

Genetically based population divergence of *Silene latifolia* from two climate regions

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ABSTRACT

Background: Plants in hot and dry climates often flower earlier, make thicker leaves, and produce fewer flowers than conspecifics from relatively wet, cool climates. *Silene latifolia*, a dioecious, short-lived, flowering perennial, grows in both of these climates in Europe.

Question: Is variation in traits seen among populations with divergent climates a result of genetic changes in response to local environmental conditions, differences in the degree of sexual dimorphism, or phenotypic plasticity?

Hypothesis: Traits will differ between populations in a common garden as a result of genetic divergence, and exhibit a pattern of variation that is congruent with adaptation to climate.

Methods: Morphological and phenological measurements were taken during two flowering seasons on plants growing in Croatia (relatively wet and cool) and Spain (hot and dry). Seeds from both regions were grown to flowering in the greenhouse and several traits were measured.

Results: Significant divergence in traits existed between Croatia and Spain that persisted in the common garden (greenhouse), indicating that populations in these two regions likely represent different ecotypes. Plants from Spain flowered earlier in the field, made thicker leaves, and produced fewer flowers than plants from Croatia. Plants from Spain also showed greater sexual dimorphism than those from Croatia.

Keywords: climate, ecological divergence, ecotypes, phenotypic plasticity, sexual dimorphism.

INTRODUCTION

Regional variation in climate has been implicated as a cause of among-population divergence in plant species. Abiotic factors determine both life-history strategies and the distribution of many plants, and also lead to within-species ecotypic variation as a result of population-level changes in response to differences in precipitation, water availability, and extreme temperatures (Hall and Willis, 2006; Galloway and Burgess, 2012; Lekberg *et al.*, 2012; Chen and Schemske, 2015). In some cases, both population divergence and phenotypic plasticity have been shown to contribute to variation, with individual traits exhibiting different degrees of canalization (Baker *et al.*, 2014). One well-used method for determining whether variation seen in traits is

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a result of population differentiation or plasticity is to study populations in a common environment (see the classic study by Clausen *et al.*, 1940, for example; Kawecki and Ebert, 2004). Given that plasticity can be an important tool allowing plants to respond to environmental heterogeneity, but one that can come with fitness costs, traits are likely to vary in how canalized or plastic they are (Oke *et al.*, 2015; Palacio-Lopez *et al.*, 2015). In addition, when plant species are dioecious, with separate male and female individuals, sexual dimorphism can contribute to trait variation (Geber *et al.*, 1999) and populations can differ in their degree of sexual dimorphism (e.g. Delph *et al.*, 2002; Delph and Bell, 2008).

Vegetative, life-history, and floral traits often show a pattern of variation associated with climate. In particular, traits that reduce water loss are signatures of plants that grow in areas with low precipitation rates; for example, thicker leaves are known to use less water, which can be especially important in periods of extended drought (Wright *et al.*, 2004). Moreover, the timing of the onset of flowering can limit exposure to peak drought conditions, which can be useful for avoiding costly reproductive processes during dry periods (e.g. Friedman *et al.*, 2015). Similarly, variation in flower number, which directly influences reproductive output, may vary with both moisture and nutrient availability, with individuals in dry conditions producing fewer flowers relative to individuals in wet conditions (Lambrecht and Dawson, 2007; Herlihy and Delph, 2009; Euler *et al.*, 2012). Environmental factors have also been implicated in changes in flower size, which often trades off with flower number (Worley *et al.*, 2000; Delph *et al.*, 2004). Lastly, flower size and number also commonly also vary between males and females (Delph *et al.*, 1996; Vaughton and Ramsey, 1998; Eckhart, 1999; Meagher and Delph, 2001; Glaetli and Barrett, 2008; Waelti *et al.*, 2009).

In this study, we compared several morphological and phenological traits for the dioecious herbaceous plant *Silene latifolia* from two native European regions with dramatically different climate profiles. First, we documented differences in morphology and phenology between plants growing in the wild from the two regions. We then determined whether differences observed between the two regions persist when grown in a common garden, and measured additional traits that vary with plant age. We asked whether trait variation was a consequence of population divergence, sexual dimorphism, and/or plasticity. Previous studies of *S. latifolia* have shown that significant sex differences in allocation to biomass and reproduction occur within and among populations (Delph *et al.*, 2005; Delph and Bell, 2008), so we also evaluated whether the two regions exhibit similar levels of sexual dimorphism.

MATERIALS AND METHODS

Study system and sites

Silene latifolia is a dioecious, short-lived, herbaceous perennial widely distributed throughout Europe (Baker, 1948). It is most commonly associated with disturbed habitats such as roadsides, agricultural fields, and semi-maintained public spaces such as parks and cemeteries. The European range of *S. latifolia* extends from the coast of Portugal across continental Europe and into Russia, and from southern Italy to as far north as Scandinavia (Taylor and Keller, 2007). Extensive sampling throughout its range has revealed haplotype clustering from eastern and western populations, but also high gene diversity across the range (Mastenbroek *et al.*, 1983; Vellekoop *et al.*, 1996; Taylor and Keller, 2007). To date, no clear pattern of geography associated with the patterns observed in trait means has emerged (Mastenbroek *et al.*,

1983; Delph *et al.*, 2002). Within *S. latifolia*, the sexes are sexually dimorphic, with males making many small flowers and smaller leaves with an up-regulated ecophysiology relative to females; males also live shorter lives (see review in Delph, 2007; Delph and Herlihy, 2012). We sampled *S. latifolia* from various sites near Zagreb, Croatia and Xativa, Spain. The two regions exhibit differences in climate. The climate of Zagreb, Croatia is classified as a maritime temperate region that lacks a dry season and has warm summers based on the Köppen-Geiger (KG) climate classification [Cfb (Peel *et al.*, 2007)]. The region near Xativa, Spain is characterized as arid, with most precipitation occurring in winter when temperatures are cooler [characterized by KG as Bsk (Peel *et al.*, 2007)]. Figure 1(A, B) shows climate variables averaged over 50 years (1950–2000) for the two regions (retrieved from www.WorldClim.org; Hijmans *et al.*, 2005), while Fig. 1(C) shows monthly precipitation from 2014 (data retrieved from www.weatherunderground.com). Note that temperatures in Spain are higher than in Croatia throughout the year, but especially in the winter months. Moreover, rainfall is lower in Spain (48.9 cm per year in Spain averaged over 1950–2000 vs. 97.2 cm in Croatia), especially during the hottest months of the year (Fig. 1). In other words, Spain is relatively hot and dry during the growing season (spring and summer), whereas Croatia is especially colder during the winter and early spring.

Natural populations in the field

In 2013 and 2014, plants were located during the flowering season and measured at two or three sites both near Xativa, Spain and Zagreb, Croatia. We measured the width of the main flowering bolt with digital calipers and collected a leaf sample from a leaf two nodes down from the node containing the first flower to open. Hole-punches were taken from this leaf and specific leaf area (SLA) was calculated by dividing the area of two punches (0.66 cm²) by their dry weight (g) [thus thicker leaves have lower SLA values (Milla *et al.*, 2008)]. For a single open flower from each plant, we measured several dimensions of flower size using digital calipers; these measures included calyx width, calyx length, petal-limb length, and petal-claw length (Fig. 2). Flower measurements were taken early in the flowering season (see below), which in Spain was mid-April and in Croatia mid-June.

Greenhouse measurements

Silene latifolia seeds were collected from maternal families from a population near the field sites in both Spain and Croatia. Crosses within each population were made among five maternal families for at least one generation in the greenhouse to reduce maternal effects. Seeds were sown in the greenhouse at Indiana University in mid-March 2013 in 128-well seed trays, and 10 seedlings per family were moved to 5-inch (127-mm) clay pots in a 1:1 Metromix and soil mixture after they developed two sets of true leaves. Plants were randomly assigned to blocks, and each week blocks were rotated to reduce greenhouse effects. The SLA of a rosette leaf was sampled using a hole-punch (as above) approximately 4 weeks after germination. When the first flower on each plant opened, we recorded the sex, the number of basal branches the plant had, and the height to the node containing the first flower to open. All flowers on females were hand pollinated on the first or second day of flower opening. We measured floral traits as above on the second and third flower to open, and averaged the two measurements. We collected the second flower, dried it in an oven at 80°C, and weighed it. The timing of the opening of the third flower was noted, and this

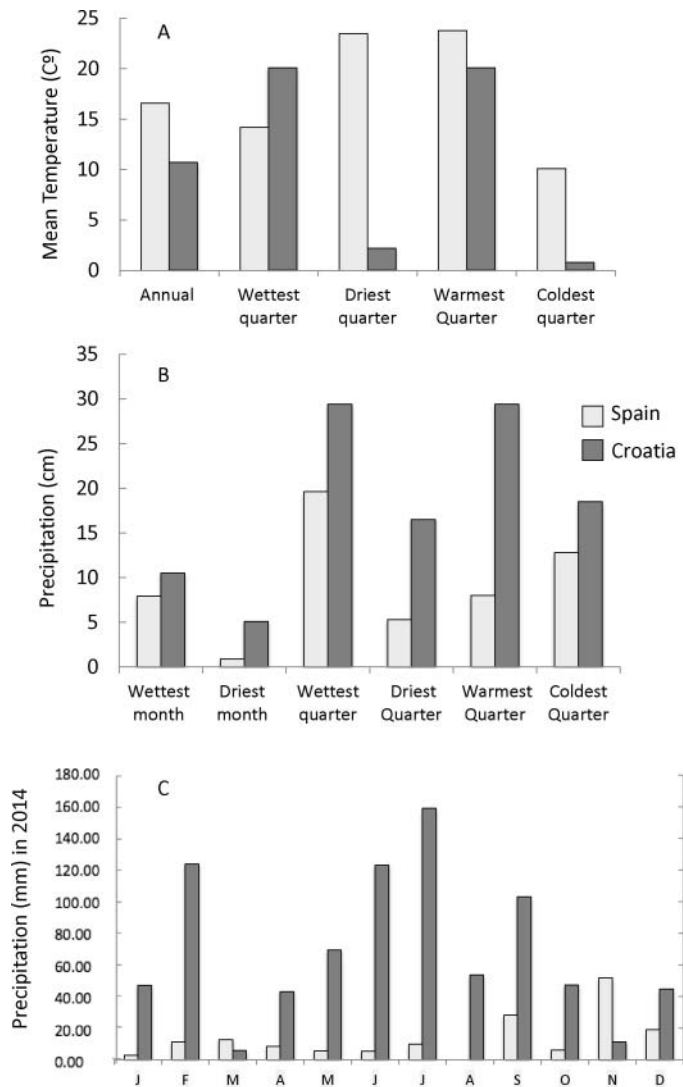


Fig. 1. (A) Mean temperature and (B) precipitation from 1950 to 2000, and (C) precipitation by month in 2014 are shown for Xativa, Spain and Zagreb, Croatia.

flower was collected for gamete quantification – ovule number for females, pollen-grain number and viability for males. Pollen number was quantified using an Elzone II particle counter (Micromeritics). Pollen viability was estimated by preserving pollen in 500 μ L 3:1 lactic acid–glycerol solution. Following mixing by use of a vortex, 20 μ L of this solution was placed on a slide, a drop of Alexander’s stain was added, a coverslip was applied, and the slide was incubated overnight at 32°C. Counts of 50 pollen grains were made and categorized as viable or inviable with the use of a digital microscope, with viable pollen appearing dark purple and inviable pollen appearing light green and usually smaller. Forty

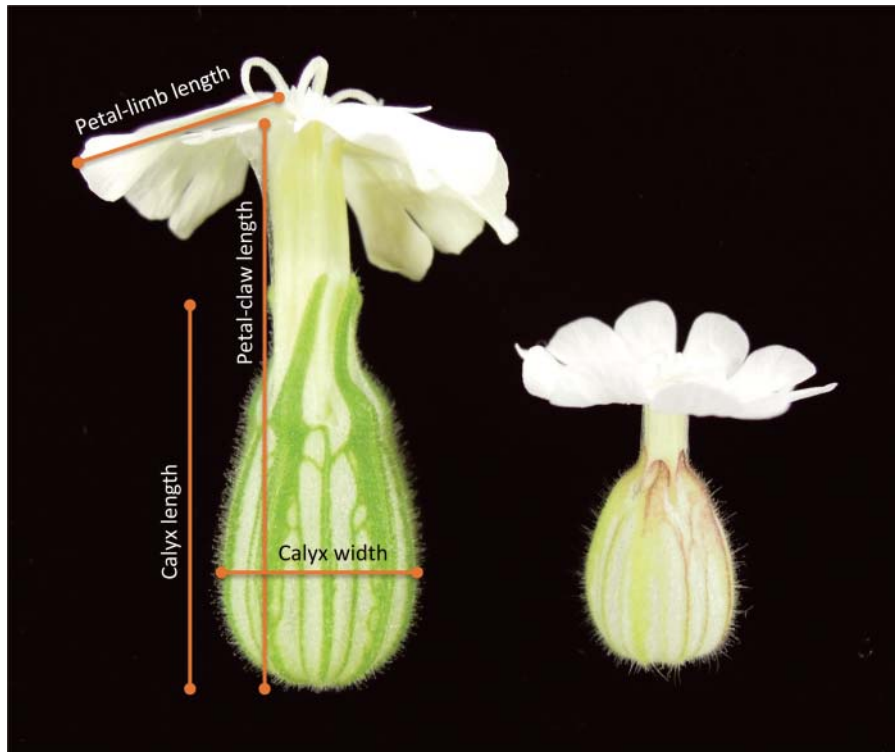


Fig. 2. Floral measurements taken on males and females included calyx length, calyx width, petal-claw length, and petal-limb length. A flower from a Spanish female is shown on the left and a Croatian female on the right.

days after the first flower opened, we counted the total number of flowers that had opened during this time by counting pedicels (which remain on the plant after the flower falls off), open flowers, and flowers that would open that evening.

Statistical analyses

Inflorescence leaf SLA and the four floral measurements, which were taken on plants in both the field and greenhouse, were used as dependent variables in three-way analyses of variance (ANOVAs). Population (Spain vs. Croatia), sex (male vs. female), and location of measurement (wild vs. greenhouse) were the independent variables, and the model included all two-way interactions and the three-way interaction, except that two-way interactions were removed from the model when $P > 0.25$, and the model was re-run. Differences in traits for plants grown only in the greenhouse were analysed using two-way ANOVAs, with population and sex as the independent variables. Main-effects results are presented in Tables 1 and 2, and least squared means and standard errors from the ANOVAs are presented in Figs. 3 and 4. Two-way interactions and associated least square means are mentioned when significant in the text below. *Post-hoc* differences were evaluated with *t*-tests. All statistical tests were run using JMP version 12.0.

RESULTS

Population divergence, sexual dimorphism, and plasticity

Flowering in the natural populations in Spain began around April and continued until July, with a small number of plants sporadically flowering later in the summer. In Croatia, flowering during the three years began in June, and continued into October.

All six traits measured in both the field and greenhouse exhibited significant population divergence; five of these were also significantly sexually dimorphic, and four differed significantly depending on the location of measurement (Fig. 3). While most of the variation in SLA was explained by the location (wild vs. greenhouse) of measurement (i.e. plasticity), the population the plants came from (i.e. population divergence) had the greatest effect on the other five traits, ranging from a 1.22- to a 1.58-fold difference between plants from Spain and Croatia (Table 1).

None of the two-way interactions for SLA were significant and were removed from the model. In terms of main effects (Fig. 3), SLA was significantly lower in Spain than in Croatia – that is, leaves from plants in Spain were thicker. Males had significantly greater SLA than females, and plants growing in the greenhouse had significantly greater SLA than those growing in the wild.

All three main effects were significant for the width of the main flowering stalk, which was significantly larger in Spain, females, and in the wild. In addition, both the population $\times$ sex and sex $\times$ location of measurement interactions were significant ($F_{1,193} = 15.31$, $P < 0.0001$ and $F_{1,193} = 10.54$, $P < 0.0014$, respectively), with males having significantly narrower main stalks than females in Spain (3.93 ± 0.185 vs. 5.2 ± 0.16 mm) but not Croatia (3.02 ± 0.146 vs. 3.03 ± 0.151 mm), and males being significantly smaller than females when measured in the wild (3.70 ± 0.132 vs. 4.87 ± 0.124 mm) but not the greenhouse (3.25 ± 0.195 vs. 3.37 ± 0.182 mm).

All four floral dimensions measured were significantly larger in Spain than in Croatia, and all but petal-limb length were significantly larger in females than males. In contrast, only calyx width and petal-limb length exhibited significant plasticity. In terms of two-way interactions, the sex $\times$ location of measurement interaction was significant for calyx width ($F_{1,661} = 23.50$, $P < 0.0001$), with females having significantly larger calyces in the greenhouse than in the wild (11.5 ± 0.23 vs. 9.7 ± 0.09 mm), but this was not the case for males (7.4 ± 0.22 vs. 7.1 ± 0.08 mm). The population $\times$ location of measurement interaction was significant for petal-limb length ($F_{1,653} = 22.31$, $P < 0.0001$), with plants from Spain exhibiting petal-limbs that were longer in the wild than in the greenhouse (11.9 ± 0.09 vs. 10.3 ± 0.33 mm), but this was not the case for plants from Croatia (9.4 ± 0.30 vs. 8.8 ± 0.15 mm). The population $\times$ sex interaction was significant for both calyx length ($F_{1,661} = 30.90$, $P < 0.0001$) and petal-claw length ($F_{1,659} = 95.37$, $P < 0.0001$). In both cases, females had significantly longer floral parts than males in Spain, but not Croatia, indicating that the degree of sexual dimorphism varied between populations for these two traits. Calyces of females were 1.17 times longer than those of males in Spain (23.9 ± 0.25 vs. 20.2 ± 0.27 mm), but only 1.01 times longer in Croatia (16.5 ± 0.30 vs. 16.1 ± 0.24 mm). Similarly, petal-claws (the portion of the petal within the tube formed by the calyx) of females were 1.28 times longer than those of males in Spain (31.2 ± 0.26 vs. 24.4 ± 0.30 mm) versus only 1.04 times longer in Croatia (17.9 ± 0.33 vs. 17.2 ± 0.26 mm).

Table 1. For traits measured in both the wild and the greenhouse, we indicate whether or not significant differences were seen between populations (population divergence), sexes (sexual dimorphism), or measurement locations (plasticity), and provide *F*-ratios and *P*-values for each effect from three-way ANOVAs. Below these the direction of the difference is given, and the fold difference in the values being compared is shown in parentheses (with the largest effect indicated in **bold** for each trait)

Trait	Population divergence	Sexual dimorphism	Plasticity
Specific leaf area	Yes: $F_{1,252} = 18.91$, $P < 0.0001$ Croatia > Spain (1.47)	Yes: $F_{1,252} = 4.02$, $P = 0.0459$ Male > Female (1.23)	Yes: $F_{1,252} = 385.77$, $P < 0.0001$ Wild < Greenhouse (2.19)
Branch width	Yes: $F_{1,193} = 92.07$, $P < 0.0001$ Croatia < Spain (1.51)	Yes: $F_{1,193} = 15.80$, $P < 0.0001$ Male < Female (1.18)	Yes: $F_{1,193} = 36.74$, $P < 0.0001$ Wild > Greenhouse (1.30)
Calyx width	Yes: $F_{1,661} = 409.69$, $P < 0.0001$ Croatia < Spain (1.49)	Yes: $F_{1,661} = 397.40$, $P < 0.0001$ Male < Female (1.47)	Yes: $F_{1,661} = 60.96$, $P < 0.0001$ Wild < Greenhouse (1.12)
Calyx length	Yes: $F_{1,663} = 860.47$, $P < 0.0001$ Croatia < Spain (1.35)	Yes: $F_{1,663} = 85.20$, $P < 0.0001$ Male < Female (1.10)	No: $F_{1,663} = 1.66$, $P = 0.1978$ Wild = Greenhouse (1.02)
Petal-limb length	Yes: $F_{1,651} = 71.22$, $P < 0.0001$ Croatia < Spain (1.22)	No: $F_{1,651} = 1.56$, $P = 0.2117$ Male = Female (1.02)	Yes: $F_{1,651} = 4.79$, $P = 0.0290$ Wild > Greenhouse (1.05)
Petal-claw length	Yes: $F_{1,659} = 2404.53$, $P < 0.0001$ Croatia < Spain (1.58)	Yes: $F_{1,659} = 145.56$, $P < 0.0001$ Male < Female (1.18)	No: $F_{1,659} = 0.78$, $P = 0.3759$ Wild = Greenhouse (1.01)

Traits measured only in the greenhouse

All seven traits measured only in the greenhouse in both sexes differed significantly between plants from Spain and Croatia (Table 2, Fig. 4). The population the plants came from had more of an effect on variation exhibited by five of these traits than did sex, ranging from a 1.31- to a 5.03-fold difference. However, five of these traits were significantly sexually dimorphic, with variation in flower mass and the number of flowers produced over the first 40 days of flowering being explained more by the main effect of sex than population.

There was a significant effect of population on rosette leaf SLA (measured prior to flowering) and the number of branches present at the time of first flowering, but the sex effect was not significant: plants from Spain made relatively thicker leaves and fewer branches than those from Croatia. The onset of flowering in plants from Spain averaged over five times longer than plants from Croatia, with many overwintering prior to flowering, and subsequent flowers were also produced at a significantly slower rate. Both traits were also significantly sexually dimorphic (significant sex effect), with males taking longer to flower but producing flowers at a faster rate than females. Days to first flowering also showed a significant population $\times$ sex effect ($F_{1,70} = 8.88$, $P < 0.0040$), with no significant difference between the sexes from Croatia, but Spanish males taking 1.37 times longer to flower than Spanish females. The main effects of population and sex were significant for height to the first flowering node, with Spanish plants being over twice as tall as Croatian plants, and females being taller than males.

While flower mass and flower production over 40 days differed significantly between populations, the main effect of sex explained more of the variation, and the population $\times$ sex interaction was significant for both traits ($F_{1,69} = 55.74$, $P < 0.0001$ and $F_{1,71} = 35.36$,

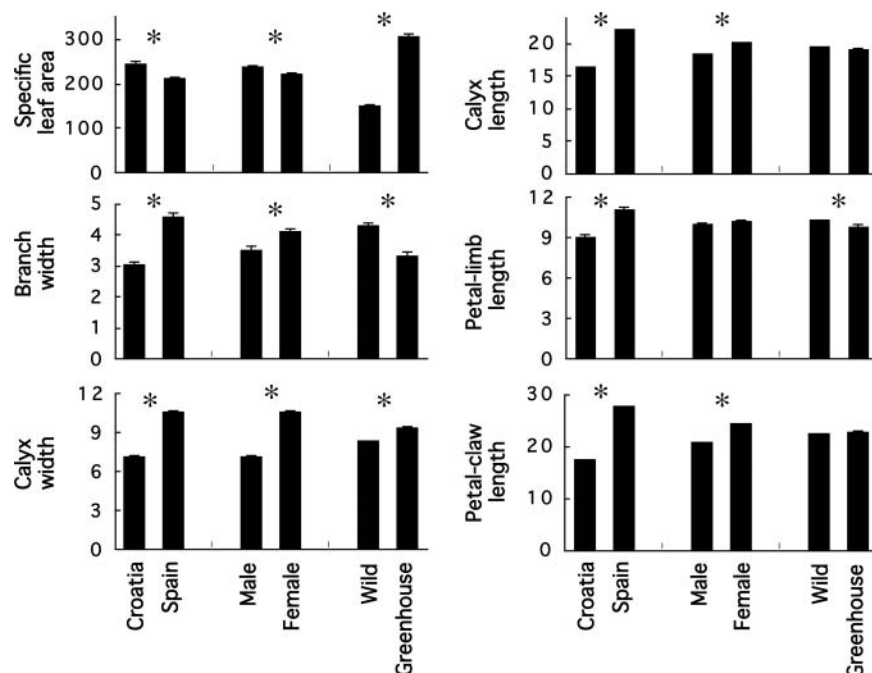


Fig. 3. Least square trait means (± 1 SE) for specific leaf area ($\text{mm}^2 \cdot \text{g}^{-1}$), width of the main inflorescence branch (mm), calyx width (mm), calyx length (mm), petal-limb length (mm), and petal-claw length (mm), based on three-way ANOVAs (see Table 1) for population of origin (Croatia vs. Spain), sex (male vs. female), and location of measurement (wild vs. greenhouse), for plants measured in both the wild and the greenhouse. Asterisks indicate significant differences.

$P < 0.0001$, respectively). These significant interaction terms indicated that the degree of sexual dimorphism varied significantly between populations, being higher in Spain. Spanish females produced flowers that were over three times heavier than Spanish males (60.3 ± 1.60 vs. 19.7 ± 1.91 mg) – both of which were larger than their Croatian counterparts – and Croatian females produced flowers over two times heavier than Croatian males (26.2 ± 1.85 vs. 11.2 ± 1.46 mg). In accordance with a flower size/number trade-off, Spanish females produced the fewest flowers, followed by Croatian females, Spanish males, and Croatian males. Sexual dimorphism for this trait was greater than all other traits measured, with males from Spain producing nearly nine times more flowers than females from Spain (201 ± 48.7 vs. 23 ± 42.2), and Croatian males making over six times more flowers than Croatian females (838 ± 37.7 vs. 130 ± 48.7).

The three sex-specific reproductive traits – ovule number, pollen number, and pollen viability – all differed significantly between populations. Spanish females produced significantly more ovules per flower than Croatian females (557.7 ± 20.00 vs. 458.5 ± 23.10 ; $F_{1,34} = 10.53$, $P = 0.0027$), but the reverse was true for pollen ($1.68 \times 10^5 \pm 2907$ vs. $2.56 \times 10^5 \pm 2252$; $F_{1,39} = 5.66$, $P = 0.0225$). Spanish males also had a significantly lower proportion of viable pollen per flower than Croatian males (0.79 ± 0.04 vs. 0.89 ± 0.20 ; $F_{1,30} = 4.46$, $P = 0.0435$).

Table 2. For traits measured in both sexes but only in the greenhouse, we indicate whether or not significant differences were seen between populations (population divergence) and/or sexes (sexual dimorphism), and provide *F*-ratios and *P*-values for each effect. Below these the direction of the difference is given, and the fold difference in the values being compared is shown in parentheses (with the largest effect indicated in **bold** for each trait)

Trait	Population divergence	Sexual dimorphism
Rosette specific leaf area	Yes: $F_{1,71} = 100.78$, $P < 0.0001$ Croatia > Spain (1.97)	No: $F_{1,71} = 0.03$, $P = 0.86$ Male = Female (1.01)
Number of branches at first flowering	Yes: $F_{1,71} = 4.76$, $P = 0.0324$ Croatia > Spain (1.31)	No: $F_{1,71} = 3.47$, $P < 0.0667$ Male = Female (1.26)
Number of days to first flower opening	Yes: $F_{1,70} = 229.35$, $P < 0.0001$ Croatia < Spain (5.03)	Yes: $F_{1,70} = 8.49$, $P < 0.0048$ Male > Female (1.30)
Number of days to third flower opening	Yes: $F_{1,69} = 22.93$, $P < 0.0001$ Croatia < Spain (2.12)	Yes: $F_{1,69} = 8.70$, $P = 0.0043$ Male < Female (1.57)
Height to first flower node	Yes: $F_{1,69} = 72.71$, $P < 0.0001$ Croatia < Spain (2.05)	Yes: $F_{1,69} = 9.33$, $P = 0.0032$ Male < Female (1.28)
Flower mass	Yes: $F_{1,69} = 154.77$, $P < 0.0001$ Croatia < Spain (2.08)	Yes: $F_{1,69} = 263.39$, $P < 0.0001$ Male < Female (2.80)
Number of flowers produced over first 40 days of flowering	Yes: $F_{1,71} = 69.58$, $P < 0.0001$ Croatia > Spain (4.32)	Yes: $F_{1,71} = 98.74$, $P < 0.0001$ Male > Female (6.81)

DISCUSSION

We measured 17 morphological and phenological traits for *S. latifolia* from two regions that vary markedly in climate, and found significant differences between the regions for all traits in both the wild and in the greenhouse. The persistence of significant differences in the greenhouse indicates a genetic basis to the between-population divergence. Overall, the pattern of differentiation observed between the two regions followed our predictions based on the differences in their pattern of rainfall and yearly variation in temperature. Relative to plants from Croatia, those from Spain had thicker leaves, began flowering earlier in the season in the wild (i.e. April vs. June), had fewer branches at the onset of first flowering, were older at first flowering, had thicker stems on the main inflorescence branch, had flowers that were larger in both dimension and weight, had taller inflorescence branches, were slower at producing flowers, and had flowers that contained more ovules (females) and fewer viable pollen grains (males) per flower.

Of the 13 traits that were measured in the field and/or greenhouse and present in both sexes, the main effect of population explained more of the variation in 10 of these than did the main effects of sex and/or location of measurement. Exceptions to this pattern included SLA of inflorescence leaves, which was highly plastic, such that leaves were over two times thinner in the greenhouse compared with the wild. In addition, although the dimensions of the calyx and petals varied more between populations than between the sexes, variation in flower mass was mostly explained by sex, with female flowers being nearly three times heavier than male flowers, indicating that the weight of the ovary was the main factor in this sexual dimorphism. Lastly, although the number of flowers produced over the first 40 days

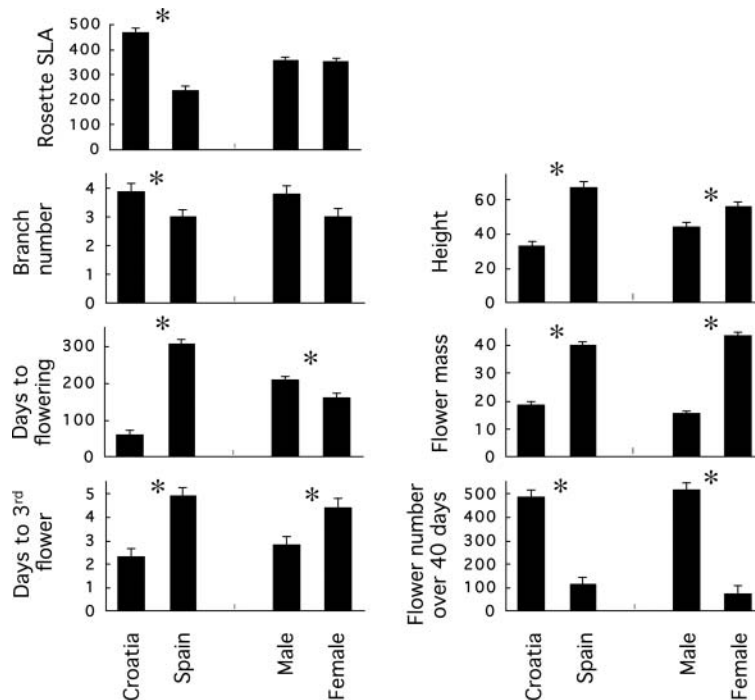


Fig. 4. Least square trait means (± 1 SE) for specific leaf area of a rosette leaf ($\text{mm}^2 \cdot \text{g}^{-1}$), number of branches at the onset of first flowering, number of days to first flowering, number of days between the first flower to open and the third flower to open, height to the first flowering node at the time of first flowering, flower mass (mg), and number of flowers produced during the first 40 days of flowering, based on two-way ANOVAs (see Table 2) for population of origin (Croatia vs. Spain) and sex (male vs. female). Asterisks indicate significant differences.

of flowering varied over four-fold between the populations, it varied even more – nearly seven times – between the sexes, and reflected a flower size/number trade-off.

The direction of divergence seen in several of the traits between *S. latifolia* from these two regions is congruent with differentiation seen among other plant species in response to climatic variables. Long periods without precipitation is thought to exert strong selection on plant morphology and physiology, and plants experiencing extended periods of high temperatures and low rainfall often exhibit trait variation similar to that seen in our Spanish population. For example, in a study of six *Arabidopsis lyrata* populations, thick leaves and lowered flowering propensity were found to vary and confer tolerance to drought (Sletvold and Ågren, 2012). Similarly, multiple studies of local adaptation in *Mimulus guttatus* have revealed that adaptation to summer drought avoidance includes flowering early in the season (Hall and Willis, 2006; Mojica *et al.*, 2012; Friedman *et al.*, 2015; Kooyers *et al.*, 2015). Lastly, *Boechera holboellii* populations from a dry population had thicker leaves than those from a relatively mesic population (Knight *et al.*, 2006).

In terms of floral traits, Spanish plants produced flowers that were significantly heavier as well as larger than those of Croatian plants in all four of the dimensions measured. Moreover, petal-limb length was the only part that was not significantly larger in females

compared with males, which may reflect optimization for attracting pollinators, as this is the floral part seen by moths as they approach the flowers (see also Delph *et al.*, 2004, 2005). Petal-claw length in Spanish females was especially exaggerated relative to other flower dimensions and, in the greenhouse, was 72% larger than the claws of Croatian females. One potential explanation is that the long claws of Spanish females may influence success of the nursery pollinator *Hadena bicruris*. In a common garden study of eight *S. latifolia* populations of varying flower sizes, plants originating from populations with longer claws prevented female moths from laying eggs inside the flower, which reduced seed predation costs without affecting pollinator efficiency (LaBouche and Bernasconi, 2013; Magalhaes and Bernasconi, 2014). In an arid region like Spain where flowers are larger and energetically costly (and fewer flowers are made), reducing fruit attacks may be especially important for female fitness. Moreover, in a study of artificial selection lines, small flowered plants were more likely to abort attacked fruits than large flowered plants (Burkhardt *et al.*, 2012), which suggests that female plants with dissimilar floral sizes may use different strategies to reduce seed herbivory.

In both populations we observed a significant increase in calyx width in females in the greenhouse, which may indicate that plants in ideal conditions are able to allocate more resources to reproduction at the individual flower level. Previous studies have demonstrated that female calyx width is directly proportional to ovule number (Delph *et al.*, 2004), which is in line with our finding that Spanish females produce significantly more ovules than Croatian females.

Differences in floral display size between plants from Spain and Croatia may also reflect different life-history strategies, which mirror strategies previously described in differences between males and females. Overall, Croatian plants make many more small flowers relative to Spanish plants, and males make many more flowers than females from the same population. This trade-off between flower size and flower number is thought to be the result of a genetic constraint, with plants making either many small flowers or few large flowers (Herlihy and Delph, 2009; Vasconcelos and Proença, 2015). Moreover, sexual dimorphism is expected to be greater in more arid environments (Barrett and Case, 2006; Herlihy and Delph, 2009), and our finding that a greater number of traits are more sexually dimorphic in plants from Spain relative to plants from Croatia supports this premise. In our study, plants from a region known to experience long periods of high temperatures and drought produced fewer large flowers, and differences in flower number were greater between males than females. When plants were grown in the greenhouse, males from Croatia produced many more flowers than males from Spain and Croatian males produced significantly more pollen and had a higher percentage of viable pollen compared with Spanish males. Previous studies in *S. latifolia* have found that pollen production is highest in many-flowered individuals under ideal conditions, but under stressful conditions plants that make many flowers make the fewest number of pollen grains (Herlihy and Delph, 2009). When multiple traits act in concert to increase reproductive output or improve survival in stressful conditions, these traits may become genetically integrated (Delph *et al.*, 2005, 2010; Gomez *et al.*, 2009). Future studies of crosses between these two populations to assess the genetic architecture of traits and how they assort will be useful for understanding whether traits known to increase drought tolerance are genetically linked.

While plants in the natural field populations in Spain flower earlier in the year, the late onset of flowering in Spanish plants in the greenhouse suggests that Spanish plants do not flower immediately after germinating. If seeds in Spain germinate in the fall (when rainfall resumes) and overwinter as a basal rosette, this strategy would be beneficial. In contrast,

plants originating from Croatia are likely to germinate and flower within the same season, consistent with their relatively fast time to flowering in the greenhouse. Studies of seed germination in these two regions are needed to test these predictions.

CONCLUSIONS

Our finding of genetically based trait variation between regions is congruent with ecotypic variation in response to climate. Previous studies of differences among *S. latifolia* populations have focused on post-glacial colonization (Hathaway *et al.*, 2009; Magalhaes *et al.*, 2011; Magalhaes and Bernasconi, 2014) or forces exerted by pollinators (LaBouche and Bernasconi, 2013). While these differences are important to informing our understanding of population differentiation, they have not taken into account the vast differences in local climate variables. Given its widespread distribution and weedy characteristics, *S. latifolia* seems to thrive under a wide variety of habitat conditions. Moving forward, studies that consider ecotypes, or groups of populations that occur in similar environmental conditions (Lowry *et al.*, 2014), are likely to provide a good framework in which to consider the genetic architecture of the diversity of phenotypes observed in *S. latifolia*.

ACKNOWLEDGEMENTS

This work received funding from the National Science Foundation (DEB-1353970 to L.F.D.). The authors thank E. Allaby, A. Aungst, M. Burger, G. Crain, H. Luton, K. Mitman, and J. Swank for help with data collection and sample processing.

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