content; %C: leaf Carbon content; LDCM: Leaf dry matter content (mg/g); LD: Leaf density ( $\text{g}/\text{cm}^3$ ); SLA: Specific leaf area ( $\text{cm}^2/\text{g}$ ); LA: Leaf area ( $\text{cm}^2$ ); AB, Aerial biomass (g); RGR: Relative growth rate; TSM: Total seed mass (mg); TSN: Total seed number; ISM: Individual seed mass (mg); NI: Number of inflorescences; RB: Reproductive biomass (g).



**Figure 4.** Phenotypic variation of populations across watering treatments (well-watered and drought) in ecophysiological traits: a) Flowering onset (days); b) Specific leaf area ( $\text{cm}^2/\text{g}$ ); c) Leaf dry matter content ( $\text{mg}/\text{g}$ ); d) Leaf density ( $\text{g}/\text{cm}^3$ ); e)  $\delta^{13}\text{C}$ , leaf Carbon isotope ratio (‰); f) Individual seed mass (mg); g) Total seed number; and h) Total seed mass (mg). Each line represents the norm of reaction of each population for a given trait. Mean values and standard error of each population in each treatment are shown (9 maternal families per population and 2-3 half-siblings per family and treatment). See Table S2 for more details of results of linear mixed models for each trait. Significance of population (P), treatment (T) and their interaction ( $P \times T$ ) are shown for each trait. Significance levels: n.s. = not significant; †  $0.05 < P < 0.1$ ; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).



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### **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.

### **Highlights**

- Populations of a Mediterranean plant show high but similar adaptive drought plasticity
- We found significant phenotypic differentiation between populations and regions
- Contrary to expectations, differentiation was not linked to environmental gradients
- Regional trait differences suggest the role of neutral processes on phenotypic differentiation