RESEARCH PAPER

Heat and drought determine flower female allocation in a hermaphroditic Mediterranean plant family

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ABSTRACT

- In animal-pollinated hermaphroditic species, larger and xenogamous flowers increase male-biased resource allocation, whereas smaller and selfing flowers invest disproportionally more resources to female function. In *Cistaceae*, an entomophilous and hermaphroditic Mediterranean family, this pattern generally follows a phylogenetic signal. However, resource allocation to carpels is independent of phylogeny, which suggests trait divergences among closely related species during the diversification into different environmental conditions.

- We tested this hypothesis across 37 species of *Cistaceae* along a temperature and precipitation gradient, including semiarid, dry, subhumid and humid sites. We quantified the proportions of dry mass and nutrient investment to carpels and tested the influence of the climatic gradient and site-specific precipitation on the interspecific variation in carpel resource allocation.

- Lowest and highest percentages of resource allocation to carpels ranged from 1.5–4.2% to 24.2–36.6%, respectively. The proportion of resources comprised in carpels significantly decreased with increasing precipitation/decreasing temperature. Thus, carpels comprised proportionally more resources under drier and hotter conditions, especially in semiarid sites.

- Our results demonstrate how the extent of climatic constraints is more important than phylogenetic relationships in determining stress-induced differences in carpel resource allocation across species of *Cistaceae* in a Mediterranean environment. We suggest that allocation of proportionally more resources to carpels in drier and hotter sites lies within a strategy to deal with the most stressful conditions by means of a high reproductive effort.

INTRODUCTION

An optimal resource allocation to floral structures determines plant reproductive success and maximises fitness rates. In animal-pollinated plants, flowers must appropriately invest the limited available carbon and nutrients into attractive corollas, protective and photosynthetic sepals, pollinator rewards, sexual structures and viable gametes (Ashman 1994; Case & Ashman 2005; Teixido *et al.* 2017). For example, the perianth requires sizeable amounts of carbon whereas sexual structures are especially costly in terms of nutrients such as nitrogen and phosphorus, due to the high protein content related to pollinator rewards, pollen tube growth, fertilisation and subsequent resource supplies to set fruit (Cruden & Lyon 1985; Ågren 1988; Roulston *et al.* 2000; Brock *et al.* 2017). Flower nutrient allocation is also influenced by site-specific nutrient availability, which may ultimately limit pollen and seed production (Arnan *et al.* 2014; Chen *et al.* 2017).

In hermaphrodites, the relative investment to each floral structure governs male–female function balance. Theoretically, sexual expression pattern is dependent on each gender's fitness gain curve, which usually entails an allocation biased towards one of the two sexes (Lloyd & Bawa 1984; Klinkhamer *et al.* 1997; Campbell *et al.* 2011). Thus, increased pollen–ovule ratios and allocation to secondary structures such as attractive corollas improves mating opportunities for the male function. Conversely, femaleness increases with a proportionally higher resource allocation to carpels and sepals, which ultimately supply the high carbon required for seed production. Most studies quantifying hermaphroditic flower sexual allocation have considered plant size-mediated intraspecific variation, with female-biased sex allocation in larger plants (*e.g.* Klinkhamer *et al.* 1997; Cao & Kudo 2008; Xiong *et al.* 2016).

Alternatively, differences among species in resource allocation to hermaphroditic flowers have been related to breeding systems and pollinator dependence, which are ultimately mediated by changes in flower size (Lloyd 1980; Cruden & Lyon 1985; Goodwillie *et al.* 2010). Hence, large and xenogamous flowers invest a higher percentage of resources in corollas and stamens, whereas smaller-flowered autogamous species assign proportionally more resources to sepals and carpels (Cruden & Lyon 1985; Teixido *et al.* 2017). Interspecific variation in floral resource allocation may also be strongly adaptive and conserved (*i.e.* phylogenetically constrained), as flowers are directly

involved in the initial phases of reproduction that determine fitness (Armbruster *et al.* 1999; Brock & Weinig 2007; Oguro & Sakai 2015). Indeed, Teixido *et al.* (2017) recently reported that dry mass and nutrient allocation to floral structures, except for carpels, was dependent on flower size, and showed a strong phylogenetic signal across related species of *Cistaceae*, a common Mediterranean hermaphroditic family. This suggests that flower size-dependent resource investment to maleness in this family is a conserved trait inherited across species diversification, while resource allocation to carpels seems instead to be labile, suggesting trait divergences among closely related species during the adaptation to different environmental conditions (see Ackerly 2009). The potential of variation in resource investment to the primary female structure of flowers among phylogenetically related lineages may not be uncommon in animal-pollinated plants, as hermaphroditic plants may independently balance allocation to carpels to assure fruit and seed production (*e.g.* Cruden & Lyon 1985; Campbell 2000; Chen *et al.* 2017).

Variations among multiple species in floral female allocation beyond differences in flower size, and alternative plausible explanations of phylogeny (*e.g.* environmental conditions), have so far been little studied and deserve further attention. Two independent studies showed that increasing elevation and subsequent cooler temperatures were associated with more attractive and pollen-rewarding, male-biased flowers in 12 species of the hermaphroditic genus *Pedicularis* on the Tibetan Plateau (Guo *et al.* 2010; Zhang *et al.* 2011), likely due to relative scarcity of pollinators (see also Crowley *et al.* 2017 for sex allocation patterns under pollen limitation). Likewise, a high reproductive effort by allocating a larger resource pool to seed production seems to be an effective strategy to deal with dry and hot conditions or nutrient-poor soils (Lloyd & Bawa 1984; Aragón *et al.* 2009; Chen *et al.* 2017). Therefore, to consider the variation of specific environmental characteristics, such as particular climate conditions at the site wherein one species occurs, is of paramount importance to fully understand female resource allocation to flowering among phylogenetically related species.

In this study, we evaluate the effects of a gradient of precipitation and temperature on the variation in direct resource investment to the primary female structure of flowers (carpels) in terms of dry mass and nutrients (N and P) across multiple species of *Cistaceae*. Dry mass mostly includes carbohydrate, protein and genetic material, comprising a reliable estimate of carbon allocation to plant organs (Niklas 1994; Lambers *et al.* 2008; Poorter *et al.* 2012). *Cistaceae* is an entomophilous and hermaphroditic family, common in Mediterranean environments, occurring across different habitats along a variable altitudinal, latitudinal and climatic gradient (Herrera 1992; Arrington & Kubitzki 2003). We used the data for resource allocation to carpels from all 23 species analysed in Teixido *et al.* (2017), and include unpublished data for 14 additional species, together with site-specific climatic information (*i.e.* precipitation and temperature). We specifically expected that species inhabiting drier and hotter sites would have increased dry mass and nutrient allocation to carpels. Such evidence would provide support to predictions of higher reproductive investment-based arguments, which could potentially explain the patterns of floral female-biased allocation under the most stressful climate conditions.

MATERIAL AND METHODS

Species and study area

Cistaceae comprises eight genera and about 200 species of herbs and shrubs from temperate sites in Europe and North America, with some species also occurring in South America (Arrington & Kubitzki 2003). The centre of diversification of the family is the Mediterranean region, especially the Iberian Peninsula, with five genera and 64 species mainly inhabiting dry and open areas (Arrington & Kubitzki 2003). The plants display hermaphroditic, disc-shaped and many single flowers, with five pink, white or yellow petals, between February and late June. Species show both outcrossing and autogamous breeding systems and contrasting flower sizes, ranging from 8 to 120 mm in diameter (Herrera 1992; Teixido *et al.* 2017). Stamens are usually numerous and free, containing variable amounts of pollen grains (Herrera 1992). The gynoecium comprises 3–12 carpels, one style and one full circular stigma, and there is large variation in the number of ovules in the ovary, ranging from ca. seven to 1200 (Herrera 1992).

A sampling of 37 species of Iberian *Cistaceae* was selected. Considering the diversity of the family in this area, this sampling is representative, as it comprises more than half of the species and an illustrative proportion of each genus necessary to test our hypothesis. The studied species also cover most climatic ranges and distribution habitats of the family (Table S1). Overall, nine species of *Cistus*, five of *Fumana*, six of *Halimium*, 14 of *Helianthemum* and three of *Tuberaria*, occurring in ten semiarid, 14 dry, nine subhumid and four humid sites, were selected (Table S1).

Resource allocation to carpels

Between 2009 and 2010, during the flowering peak of every species, 15 plants from a single population per species were randomly selected and two mature flower buds, close to opening, including the pedicel, were randomly harvested per individual and kept in 70% alcohol. Flower buds are essential to assess reliable values of resource allocation because all pollen is comprised in the anthers and no pollen grains are deposited on the stigma. Flower buds were divided into the floral structures, *i.e.* pedicels, sepals, petals, carpels and stamens. To obtain dry mass (mg), all these structures were subsequently oven-dried and weighed with a microbalance (see Teixido *et al.* 2017 for details). For dry mass (µg) and concentration (mmol·g^{-1} dry mass) of nitrogen (N) and phosphorus (P), floral structures were digested in sulphuric acid and analysed by means of a SKALAR San++ Analyzer (Skalar, Breda, The Netherlands; see also Teixido *et al.* 2017 for details). Lastly, mean percentages of dry mass, N and P allocated to carpels per species were calculated as the investment to this floral structure over the total investment to a flower for each species.

To evaluate the variation in dry mass and nutrient allocation to carpels along the climatic gradient, we assessed mean annual temperature and precipitation from each collection site using the Digital Climatic Atlas of the Iberian Peninsula (1951–1999; Ninyerola *et al.* 2005). We used mean annual temperature and precipitation instead of values during the flowering period as a suitable proxy for the particular climatic conditions of each site wherein each study species occurs for two main reasons. First,

resource allocation to reproduction is closely related to annual climatic variation, regardless of flowering season (Sletvold & Ågren 2015). Second, most Mediterranean species flower during spring following a climatic gradient (*i.e.* flowering time is delayed with increasing mean annual precipitation/decreasing mean annual temperature: Arroyo 1990; Thompson 2005). Therefore, site-intrinsic microclimatic conditions are similar during flowering periods (see Fig. S1 for our study species). Nevertheless, due to the presence of five annual species in our dataset, we included further analyses considering temperature and precipitation during the flowering period. For this, we used mean temperature and precipitation for the month corresponding to the flowering peak from each collection site (Ninyerola *et al.* 2005). Following the precipitation-based climatic conditions, we subsequently classified the sites wherein each study species occurs as semiarid, dry, subhumid or humid (Rivas-Martínez *et al.* 2017; see Table S1 for details).

Statistical analysis

To determine the effects of the gradient of precipitation and temperature on interspecific variation in resource allocation to carpels, we used regression models. First, we estimated phylogenetic signal for precipitation and temperature across the phylogeny previously reconstructed for 23 species (Teixido *et al.* 2017). We applied Blomberg's K-statistics (Blomberg *et al.* 2003) implemented in the PICANTE R library (Kembel *et al.* 2010), the most frequently used model-based approach in comparative studies. Neither precipitation (K = 0.17, $P = 0.320$) nor temperature (K = 0.22, $P = 0.306$) showed phylogenetic signals. As dry mass and nutrient allocation to carpels did not show phylogenetic conservatism (Teixido *et al.* 2017), we considered the independence among the set of species in our analyses.

Mean annual precipitation and temperature were highly negatively related (Spearman's rank correlation = –0.73, $P < 0.001$, n = 37). To include both variables in our model and avoid collinearity problems, we conducted a principal components analysis (PCA), which combined precipitation and temperature in a single PC component (PCA1). Variables were standardised using the *rda* function of the VEGAN package in R (Oksanen *et al.*, 2019). As PCA1 explained 86.2% of variance, we regressed each response variable related to resource allocation to carpels against PCA1 scores as a proxy for climatic conditions at each site. For N and P concentration, we used linear regressions, and for percentages of dry mass, N and P, we conducted quasi-Poisson regressions. In this case, pseudo-coefficients of determination (R^2) were estimated as $1 - (D_{model}/D_{null})$, where D_{model} and D_{null} are the residual deviances of the regression model and of the corresponding null model, respectively. Precipitation and temperature during the flowering period did not relate to each other (Spearman's rank correlation = –0.27, $P = 0.171$, n = 37) or show a phylogenetic signal (K = 0.26, $P = 0.122$ for precipitation and K = 0.11, $P = 0.723$ for temperature). Therefore, we separately regressed each response variable related to resource allocation to carpels against precipitation and temperature. We used the same regression models and estimated R^2 for each response variable as stated above.

We additionally tested for significant differences in resource allocation to carpels among sites (*i.e.* semiarid, dry, subhumid

and humid; fixed factor) by fitting ANOVAs and GLMs, depending on the response variable (*i.e.* percentage of dry mass, N and P, and N and P concentration). For N and P concentration, we fitted general linear model ANOVAs. Assumptions of normality and homogeneity of variance were tested using Shapiro–Wilks test and Levene test, respectively. For percentages of dry mass, N and P, we fitted GLM considering quasi-Poisson distribution to reduce variance overdispersion (Ver Hoef and Boveng 2007). Significant differences among sites were tested using Tukey *post-hoc* tests. All analyses were performed with the R software (R Development Core Team 2017).

RESULTS

On average, carpels in *Cistaceae* comprised 5.8% (range: 1.5–26.7%; Table S2) of the total dry mass allocation to flowers. Resource allocation to carpels along the climatic gradient, except for nutrient concentration, significantly decreased with increasing mean annual precipitation and temperature (Fig. 1A–E; but see marginal differences for the percentage of P allocation; Fig. 1C). Precipitation during the flowering period showed significant differences among semiarid and humid sites ($\chi^2 = 8.02$, $P = 0.046$), but temperature was similar ($\chi^2 = 1.93$, $P = 0.588$; Fig. S1). Accordingly, resource allocation was not related to precipitation or temperature during the flowering peak (Table S3).

Percentage dry mass showed an almost twofold significant increase in species inhabiting drier and hotter sites (*i.e.* semiarid and dry, Fig. 2; see also Table 1 for the full model). For nutrients, the percentage N allocation was significantly lower than the percentage P allocation (mean: 10.2% *versus* 14.0% for N and P, respectively; paired *t*-test$_{1,36}$ = –7.28, $P < 0.001$). However, the site-dependent interspecific variation range (1.7–24.2% for N and 4.2–36.6% for P) was only significant for N (marginal significance; Table 1). In this case, N allocation significantly increased ca. 60% in semiarid sites in relation to subhumid and humid sites (Fig. 2). Relative to concentration, both N and P showed significant differences among sites (Table 1), increasing approx. 35–55% in the semiarid site (Fig. 2).

DISCUSSION

We demonstrate that resource allocation to carpels varies in relation to a precipitation and temperature gradient across *Cistaceae* species, a typical entomophilous and hermaphroditic Mediterranean family. We confirm that female-biased allocation at the flowering stage increases in species inhabiting drier and hotter sites. Interspecific variation in resource allocation to the primary female structure of flowers has previously been associated with intrinsic factors such as breeding systems (Cruden & Lyon 1985) and phylogeny-dependent variation in flower size (Teixido *et al.* 2017). Similar to our results, two independent studies have also reported the importance of climate conditions on the proportion of resource allocation per flower to female function, which decreased with elevation across 12 species of *Pedicularis* in an alpine habitat (Guo *et al.* 2010; Zhang *et al.* 2011). However, these studies are limited in that the effects of elevation on carpel allocation did not consider the history of divergence in the genus. Our multispecies
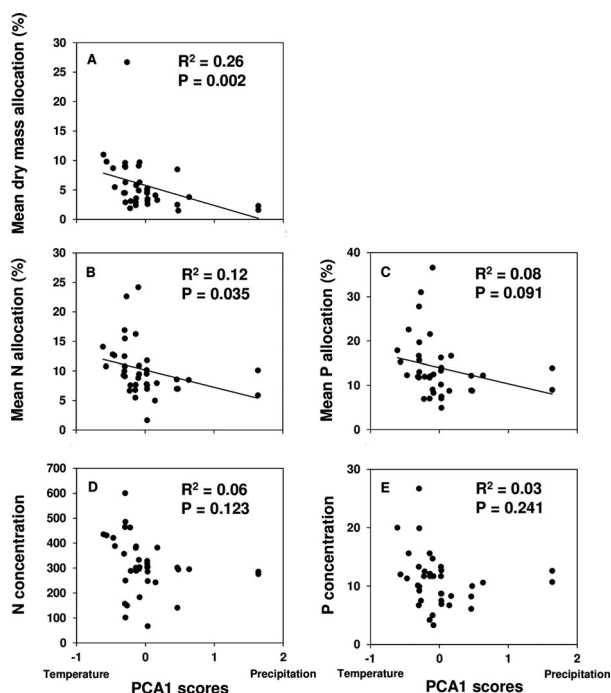
Fig. 1. Regression analyses for (A) percentage dry mass, (B) percentage N, (C) percentage P, (D) N concentration and (E) P concentration in carpels *versus* mean annual precipitation per site in the 37 studied species of *Cistaceae*. N and P concentration were tested by means of linear regressions with *F*-tests. Percentages of dry mass, N and P were tested using quasi-Poisson regressions with χ^2.

approach confirms that resource allocation to carpels is a labile and non-conservative trait that converges among distantly related species that have diversified into similar ecological niches.

As predicted, species from drier and hotter sites, especially the semiarid, allocated more resources to carpels. This pattern indicates how the extent of climatic constraints is more important than phylogenetic relationships in determining stress-induced differences in female resource allocation. These findings support the assumption that allocation of proportionally more resources to carpels may guarantee high reproductive output under the most stressful conditions (Lloyd & Bawa 1984; Galen 2000; Zhao *et al.* 2008; Chen *et al.* 2017). This is especially relevant for N and P, as female-biased nutrient allocation to flowers increases fruit and seed production in stressful environments (Case & Ashman 2005; Arnan *et al.* 2014; Chen *et al.* 2017). We also measured nutrient concentration, which provides valuable information about differential allocation among species, regardless of allocation in terms of mass (*i.e.* interspecific nutrient concentration can be similar despite increasing nutrient mass allocation). Although we did not detect any difference in nutrient concentration along the precipitation and temperature gradient, our results still reinforce the importance of investing more nutrients per unit mass to carpels and potentially to seed production in the semiarid area (Fig. 2).

Our results also emphasise the significance of flowering costs and constraints imposed by heat and drought in a Mediterranean ecosystem (*e.g.* Thompson 2005). To endure stress,

large and highly persistent seed banks are not uncommon for plants occurring in semiarid Mediterranean regions (Caballero *et al.* 2005; Olano *et al.* 2012; Escudero *et al.* 2015). In fact, this seems to be the strategy of *Helianthemum squamatum*, a perennial shrub of *Cistaceae* inhabiting semiarid environments, combined with annual-like behaviour (*i.e.* high reproductive output at the expense of survival; Aragón *et al.* 2009). Therefore, the precipitation-dependent female-biased allocation to flowers across *Cistaceae* species may be a particular adaptive response to semiarid conditions among distantly related taxa.

However, a caveat here is that we did not detect significant differences in the percentage of resource allocation to carpels between semiarid and dry sites. Although nutrients did show a quantitative tendency for increasing allocation in the semiarid sites (about 20% higher than dry sites; Fig. 2), dry mass was similar. Specifically, a plausible explanation for this pattern is the exceptionally high percentage of dry mass allocation to carpels reported for the annual selfing *Tuberaria guttata* in the dry site (about five-fold higher than the family mean; Table S2). In this species, selfing-mediated reproductive assurance largely increases seed production when pollinators are scarce or excluded (Herrera 1992, 2004; Teixido & Aizen 2019). A proportionally higher female-biased allocation to flowers in annual species that guarantees seed production by selfing may be relevant to maximise reproduction when subsequent survival is zero. In this regard, the relative reproductive effort (seed number·mg^{-1} plant mass) in *T. guttata* is remarkably high when compared to perennial and xenogamous *Cistus* species (Herrera 1992). Life form and mating system might be two relevant intrinsic factors, strongly related and phylogenetically determined across seed plants worldwide (Moeller *et al.* 2017), which regulate dry mass allocation to carpels in our study system, with increased percentages in annual and selfing species (Cruden & Lyon 1985; Goodwillie *et al.* 2010; see Teixido *et al.* 2017 for details in *Cistaceae*). Either way, our results provide compelling evidence that precipitation and temperature determine female gender allocation to flowers beyond phylogenetic relationships across *Cistaceae* species.

Other aspects of differences in resource allocation to carpels to be considered are the availability of nutrients in the soil and the plant size. In our study system, semiarid sites are on nutrient-poor gypsum soils (Escudero *et al.* 2015) and this may ultimately increase female reproductive success in hermaphroditic species mediated by a disproportionate increase in nutrient allocation to carpels and subsequent seed production (Chen *et al.* 2017). Nutrient availability in soil across sites may also influence plant size, which in turn determines resource allocation to flowers. However, plant size-mediated sex allocation has exclusively been reported at specific level (*e.g.* Klinkhamer *et al.* 1997; Cao & Kudo 2008; Xiong *et al.* 2016). In *Cistaceae*, interspecific variation in plant size follows a phylogenetic signal (Guzmán & Vargas 2009) whereas carpel allocation does not show any conservatism across lineages (Teixido *et al.* 2017). In addition, species of this family occur across climatically variable sites regardless of plant size (*e.g. Cistus ladanifer*, one of the largest-sized species, occurs simultaneously with *Tuberaria guttata*, a small annual herb, in dry sites; Herrera 1992; Table S1). We suggest that interspecific variation in plant
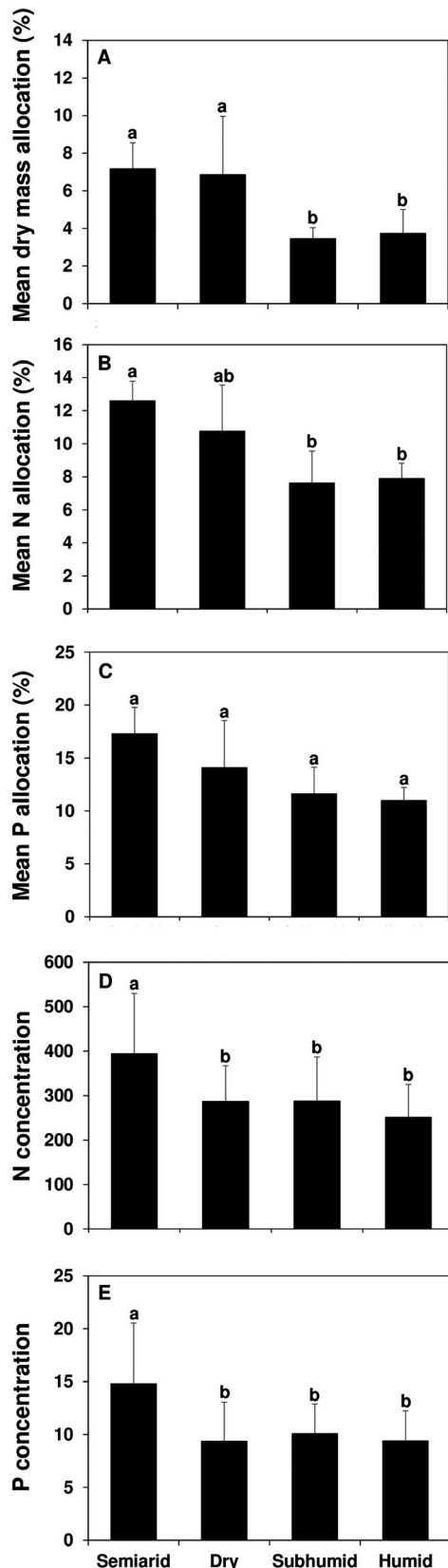
Fig. 2. Mean ± SE (A) percentage dry mass, (B) percentage N, (C) percentage P, and mean ± SD (D) N concentration and (E) P concentration in carpels per site across *Cistaceae* species. Different letters on each bar indicate significant differences (*P* < 0.05).

Simultaneously to the patterns of resource allocation under dry and hot conditions, a decreased proportion of dry mass and nutrient allocation to carpels in cooler and wetter sites may be associated with increased allocation to the male function under pollinator unpredictability. In this regard, larger and more attractive, male-biased flowers with high pollen–ovule ratios enhance male fitness through providing more mating opportunities (*i.e.* male function hypothesis: Ashman & Morgan). Indeed, in large-flowered and xenogamous *Cistus* species from pollen-limited populations, pollinator-mediated phenotypic selection favours larger corollas through male function (reviewed in Teixido *et al.* 2016). However, this microevolutionary pattern is somewhat inherent within species among different populations, whereas, at family level, phylogeny strongly drives flower size-dependent male allocation to flowers in *Cistaceae* (Teixido *et al.* 2017). Consequently, male-biased allocation at the expense of reducing resource investment to carpels is inconsistent with a divergence between phylogenetically related species diversified into different environmental conditions. In agreement with this, further regression analyses for our 37 species dataset show that both flower size and nutrient concentration and percentage of resource allocation to corollas and stamens are independent of precipitation and temperature (see Table S5).

In conclusion, the present work follows an unexplored approach based on alternative explanations of phylogeny to account for resource allocation patterns to the primary female structure of flowers. Our results demonstrate that dry mass and nutrient allocation to carpels show site-specific differences following a precipitation and temperature gradient, with species inhabiting drier and hotter scenarios allocating proportionally more resources to flowers of female gender among related species within this typical entomophilous and hermaphroditic Mediterranean family. We suggest that this pattern lies within a strategy to deal with the most stressful conditions by means of high reproductive effort. From an evolutionary perspective, the results suggest that similar selection pressures play an essential role in determining convergent female-biased

Table 1. Results of analyses testing the effects of site (semiarid, dry, subhumid and humid; see Fig. 2 for details) on resource allocation to carpels in *Cistaceae* (n = 37 species). Percentage dry mass, N and P were tested by fitting GLMs with quasi-Poisson distribution and with a chi-squared test (χ^2). N and P concentrations were tested by fitting an ANOVA with a *F*-test.

Resource	df	Test value	*P*
Dry mass (%)	3, 33	7.58	**0.047**
Nitrogen (%)	3, 33	7.47	0.052
Phosphorus (%)	3, 33	4.33	0.228
[Nitrogen]	3, 33	3.02	**0.043**
[Phosphorus]	3, 33	3.91	**0.017**

Significant differences among sites were tested with Tukey *post-hoc* tests (Fig. 2) and significant *P*-values are marked in bold.

size may be irrelevant in determining resource allocation to carpels across sites and species, but further studies are required to obtain solid conclusions.

allocation traits across distantly related *Cistaceae* species in a Mediterranean ecosystem.

SUPPORTING INFORMATION

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

Figure S1. Mean ± SE (A) mean precipitation and (B) mean temperature during the flowering period (month corresponding with flowering peak) per site across *Cistaceae* species.
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Table S1. Location, site and climatic data of the 37 species of *Cistaceae* used in the study.

Table S2. Mean ± SE (%) dry mass, N and P allocation, and mean ± SD concentrations of N and P allocation (mmol/g dry mass) to carpels in the 37 studied species of *Cistaceae*.

Table S3. Results of the regressions testing the effects of mean precipitation and mean temperature during the flowering period (month corresponding with flowering peak) on resource allocation to carpels in *Cistaceae* (N = 37 species).

Table S4. Mean ± SE (%) dry mass, N and P allocation, and mean ± SD concentrations of N and P allocation (mmol/ g dry mass) to stamens in the 37 studied species of *Cistaceae*.

Table S5. Regression analyses for flower size, percentage of dry mass, nutrient allocation, and concentration of nutrient allocation to corollas and stamens vs PCA1 scores per site in the 37 studied species of *Cistaceae*.

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