



Past selection shaped phenological differentiation among populations at contrasting elevations in a Mediterranean alpine plant[☆]



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ABSTRACT

Flowering phenology is an important life-history trait strongly influenced by the environment that directly affects plant fitness. Climate change is bringing about shifts in flowering time caused by adaptive evolution and phenotypic plasticity, but their relative contributions and effects are poorly understood. This is especially critical in Mediterranean alpine species, which experience steep environmental gradients at short distances characteristic of alpine environments and an intense summer drought period derived from the Mediterranean climate. Moreover, tracking climate change through migration to higher elevations is not always possible for alpine species since many already find their optimal niche at mountain summits. In this study, we aimed to determine if flowering phenology is genetically differentiated among populations inhabiting contrasting environmental conditions and ascertain if it has been subjected to past selection. This knowledge is crucial to understanding adaptation of alpine plants to current environmental gradients and to provide insight about what adaptations may be necessary to cope with future and ongoing climate warming. We used a common garden experiment to analyze genetic differentiation in phenological traits of nine populations of *Silene ciliata* Pourret (Caryophyllaceae) distributed at two environmentally differentiated areas (optimal and marginal habitat suitability) in three mountain ranges of Central Spain. Environmentally optimal areas for this species are close to the mountain tops, whereas marginal areas are found at the lower distribution edge. We also studied the relation between neutral genetic differentiation (F_{ST}) and quantitative trait differentiation (P_{ST}) to infer past selection on characters under contrasting environmental conditions. We found genetic differentiation for the onset, peak and end of flowering between populations in optimal and marginal areas in the presence of substantial gene flow. This finding highlights the strong diverging selection pressures between the different Mediterranean alpine environments. We also found evidence of past diversifying selection for flowering peak and end of flowering. This evidence of past adaptation in addition to adaptive phenotypic plasticity to advance flowering dates under warmer temperatures suggest that adaptation of flowering phenology to current and future warming should be feasible, especially for populations inhabiting optimal areas.

1. Introduction

Species distributions are moving towards higher latitudes and/or elevations in response to climate change, thereby tracing their optimal environmental conditions (Davis, 2001; Lenoir et al., 2008; Walther, 2003; Walther et al., 2002). Such movements are particularly

conspicuous in mountain ecosystems where an upward shift of species is happening over short distances (Gottfried et al., 2012; Pauli et al., 2012; Steinbauer et al., 2018). However, for those populations that already inhabit the peaks of the alpine mountain systems, upward migration is no longer possible (Marris, 2007; Steinbauer et al., 2018; Walther, 2003; Walther et al., 2005). This may result in species

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extinctions, if populations are not able to genetically adapt to changing conditions. Alpine plant species usually have sharp upper and lower elevational boundaries, with locally adapted populations along the elevational gradient (Byars et al., 2007; Giménez-Benavides et al., 2007a; Scheepens and Stöcklin, 2013). For genetic adaptation to succeed there needs to be sufficient genetic variation (Kawecki and Ebert, 2004), either already present in the population or acquired through gene flow from other populations. It is worth noting, though, that gene flow can both favour and obstruct genetic adaptation to local conditions depending on the adaptive or maladaptive nature of the new incoming alleles (Lenormand, 2002). Species phenology is tightly adapted to specific environmental conditions, thereby optimizing the capacity of species to grow, reproduce and survive under them (Anderson et al., 2012; Haggerty and Galloway, 2011; Kawai and Kudo, 2011; Kudo and Hirao, 2006). One of the most common responses of plants to climate change is the adjustment of flowering phenology to the new conditions (Cleland et al., 2007; Franks et al., 2007). Both phenotypic plasticity and adaptive evolution can underlie rapid phenological shifts in response to climate change, but their relative contributions are poorly understood (Anderson et al., 2012).

In alpine ecosystems, the vegetative growth and reproductive period are constricted to a short time span, mainly by the long snow cover duration, and late spring and early autumn frosts (Körner, 2003; Molau et al., 2005; Sedlacek et al., 2015; Stinson, 2004). Mediterranean alpine ecosystems have some extraordinary peculiarities that affect flowering phenology of species inhabiting these areas (García-Camacho and Escudero, 2009; Giménez-Benavides et al., 2008): water deficit affects plants during the summer and becomes more intense towards the end of the season (Aschmann, 1973) shortening even more the growing and reproductive periods (Giménez-Benavides et al., 2007b, 2018). Shifts in the timing of such crucial events may result in a maladaptive phenology (Scheepens and Stöcklin, 2013).

Mediterranean alpine ecosystems arise as a study case of great interest since species inhabiting those ecosystems are already facing higher temperature and drought conditions expected by climate change. Since most phenological differentiation studies have been performed in alpine ecosystems, we currently lack knowledge about the evolutionary consequences that these limiting factors may have imposed on phenological responses in Mediterranean alpine environments. *Silene ciliata* Pourret (Caryophyllaceae) is a dominant member of Mediterranean alpine ecosystems (Kyrkou et al., 2015; Tutin, 1995). Previous demographic studies on the southernmost populations of this species have shown that populations at the low edge of the species elevational range are experiencing declining growth rates and risk local extinction, in contrast with the stable growth rate found in populations at higher elevations (Giménez-Benavides et al., 2011a). Hence, this suggests the presence of marginal conditions at the lower edge and of optimal conditions at the upper edge (Giménez-Benavides et al., 2018). Giménez-Benavides et al. (2007b) observed significant variation in flowering phenology and reproductive success among populations of *S. ciliata* along the elevational range. They also found that early flowering within populations improved plant fitness (Giménez-Benavides et al., 2011b). Thus, flowering phenology in this Mediterranean alpine species may be under selection and subject to local adaptation processes.

In this study, we aim to determine if flowering phenology of *S. ciliata* is genetically differentiated among populations inhabiting contrasted environmental conditions and ascertain if phenological traits have been subjected to selection in the past, in order to provide insight into evolutionary responses of phenology in Mediterranean alpine environments under ongoing climate change. We combine a common garden experiment with information on intra-range habitat quality (environmental classification) of the species. We specifically ask the following questions: (i) Is flowering phenology genetically differentiated among our study populations? (ii) If so, is this differentiation associated with optimal vs. marginal environmental conditions and/or spatial location? (iii) Is there any evidence of past diversifying selection

on phenological traits? (iv) What are the implications of genetic differentiation of flowering phenology given current and future global warming?

2. Material and methods

2.1. Study species

Silene ciliata Pourret (Caryophyllaceae) is a cushion plant up to 2 cm high and 15 cm in diameter which inhabits a marked environmental gradient in Mediterranean alpine habitats. This long-lived perennial (longevity estimated between 23–147 years inferred from average transition matrices by Giménez-Benavides et al. (2011a)) occurs in areas above the tree line in the Mediterranean mountain ranges of Southern Europe, from the Sistema Central in the center of the Iberian Peninsula to the Massif Central in France, the Apennines in Italy and the Balkan Peninsula (Kyrkou et al., 2015; Tutin et al., 1995). In the Sistema Central this species reaches its southernmost distribution where populations are isolated from more northern populations in the Iberian Peninsula. *Silene ciliata* has a late flowering period in comparison to other species of the same community, flowering from the end of June until mid-September. Although the species is self-compatible, autogamy is restricted by a pronounced protandry (García-Fernández et al., 2012).

In a previous study conducted in the Sistema Central, similar levels of genetic diversity and low genetic differentiation were found among *S. ciliata* populations located along an elevational gradient, indicating the existence of substantial gene flow across different elevations (García-Fernández et al., 2012; Lara-Romero et al., 2016).

2.2. Study region and source populations

The Sistema Central is a southwest-northeast oriented mountain range of approximately 500 km, located in the center of the Iberian Peninsula. *Silene ciliata* occurs in the Oromediterranean and Cryoromediterranean bioclimatic belts above the tree line which is located around 1900 m.a.s.l. (Rivas-Martínez and Loidi, 1999). Vegetation composition of these habitats is rather homogeneous throughout the Sistema Central (Escudero et al., 2004). The climate combines Alpine and Mediterranean features, including strong daily and seasonal temperature fluctuation, a long period of snow cover and a marked drought in summer.

The study focused on *S. ciliata* populations from the three mountain ranges located along the Sistema Central: Béjar, Gredos, and Guadarrama (Fig. 1). Environmental variation within the distribution area of *S. ciliata* in the Sistema Central was studied by modeling the potential habitat suitability using the MAXENT algorithm (Phillips et al., 2006) and was based on the occurrence probability of this species in each mountain range (Morente-Lopez et al., unpublished data). We defined optimal and marginal environments based on the environmental differentiation summarized by the habitat suitability model. The "optimal" category was assigned to the areas with habitat suitability values in the highest 33rd percentile of the distribution whereas the "marginal" category was composed of the areas with habitat suitability values in the lowest 33rd percentile of the distribution. Optimal and marginal populations essentially correspond to those that occur at the high and low edge of the elevational range, respectively. This classification is congruent with demographic trends observed by Giménez-Benavides et al. (2011a). Our study comprised nine populations of *S. ciliata*, with one population located in an optimal area and two populations in marginal areas for each of the three mountain ranges of the Sistema Central (Table 1, Fig. 1).

2.3. Plant and data collection

At the end of the summer of 2013 we collected a minimum of thirty randomly selected mother plants from the nine populations. The

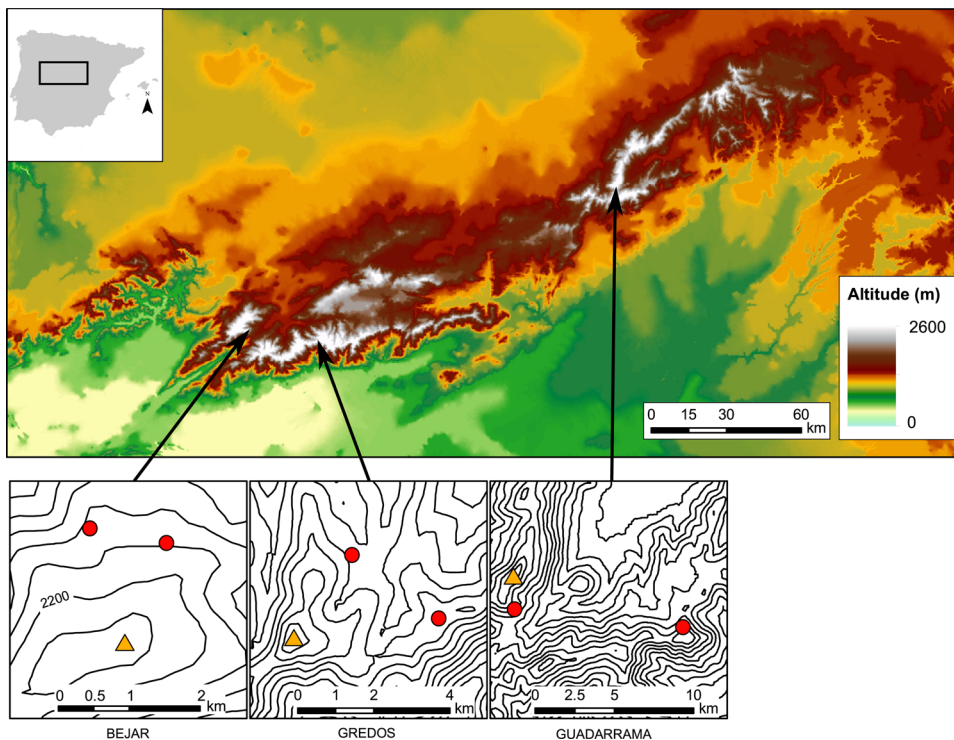


Fig. 1. Study sites of *Silene ciliata* in the Sistema Central of the Iberian Peninsula. Yellow triangles represent each of the three populations located in optimal areas and red dots represent each of the six populations located in marginal areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

collected mother plants were divided in a variable number of cuttings (depending on the size of the plant) to obtain a greater number of individuals. *Silene ciliata* is a slow growing species that requires at least two years to obtain adult plants from seeds. Thus, propagation by cuttings is an effective way to acquire reproductive individuals. The resulting individuals were then planted in 88 mL plastic pots filled with a commercial potting substrate enriched with NPK fertilizer. The whole collection was kept in the Universidad Rey Juan Carlos CULTIVE laboratory greenhouse (690 m a.s.l.) to let individuals root and grow for three months. The resulting collection was brought outside to the CULTIVE experimental field in January 2014. Plants were grown in common garden conditions for seven months before data collection, in order to minimize carry-over effects from the original environment related with the environmental conditions found at the natural populations (Bischoff and Müller-Schärer, 2010). In March 2014, a total of 2200 individuals were transplanted into 2.5 L pots. The total number of individuals and mother plants in the experiments was slightly reduced due to mortality.

Data on flowering phenology were collected in the growing season of 2014 from two clonal individuals of each mother plant when possible, yielding a minimum of 54 individuals from 29 mother plants from each population (Table 1). After the first flower appeared during the

experiment, the rosette diameter of all individuals was measured. We counted the total number of new flowers of each individual weekly from 15 April 2014, when the first flower appeared, until 25 September 2014, when the last new flower appeared, during a total of 23 censuses. From these data we calculated five individual-based flowering-related traits: flowering onset, end, duration, and peak, and total number of flowers. Flowering onset and end were defined as the number of days elapsed between 1 January 2014 and the dates in which the first and last flower of the individual appeared, respectively. Flowering duration was estimated as the number of days elapsed between the onset and end of flowering. Flowering peak was defined as the number of days elapsed between the date of flowering onset of an individual and the date with the greatest number of open flowers on that individual. The total number of flowers was estimated as the number of flowers an individual had produced by the end of the flowering period.

2.4. Trait differentiation analysis

Phenotypic trait differentiation was analyzed among populations, among the three mountain ranges and between optimal and marginal environments. The variables flowering onset, peak, duration and end are time-to-event data, so we applied Kaplan-Meier survival curve

Table 1

Studied *S. ciliata* populations of the Sistema Central, Central Spain. For each population its ID, mountain range of origin, environmental classification, mean annual temperature (MAT), geographical coordinates and sample size (number of mother plants collected in the field and number of individuals generated from cuttings) are provided.

ID	Mountain range	Environment	MAT (°C)	Latitude	Longitude	Elevation (m)	№ Mother Plants	№ Individuals
NEG	Béjar	Optimal	4.99	40°20'19.97''N	5°41'22.27''W	2360	31	54
RUI	Béjar	Marginal	6.11	40°21'7.03''N	5°40'59.71''W	2000	31	125
AGI	Béjar	Marginal	6.46	40°21'12.36''N	5°41'46.52''W	1950	46	79
ZON	Gredos	Optimal	5.56	40°14'57.5''N	5°16'8.3''W	2380	29	57
SES	Gredos	Marginal	6.93	40°16'24.45''N	5°14'54.93''W	1900	33	103
CAM	Gredos	Marginal	6.18	40°15'42.63''N	5°12'55.74''W	2000	46	83
PEÑ	Guadarrama	Optimal	3.94	40°51'2.11''N	3°57'24.02''W	2400	35	65
NAJ	Guadarrama	Marginal	6.78	40°49'23.46''N	3°49'52.53''W	1850	43	74
MOR	Guadarrama	Marginal	5.80	40°50'11.82''N	3°57'0.91''W	1980	29	80

comparisons based on Cox proportional hazards regression models to our flowering data. We used the *coxph* function from R-package *survival* version 2.38 (Therneau, 2016) to investigate whether our studied populations phenologically differed from each other. To answer this specific question the variable population must be considered as a fixed effect. Plant size was included as a covariate to control its possible effect on phenological traits. Then we used the function *coxme* from R-package *coxme* version 2.2–7 (Therneau, 2018), which allows the analysis of mixed effects Cox models, to study if the phenological differentiation is related with geographical and/or environmental differences while accounting for random grouping variables. Thus, we included mountain range of origin, environment (optimal vs. marginal) and their interaction as fixed factors, and population as a random factor. Plant size at the beginning of the flowering season was included as a covariate. Following the same above-mentioned model specifications, total number of flowers was analyzed using a generalized linear model (*glm* function from R package *stats* version 3.4.3) and thereafter using generalized linear mixed-effects models implemented in R function *glmmTMB* (package *glmmTMB* version 0.2.3, Bates et al., 2014) considering in both cases that this variable adjusted to a Poisson distribution. For all the above explained models Wald χ^2 tests were calculated for each fixed effect and *P*-values were calculated using function *Anova* from R package *car* version 3.0–4 (Fox et al., 2012). An ANOVA type III was used to deal with the collinearity between variables inherent to our experimental design. Benjamini and Hochberg (1995) correction for multiple testing was applied using the *p.adjust* function from R package *stats* version 3.4.3. for models made for time-to-event variables. Since substantial correlation between phenological variables was expected, we developed a correlation matrix between the four flowering traits (Appendix A.5 in Supplementary material) using *cor* function from R package *stats* version 3.4.3. We excluded from the above analyses 72 individuals that did not flower, because the lack of flowering was associated to random local incidence of malfunctioning irrigation drippers, fungal disease, herbivory, or an unfavorable status of the original cutting, and they were not likely to flower at a later time.

In order to project the flowering phenology observed in the common garden to timing of phenological events under field conditions, we obtained mean daily temperature from the nearest weather stations of the Spanish Meteorological Agency (AEMET: http://www.aemet.es/es/datos_abiertos) from the common garden site and the natural populations at Sierra de Guadarrama to calculate cumulative temperature curves. This analysis was exclusively performed for Sierra de Guadarrama because it is the only mountain range in which both climatological and phenological data for the species are available under field conditions. The temperatures corresponding to the optimal and marginal natural populations were then estimated using a correction factor derived from the geographic coordinates of each site and the interpolations used for this area by the Climatic Digital Atlas of the Iberian Peninsula (ACDPI: <http://opengis.uab.es/wms/iberia/>). Flowering onset of optimal and marginal populations in the common garden was depicted on the cumulative temperature curves. The cumulative temperatures for the onset of flowering observed under common garden conditions were then projected applied to the cumulative temperature curves under field conditions in order to project flowering onset under optimal and marginal field conditions. The projected dates of flowering were compared to the flowering periods observed in the field by Giménez-Benavides et al. (2007b).

2.5. Molecular analysis

We collected leaves from 20 previously sampled genets from each population ($n = 180$) and extracted DNA using the DNeasy Plant minikit (QIAGEN, Valencia, USA) from 10 to 20 mg of dried *S. ciliata* leaf tissue. Eight microsatellite loci were selected for genotyping based on a previous study of García-Fernández et al. (2012): Sci1224, Sci1208, Sci0106, Sci1443, EST-2HTS, EST-37HTS, EST-G34D06 and

EST-G47A02. PCR was performed according to the protocol from the same study. An automated DNA sequencer (ABI PRISM 3730, Applied Biosystems, California, USA) was used to genotype all samples in Parque Científico de Madrid (Madrid, Spain). We used GeneMarker version 1.85 (SoftGenetics, StateCollege, Pennsylvania, USA) for fragment size determination. We evaluated the genotyping accuracy by re-amplifying and re-scoring 20% of the samples. To assess the frequency of null alleles, and allelic dropout we used MICRO-CHECKER (Van Oosterhout et al., 2004). Null alleles were found in most of the populations, but as they were not related to a particular locus across populations, we kept all the markers. No allelic dropout was found.

F_{ST} is an index of genetic differentiation between groups of individuals (Wright, 1950). We calculated three different F_{ST} values based on microsatellite marker data: (1) Overall genetic differentiation among the nine populations (F_{ST_Pop}); (2) genetic differentiation among mountain ranges while taking into account the genetic variance among populations within mountain ranges (F_{ST_Mt}); (3) genetic differentiation between environments (optimal vs. marginal) while taking into account the genetic variance among populations within environments (F_{ST_Env}). R package *hierfstat* version 0.04–22 (Goudet and Jombart, 2015) was used to estimate F_{ST} values and their corresponding 95% confidence intervals (CI) based on 1000 distance matrices bootstrapped over loci using the functions *varcomp.glob* and *boot.vc*. GenAlEx v6.502 (Peakall and Smouse, 2012) was used to perform an analysis of molecular variance (AMOVA) with the same above-mentioned designs to cross-check the results obtained from *hierfstat*.

2.6. P_{ST} – F_{ST} comparisons: past selection

In order to infer past selection on traits, comparisons between neutral genetic differentiation (F_{ST}) and quantitative trait differentiation (Q_{ST}) are widely used (e.g. Merilä and Crnokrak, 2001; Preite et al., 2015; Ye et al., 2013). Analogous to F_{ST} , Q_{ST} measures the genetic differentiation in quantitative traits (Merilä and Crnokrak, 2001). In order to calculate so-called narrow-sense Q_{ST} , it is necessary to estimate the additive variance of the traits, for which information on the relatedness among individuals within a population is needed. To this aim, intra-family phenotypic measurements of half-sibs or full-sibs are generally conducted (Volis et al., 2005). In our study we lack the necessary design and kinship information, but Q_{ST} can still be approximated without knowledge of kinship by considering a range in average relatedness among individuals. Leinonen et al. (2006), coined this approximation P_{ST} and promoted its use in quantitative genetics on wild-collected specimen, which are affected by environmental effects. In our study we largely controlled environmental variability in the common garden, so our approach can be seen as an environmentally-controlled P_{ST} analysis. We developed three P_{ST} values mirroring the three F_{ST} values: (1) phenotypic differentiation among all nine populations (P_{ST_Pop}), (2) among mountain ranges (P_{ST_Mt}) and (3) between the two environments (P_{ST_Env}), the latter two simultaneously accounting for the population phenotypic variance by including populations as a random variable in the models. P_{ST} was calculated using variance components obtained from random-effects models: $P_{ST} = c/h^2 \times VC_i / (c/h^2 \times VC_i + 2 \times VC_{error})$, where VC is phenotypic variance component of factor $i =$ population, mountain ranges or environments, VC_{error} is the residual variance, h^2 the proportion of phenotypic variance that is due to additive genetic effects within populations, and c the proportion of total variance that is presumed to be due to additive genetic effects among populations (Brommer, 2011). When variance components related to the three mountain ranges or the two environments (marginal vs. optimal) were computed, the variance component of populations within mountain range or environment was simultaneously assessed in the model, respectively. The ratio between the unknown variables h^2 and c was used to calculate P_{ST} across a range of c/h^2 from 0.0 to 2.0. The true value of c/h^2 is generally below 1.0 since trait heritability is usually lower among populations than within populations (Brommer,

Table 2

Results of the Kaplan-Meier models with population as a fixed factor and plant size as a covariate on four phenological variables of *Silene ciliata* measured in a common garden experiment.

	df	Onset χ^2	Peak χ^2	Duration χ^2	End χ^2
Plant size	1	6.0	2.2	1.8	2.3
Population	8	56.9***	35.8***	79.3***	105.9***
R ²		0.48	0.46	0.52	0.55

*** P < 0.001.

2011). However, since very low values are unlikely, we inferred past selection by comparing P_{ST} and F_{ST} for c/h^2 over the range 0.5–1.0. We computed P_{ST} confidence intervals by 999 bootstrapping iterations of the original data. Past diversifying selection is inferred if P_{ST} values are significantly higher than F_{ST} for c/h^2 between 0.5 and 1. No significant difference between P_{ST} and F_{ST} means that any phenotypic differentiation may have been caused by neutral processes alone. If P_{ST} values are lower than F_{ST} values, past stabilizing selection can be inferred.

3. Results

3.1. Phenological trait differentiation

All flowering traits showed significant differences among populations for all phenological traits (Table 2). A strong significant effect of environment for the onset ($P < 0.001$), peak ($P < 0.01$) and end of the flowering ($P < 0.001$), but not for the duration was found in the mixed effects models (Table 3). A significant effect of the mountain range of origin was found only for end ($P < 0.001$) of flowering. None of the models showed a significant mountain range x environment interaction for the phenological traits and no significant effect of plant size was found on phenological traits. Populations from optimal environments (upper edge) showed an advanced flowering phenology, with earlier flowering onset, peak and end, compared to populations from marginal environments (lower edge) (Fig. 2A–C). Populations from optimal areas started flowering on average 10 days earlier and finished flowering 24 days earlier than populations in marginal areas. Furthermore, flowering peak was 20 days earlier in populations from optimal areas (Appendix A, Table A.1 in Supplementary material). Also, flowering end was earlier in Gredos, closely followed by Béjar and finally Guadarrama (Fig. 3A). Differences in phenological traits between mountain ranges were weaker than those found between environments, the latter showing less overlap of confidence intervals (Figs. 2 and 3). Population and plant size had a significant effect on the total number of flowers (Table 4), but no significant effects of mountain ranges or environments on the total number of flowers were found (Table 4). Parameter estimates of all the models can be found in Appendix A;

Table 3

Results of the Kaplan-Meier mixed models with mountain range, environment, and their interaction as fixed factors, population as random factor and plant size as a covariate on four phenological variables of *Silene ciliata* measured in a common garden experiment.

	df	Onset χ^2	Peak χ^2	Duration χ^2	End χ^2
Plant size	1	2.8	3.6	1.5	1.9
Mountain range	2	9.7	5.5	10.0	17.0***
Environment	1	15.0***	12.4**	5.8	11.0***
Mountain range*Environment	2	3.5	1.3	6.9	9.0
R ²		0.46	0.45	0.50	0.54

** P < 0.01.

*** P < 0.001.

Tables A.2–4 in Supplementary material. Pairwise correlation analyses between the phenological variables showed a high correlation between duration and end of flowering ($\tau = 0.72$, Table A.5 in Supplementary material).

The calculation of the cumulative temperature curve for the common garden conditions showed that flowering onset of optimal and marginal populations in Sierra de Guadarrama took place with 1697.3 and 1934.9 °C days, respectively. When these temperature thresholds were projected to the cumulative temperature curves of the optimal and marginal field conditions, the flowering onset of the optimal population was later than that of the marginal population (Fig. 4).

3.2. Genetic differentiation

When testing solely for population structure, hierarchical F_{ST} analysis showed significant genetic differentiation among populations (F_{ST_Pop}) (Mean [CI]: 0.09 [0.06, 0.15]). When mountain range structure was included, genetic differentiation among mountain ranges ($F_{ST_Mt.}$) (0.05 [0.006, 0.11]) and among populations ($F_{ST_Pop/Mt.}$) (0.06 [0.04, 0.08]) were similar. When environment structure was included, no genetic differentiation was found between the two environments ($F_{ST_Env.}$) (0.0 [-0.02, 0.02]), but populations still explained a substantial part of the genetic variation ($F_{ST_Pop/Env.}$) (0.09 [0.06, 0.15]) (Appendix B, Table B.1 in Supplementary material). Molecular variance analysis showed a very similar pattern (Appendix B, Table B.2 in Supplementary material).

3.3. Past selection pressures

Trait differentiation between environments ($P_{ST_Env.}$) indicated a signal of past diversifying selection for flowering peak and, marginally, for end of flowering, since differentiation in these traits exceeded molecular marker differentiation ($F_{ST_Env.}$) for c/h^2 0.5–1 (Fig. 5A and B). On the contrary, onset and duration of flowering did not show significant differences between $P_{ST_Env.}$ and $F_{ST_Env.}$ (Appendix C, Fig. C.1 in Supplementary material). For the four tested phenological traits, neither trait differentiation among populations (P_{ST_Pop}) nor among mountain ranges ($P_{ST_Mt.}$) exceeded the corresponding molecular marker differentiation (F_{ST_Pop} and $F_{ST_Mt.}$, respectively) (Appendix C, Fig. C.2 and 3 in Supplementary material).

4. Discussion

4.1. Genetically-based phenological differentiation

Genetically-based phenological differentiation can be the result of neutral processes as well as selection (García-Ramos and Kirkpatrick, 1997). In our case, we found evidence of genetically based differentiation and past selection on phenological traits between marginal and optimal populations at the elevational extremes, since neutral marker differentiation was virtually absent whereas phenological trait differentiation was strong. Phenological traits involving reproductive timing are likely to be under strong selection since they are vital for plant fitness (Chuine, 2010; Fox, 2003; Levin, 2006). This is in agreement with empirical studies showing that these traits generally show high heritability and evolvability (Kawakami et al., 2011; Méndez-Vigo et al., 2013; Volis, 2011) and with a meta-analysis showing consistent selection patterns (Munguía-Rosas et al., 2011). In this context, selection analyses performed under field conditions by Giménez-Benavides et al. (2011b) for the same species in the same area showed that flowering onset and duration are under selection within populations at different elevations.

Our molecular marker results and previous studies (García-Fernández et al., 2012; Morente-López et al., 2018) reveal substantial gene flow across the elevational gradient in this species and it is widely acknowledged that gene flow can counteract the effects of selection

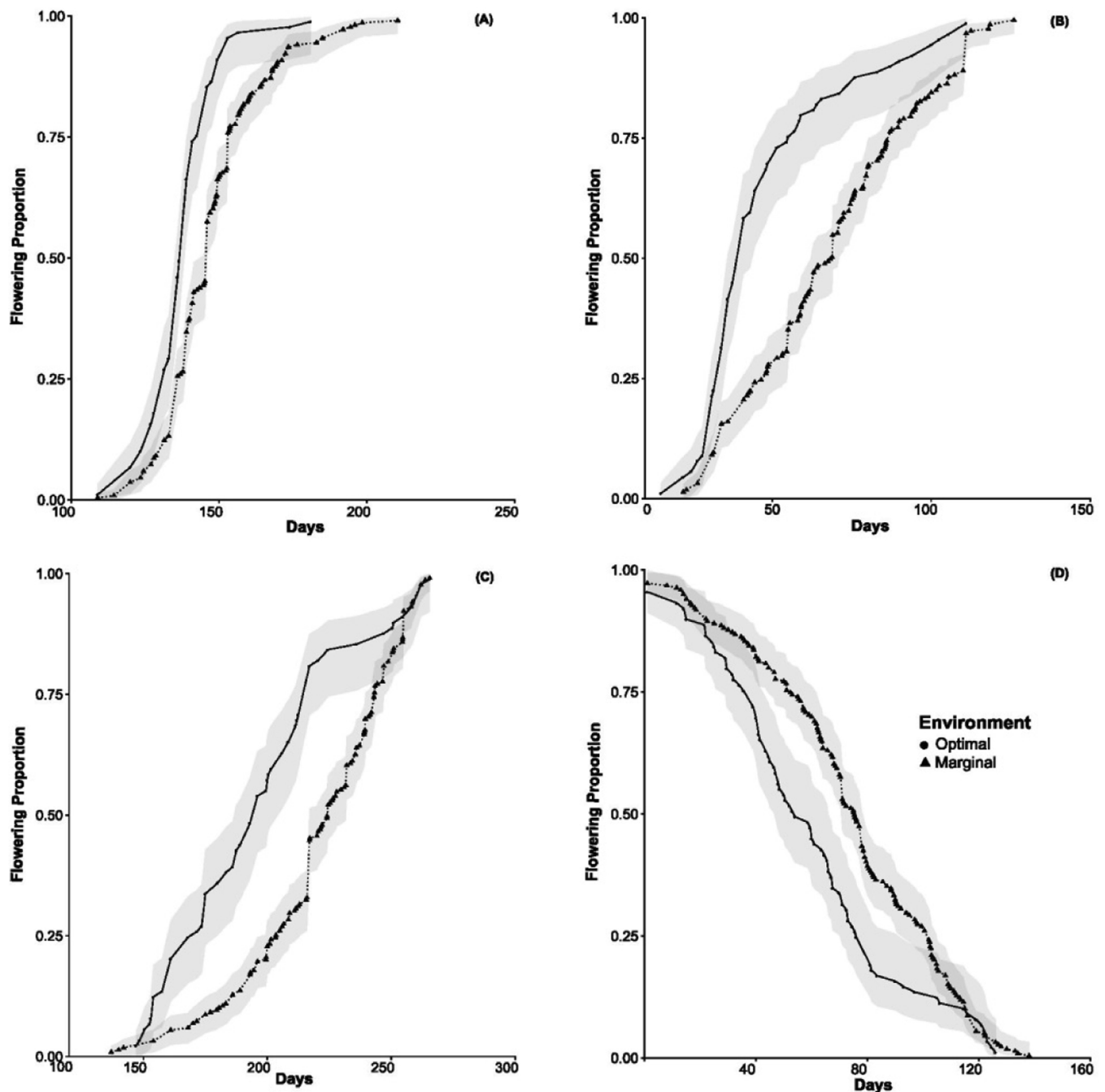


Fig. 2. Flowering curves representing the onset (A), peak (B), end (C) and duration (D) of *Silene ciliata* flowering for the optimal and marginal environments.

(Garcia-Ramos and Kirkpatrick, 1997; Lenormand, 2002; Yeaman and Whitlock, 2011). Considering that opposing effects between gene flow and local selection pressures can lead to different gene flow – selection equilibria at the different elevations (Garcia-Ramos and Kirkpatrick, 1997; Linhart and Grant, 1996), we showed that past selection acted successfully in the face of significant gene flow between populations inhabiting environmentally differentiated areas. Thus, it seems that the environmental gradient is strong enough between populations to create not only effective divergent selective pressures but also differential phenotypic values.

Genetic differentiation in phenological traits was found among

mountain ranges of origin only for flowering end, but no evidence of past selection was found. This result can be partially explained by neutral processes, such as gene flow and genetic drift. Nevertheless, given the observed significant neutral marker differentiation among mountains, phenological adaptation may have remained undetectable in our analysis, and thus the action of adaptive processes promoting differentiation among mountain ranges cannot entirely be excluded. Although mountains were included to replicate optimal and marginal conditions across the study region, we cannot rule out the existence of local variation in climate among the mountains (Scherrer and Körner, 2010) to which populations may have adapted.

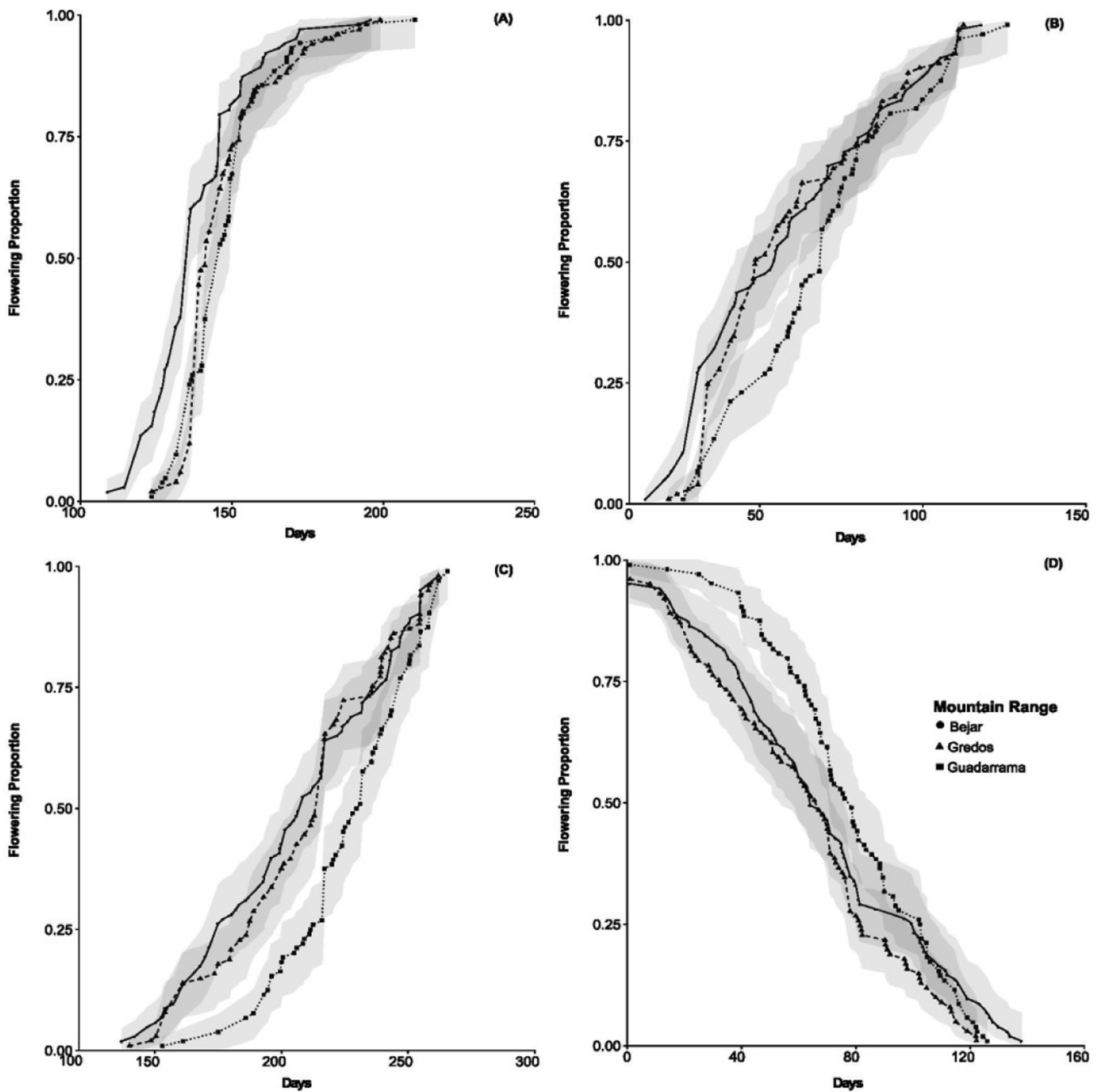


Fig. 3. Flowering curves representing the onset (A), peak (B), end (C) and duration (D) of *Silene ciliata* flowering for the three mountain ranges studied.

It should be noted that the observed genetic differences among populations have been observed in a single growing environment outside of the environmental range of the species. Therefore, results need to be interpreted carefully as phenotypes expressed in the experiment may not be the same as those that would have been expressed under natural conditions. In any case, the marked differences observed under common garden conditions provide a solid evidence of genetic differentiation between populations (Lara-Romero et al., 2014). In this sense, when the cumulative temperature for the onset of flowering observed under common garden conditions for Sierra de Guadarrama populations was projected onto the cumulative temperature curves under field conditions, flowering onset takes place earlier in the marginal field conditions and falls within the flowering period observed in the field by Giménez-Benavides et al. (2007b) (Fig. 4). Thus, despite the pattern of

genetic differentiation was observed in a different environment outside the species range, the flowering phenology of *S. ciliata* may not be so different under natural conditions.

The potential incidence of maternal effects is another factor that should be considered in this study, because the individuals of the common garden were obtained from cuttings of mother plants living in the natural populations. Nevertheless, this effect is likely to be low due to the considerable period in which they were grown in common garden conditions and to the similar flowering patterns observed with plants derived from seeds obtained under common garden conditions from these same populations (data not shown).

Table 4

Results of the linear model (LM) and the linear mixed model (LMM) of the total number of flowers of *Silene ciliata* measured in a common garden experiment. The linear model was defined with population as explanatory variable and size as a covariate. The linear mixed model was defined with mountain range, environment and their interaction as fixed factors, population as a random factor and plant size as a covariate. R²m: marginal R², i.e., proportion of variance explained by fixed factors. R²c: conditional R², i.e., the proportion of variance explained by both random and fixed factors.

Total Flowers (GLM)			Total Flowers (GLMM)		
	df	χ^2		df	χ^2
(Intercept)	1	6703.53***	(Intercept)	1	169.85***
Plant size	1	158.15***	Plant size	1	156.89***
Population	8	2067.09***	Mountain range	2	6.75
			Environment	1	0.60
			Mountain range*Environment	2	2.32
			R ² m		0.1; R ² c
					0.3

*** P < 0.001.

4.2. Diversifying selection originating from contrasting environmental conditions

Populations from the optimal environments (higher elevation) started the flowering season and reached the flowering peak earlier than populations from marginal environments (lower elevation) under common garden conditions (Figs. 2, 4 and 6). This differentiation may result from diversifying selection on threshold flowering cumulative temperature in optimal and marginal environments caused by the contrasting past environmental conditions experienced by populations. Lower threshold flowering cumulative temperature (i.e. earlier flowering in the common garden, see Figs. 4 and 6) may be linked to significantly shorter effective growing season experienced at higher elevations in terms of temperature (Nagy and Grabherr, 2009), and to the positive correlation between flowering duration and seed production found in natural populations (Giménez-Benavides et al., 2007b). Thus, earlier flowering may ensure enough time to complete the reproductive process and to increase seed production. This pattern has been related with the need to optimize fruit set and seed development as a late-flowering seed-risk strategist (Molau, 1993). Similar phenological differentiation has been previously reported for *Armeria caespitosa*, another Mediterranean alpine species (Lara-Romero et al., 2014). In contrast, in marginal populations, where the length of the growing season is not as restricted by temperature as in optimal populations, the risk of anticipated drought in summer may not have been strong enough to promote a similar advance in flowering time.

4.3. Implications of population phenological differentiation in the context of climate change

The results of this study may have relevant implications in the context of global warming in Mediterranean alpine areas. As discussed above, the common garden experiment was established in a location that has warmer conditions than those occurring in the natural populations (Figs. 5 and 6). As a result, *S. ciliata* plants started flowering more than two months earlier (mid of April) than populations in the field (between end of July and mid of August) (Giménez-Benavides et al., 2007b). This indicates that flowering phenology in this species is strongly affected by temperature and not constrained by photoperiod as suggested earlier (Giménez-Benavides et al., 2007b). This is consistent with inter-annual phenological variation found by Giménez-Benavides

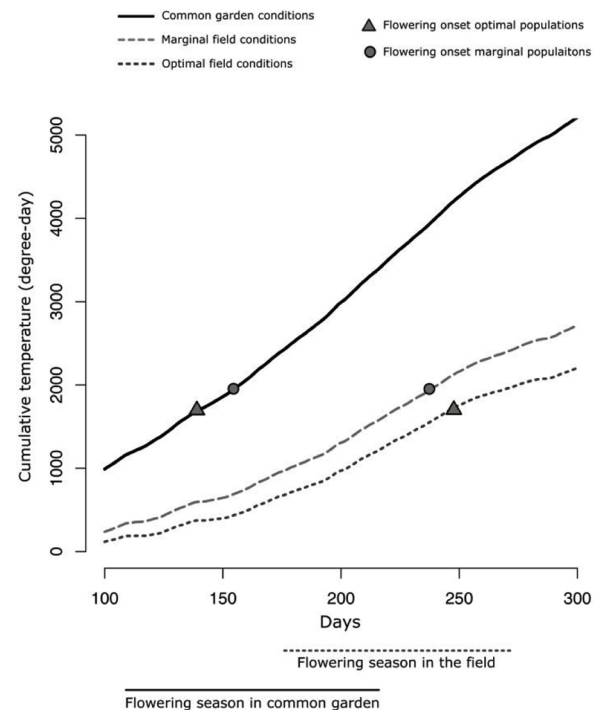


Fig. 4. Cumulative temperature curves through time in the common garden and under field conditions (optimal and marginal areas) for Sierra de Guadarrama populations. Basal temperature was set at 0 °C. The flowering onset of optimal (triangle) and marginal populations (circle) is represented under common garden conditions. A projection of the flowering onset cumulative temperature reached under common garden conditions is represented on the optimal and marginal field conditions cumulative temperature curves (triangle and circle, respectively). Under common garden conditions, optimal populations start the flowering season earlier than marginal populations, but they are projected to flower later under the different natural environmental conditions. Projected flowering onset falls within the flowering season observed in the field by Giménez-Benavides et al. (2007b) (dotted line at the bottom).

et al. (2011b) and with recent research in other Alpine species showing that flowering is strongly regulated by temperature (Bliss, 1971; Huelber et al., 2006; Kudo and Hirao, 2006; Lara-Romero et al., 2014; Scheepens and Stöcklin, 2013; Walker et al., 1995). This inherited capacity to shift flowering onset may be of adaptive value for natural populations under ongoing global warming (Chuine, 2010; Davis et al., 2005; Linhart and Grant, 1996), as it may allow them to advance their flowering period in response to warmer conditions, increasing flowering duration and reproductive performance (Giménez-Benavides et al., 2011b).

It has been hypothesized that the reproductive success of late-flowering seed-risk strategists *sensu* Molau (1993) will improve under global warming due to elongation of the reproductive period caused by fewer days of snow cover in alpine areas (Alatalo and Totland, 1997; Molau, 1993; Molau et al., 2005). However, late flowering seed-risk species inhabiting Mediterranean ecosystems may not benefit from this, because, like snowmelt date, summer drought is likely to occur earlier as well and, thereby, keep the end of the growing season constrained (Giménez-Benavides et al., 2018). In fact, *S. ciliata* populations may experience a diminished reproductive period with temperature increase and precipitation decrease as predicted by global warming scenarios (Pachauri et al., 2014). Thus, as climate change may cause an advance and a shortening of the growing season, we hypothesize that optimal populations may be better adapted because they have already been

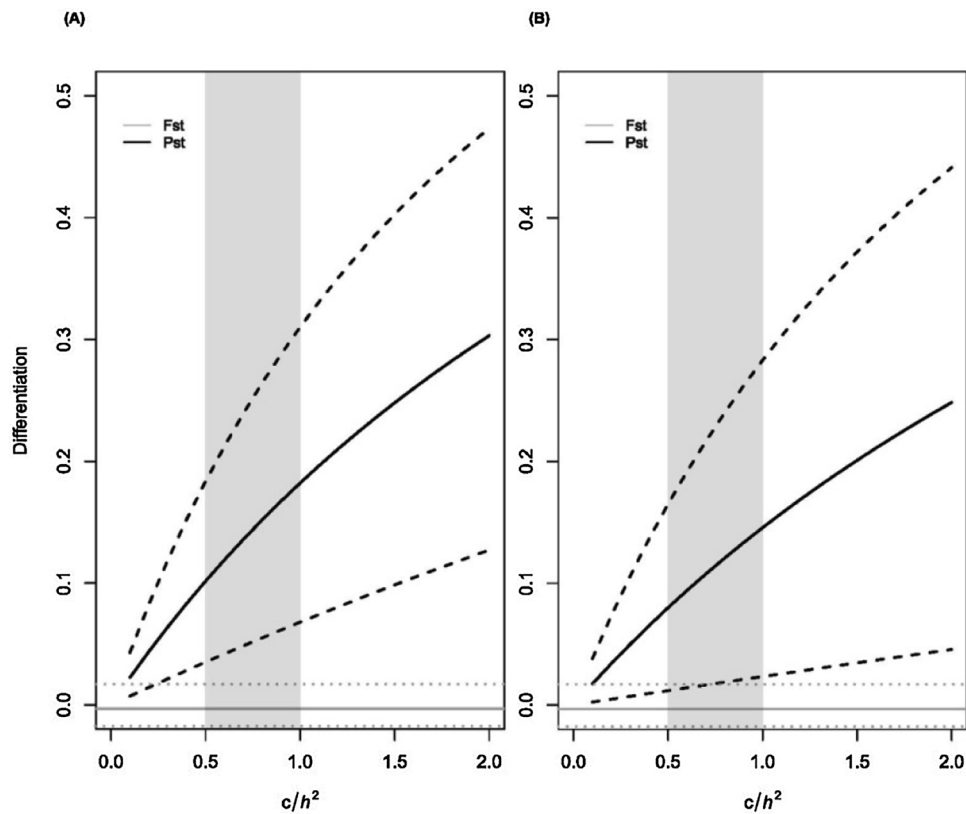


Fig. 5. P_{ST} and F_{ST} graphical comparisons for (A) flowering peak and (B) end of flowering in *Silene ciliata* populations from contrasting environments grown in a common garden experiment, depending on c/h^2 ranging from 0.0–2.0. Solid lines indicate the mean values and dotted lines the confidence intervals. The grey area indicates the more plausible range for c/h^2 between 0.5 and 1.0 (see Brommer, 2011).

selected for earlier flowering. In contrast populations inhabiting marginal environments have not been selected as strongly for early flowering in the past and therefore may have less evolutionary resilience (*sensu* Sgrò et al., 2011) to deal with environmental conditions that favor earlier flowering. This is in agreement with the declining population trends found at the marginal populations by Giménez-Benavides et al. (2011a). Further research, involving reciprocal transplants at the elevational boundaries and below the current elevational range, as well as experiments under controlled temperature conditions, are needed to confirm local adaptation in the study populations (Kawecki and Ebert, 2004) and to evaluate the evolutionary resilience of populations from both optimal and marginal environments.

5. Conclusions

Our results show that flowering phenology in *S. ciliata* is genetically differentiated among populations. We observed strong phenological differentiation between marginal and optimal populations. This differentiation among contrasting elevations is due to past selection in the presence of significant gene flow. Thus, environmental differences were strong enough to generate diversifying selection over very short distances. With ongoing climate change, the timing and length of the growing season in Mediterranean alpine ecosystems will change depending on snowfall, snowmelt and the intensification of summer drought. *Silene ciliata* populations will have to adapt to this by advancing the flowering period to complete reproduction before summer drought arrives. Populations can plastically respond to the temperature increase, advancing their flowering phenology as we observed in the common garden for both optimal and marginal populations. However, populations from optimal environments, already selected in the past to advance their flowering time, may have an adaptive advantage in the context of greater temperatures and drought, and thus be better adapted to earlier and shorter vegetative periods.

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Data accessibility

The datasets generated and used in this publication can be found in the Universidad Rey Juan Carlos data repository e-cienciaDatos.

Genetic data (doi: <https://www.doi.org/10.21950/GSTZ26>): <https://edatos.consorciomadrono.es/dataset.xhtml?persistentId=doi:10.21950/GSTZ26>.

Phenology data (doi: <https://www.doi.org/10.21950/XLYLQK>): <https://edatos.consorciomadrono.es/dataset.xhtml?persistentId=doi:10.21950/XLYLQK>.

CRediT authorship contribution statement

Javier Morente-López: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **J.F. Scheepens:** Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **Carlos Lara-Romero:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing - review & editing. **Raquel Ruiz-Checa:** Data curation, Investigation, Writing - review & editing. **Pablo Tabarés:** Data curation, Investigation, Writing - review & editing. **José María Iriondo:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

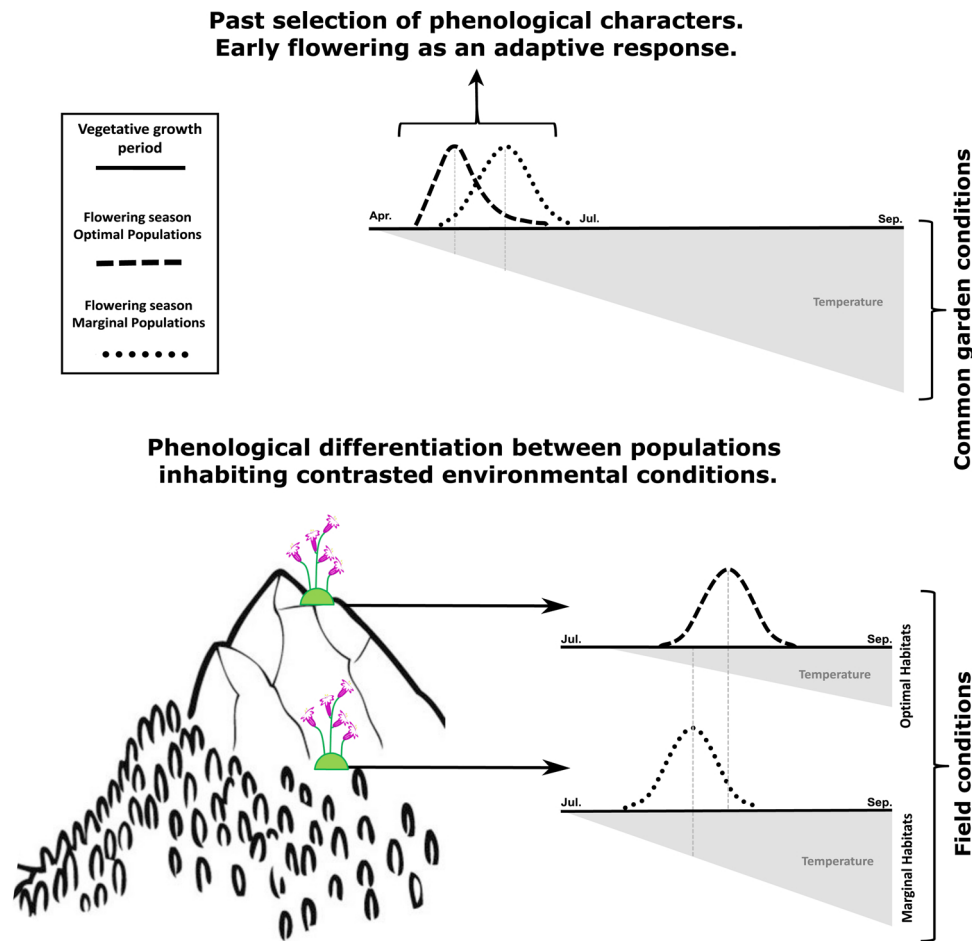


Fig. 6. Conceptual representation of phenological differentiation between optimal and marginal populations of *Silene ciliata*. In field conditions, plant populations in marginal areas show an advanced flowering phenology (onset, peak and end) compared to plant populations in optimal areas. In this study, under common garden conditions, plant populations from optimal areas show an earlier flowering phenology than plant populations from marginal areas.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.envexpbot.2019.103894>.

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