

# Phenotypic plasticity in seed germination relates differentially to overwintering and flowering temperatures

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(Received 11 March 2014; accepted after revision 15 July 2014; first published online 28 August 2014)

## Abstract

Phenotypic plasticity in seed dormancy may allow plant species to cope with rapid environmental changes, such as climate warming. In controlled experimental settings, plasticity in dormancy has been found to relate to temperature during seed maturation, but this relationship has not been tested in field conditions. Here we analyse for the first time the variation in dormancy during five successive years in wild populations of the study species *Centaureium somedanum*, to determine how this variation is related to average temperatures during specific seasons of plant activity. We performed laboratory germination experiments to measure: (1) the degree of dormancy at dispersal; and (2) the sensitivity to dormancy-breaking factors. We calculated average temperatures during four seasons of plant activity (overwintering, vegetative growth, flowering and seed maturation) for each year, and tested the relationship between these temperatures and patterns of dormancy variation, using Generalized Linear Models. Dormancy varied among sites and years, seeds being more dormant in colder years. For the degree of dormancy at dispersal, we found a positive relationship with flowering temperature and a significant effect of collection site. For the sensitivity to dormancy-breaking factors, we found no significant differences among sites, a positive relationship with flowering temperature and a negative relationship with overwintering temperature. Phenotypic plasticity in dormancy in *C. somedanum* is thus especially marked in the sensitivity to dormancy-breaking factors. Temperatures during overwintering and flowering have differential effects on this plasticity, allowing the plant to detect the gradient of seasonality, a main ecological feature of its distribution.

These results highlight the importance of taking into account more than average temperatures when assessing the response of plant phenotypic plasticity to climate change.

**Keywords:** *Centaureium somedanum*, dormancy variation, Gentianaceae, plant–climate interactions, plant regeneration, seed ecology

## Introduction

Phenotypic plasticity will be a major player in plant responses to climate change (Nicotra *et al.*, 2010; Reed *et al.*, 2011). Global warming (IPCC, 2013) can potentially disrupt the many interactions between climatic patterns and biological processes, such as the thermal control of seed germination (Probert, 2000). In seasonal climates, the timing of seed germination is closely linked to annual climatic cycles to ensure that seedling establishment occurs in the favourable season (Donohue *et al.*, 2010), but there is a risk that this relationship will be disrupted by climate warming (Walck *et al.*, 2011). Future temperatures may no longer match germination requirements (Cochrane *et al.*, 2011), or may be inadequate to fulfil dormancy-breaking requirements (Orrù *et al.*, 2012). Such effects can modify recruitment from the soil seed bank (Ooi *et al.*, 2009; Ooi, 2012; Hoyle *et al.*, 2013) and shift germination timing (Mondoni *et al.*, 2012), ultimately compromising species' survival. Nonetheless, predicting germination responses to climate change is a challenge for plant ecologists. In seeds with physiological dormancy, which are the majority in temperate regions (Baskin and Baskin, 1998), it is necessary to understand the influence of environmental temperature on four levels: germination itself, dormancy loss, phenotypic plasticity in dormancy and selection of dormancy genotypes.

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The rate of germination is a linear function of temperature between base and ceiling thermal thresholds beyond which no germination occurs (García-Huidobro *et al.*, 1982; Pritchard and Manger, 1990; Hardegree, 2006). Germination thermal thresholds depend on the level of dormancy, i.e. a seed characteristic, the degree of which defines the conditions that should be met to make the seed germinate (Vleeshouwers *et al.*, 1995; Finch-Savage and Leubner-Metzger, 2006). Physiologically, changes in dormancy are translated into changes in germination thermal thresholds, and the rate of this change is again a linear function of temperature below a thermal threshold beyond which dormancy loss stops (Pritchard *et al.*, 1996, 1999; Steadman and Pritchard, 2004). Differences among individuals and seed lots in their thermal thresholds are noticed as spatial and temporal variation in seed dormancy, either in dormancy levels (Daws *et al.*, 2004; Orrù *et al.*, 2012) or in the sensitivity to dormancy-breaking factors (Pritchard *et al.*, 1999). These differences in dormancy can be the result of local differences in environmental temperatures acting in the long and short terms. In the long term, local thermal differences can select genetically based differences in dormancy through clinal variation and local adaptation (Wagmann *et al.*, 2012; Fernández-Pascual *et al.*, 2013). In the short term, temperature variation during seed maturation can produce phenotypic plasticity in dormancy (Fenner, 1991; Gutterman, 2000; Fenner and Thompson, 2005).

In a changing climate this phenotypic plasticity becomes of the utmost importance, as it may be the key for quick plant responses to new conditions (Matesanz *et al.*, 2010; Walck *et al.*, 2011). Controlled seed maturation in experimental gardens has shown that warmer maturation environments produce less dormant seeds (Luzuriaga *et al.*, 2006; Qaderi *et al.*, 2006; Figueroa *et al.*, 2010; Chiang *et al.*, 2011), but there is little information on how this relationship holds in field conditions. A possible approach to addressing this question might be to study dormancy levels of wild populations in successive years, so the genetic background remains relatively constant and any differences can be attributed to phenotypic plasticity in response to varying weather conditions. Analysing these temporal changes in relation to climate is a key step towards understanding the effects of climate change on seed ecology (Walck *et al.*, 2011). It is, for instance, necessary to establish the specific influence of temperature during different seasons of plant growth, as climate change is expected to be focused on certain times of the year (IPCC, 2013). However, to our knowledge, no field studies have evaluated the influence of climate on seed dormancy during consecutive years.

In this work, we investigate how dormancy variation is related to climatic trends during

five successive years in the study species *Centaureium somedanum* M. Laínz (Jiménez-Alfaro *et al.*, 2013). At dispersal, seeds of *C. somedanum* have non-deep simple morphophysiological dormancy (Fernández-Pascual *et al.*, 2012). The physiological component of this dormancy varies following an altitudinal cline that is related to long-term climatic differences among sites and also to neutral genetic variation (Fernández-Pascual *et al.*, 2013). Moreover, in a previous common garden experiment we demonstrated phenotypic plasticity in seed dormancy in response to the seed maturation environment (Fernández-Pascual *et al.*, 2013). These preceding studies point to this species as a good biological system to test the influence of short-term climatic changes. To this end, we tested the relationship between environmental temperatures experienced by wild populations and two seed traits: (1) level of dormancy at dispersal; and (2) sensitivity to dormancy-breaking factors. To assess the potential role of thermal variation within each year, we tested the effect of temperatures during different seasons of plant growth.

## Materials and methods

### Study species

*C. somedanum* M. Laínz (Gentianaceae) is a perennial herb that grows in small-sedge and brown-moss communities associated with tufa-forming springs (Jiménez-Alfaro *et al.*, 2013). Its distribution is confined to a 210 km<sup>2</sup> area in the Cantabrian Mountains of north-western Spain, where discrete populations can be found from 600 to 1700 m above sea level (asl) (Jiménez-Alfaro *et al.*, 2010). The regional climate is transitional between Oceanic and Continental-Mediterranean, with local differences depending on altitude and exposure. The species is a facultative outcrosser and shows a high reproductive output: on average individuals produce 2–4 fruits with *c.* 140 seeds each, although the number of fruits can reach up to 15 (Jiménez-Alfaro *et al.*, 2005); and the proportion of viable seeds is generally over 95% (Fernández-Pascual *et al.*, 2012). Flowering peaks in July, seeds mature during August and September, dispersal lasts until October and seedlings emerge next summer (Jiménez-Alfaro *et al.*, 2010).

### Seed collection and germination

We collected seeds of *C. somedanum* during five consecutive years (2008–2012). To assess within-species variation in seed dormancy, we replicated the study in the two largest populations, La Malva (43°07′05″N, 06°15′05″W, 600 m asl, *c.* 230 reproductive

individuals) and El Valle (43°04'19"N, 06°11'49"W, 1280 m asl, c. 280 reproductive individuals). These two sites are 7.5 km apart, on the northern side of the Cantabrian Mountains, and have a strong Oceanic influence. Each year we made one collection per site at the time of seed dispersal, sampling 20 medium-sized individuals. Marking individuals was unfeasible because of the small and frail plants; each year we selected individuals randomly from the same areas. From each individual we took two capsules (dry and brownish). Capsules spent 3 weeks in our laboratory (c. 22°C, 50% relative humidity) before being cleaned and used in germination experiments.

For the experiments, seeds were sown on 1% distilled water agar held in 6-cm-diameter Petri dishes (eight dishes with 25 seeds each per collection), sealed with Parafilm to prevent desiccation. Half of the dishes (fresh seeds) went immediately into germination incubation to check dormancy at dispersal; the other half (stratified seeds) were exposed to wet-cold stratification (12 weeks, 1% agar, 3°C, darkness) before being placed in germination incubation, to assess their receptiveness to dormancy-breaking factors. Germination incubation was conducted in a growth chamber (Grow-S 360, Ing. Climas, Barcelona, Spain) programmed with a 12 h light/12 h darkness photoperiod (c.  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  during the light phase provided by six Philips TLD30W/54-765 cool fluorescent tubes) coupled with a 22/12°C thermal regime. These conditions promote *C. somedanum* germination once dormancy is broken (Fernández-Pascual *et al.*, 2012). Although this procedure does not allow for interpreting the effect of dormancy on the base and ceiling thermal thresholds for germination (Steadman and Pritchard, 2004), tests performed under a single temperature treatment offer valuable information on differences in dormancy among seed collections (Andersson and Milberg, 1998; Wagmann *et al.*, 2012; Fernández-Pascual *et al.*, 2013). During incubation, we counted and discarded germinated seeds three times

a week. Radicle protrusion was the criterion for germination. We terminated experiments after 4 weeks and opened non-germinated seeds with a scalpel, classifying them as full, empty or fungus-infected. We excluded empty ( $3.1 \pm 0.5\%$ ) and infected ( $1.9 \pm 0.4\%$ ) seeds from statistical analyses and germination percentages.

### Temperature data

To describe temperature during the study period we used climatic data recorded by the Spanish Meteorological Agency. Data for the entire study period were only available from Xinestosu meteorological station (43°03'53"N, 06°23'30"W, 1170 m asl). The station is located 15 km away from the study area, in a valley under the same climate. Daily records show a very high correlation ( $R^2 > 97\%$ ) with those obtained in the stations that are closest to the study sites, La Pola and La Peral. Therefore, although absolute values from Xinestosu are not suitable to establish a predictive model, they allow the assessment of differences among years at the regional level, as is our goal.

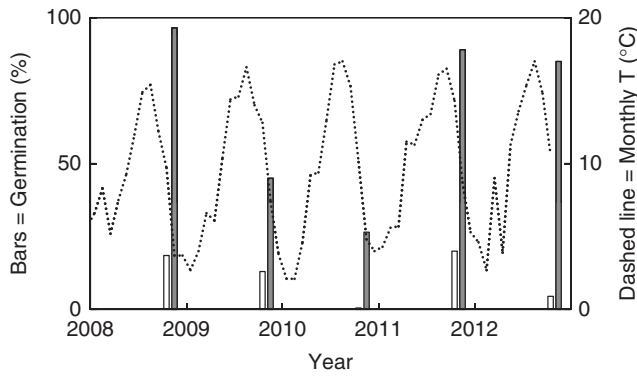
We divided the year into four seasons of plant activity: (1) overwintering (November–February), which is the unfavourable season in this area, with cold temperatures, relatively frequent snow and no plant activity; (2) vegetative period (March–May), with rising temperatures and vegetative plant growth; (3) flowering (June–July), including flower development and pollination; and (4) seed maturation (August–October) up to dispersal. Then we calculated the average temperature for each season and year (see Table 1).

### Statistical analyses

To analyse variation in germination data, we fitted a fully factorial Generalized Linear Mixed Model

**Table 1.** Temperature and germination in the five studied years. Temperatures are the average of the plant activity seasons of overwintering (O), vegetative growth (V), flowering (F) and seed maturation (S); annual average (A) is also given. Germination of fresh and stratified seeds is given for the two populations. Germination data are the mean  $\pm$  SE of four Petri dishes

Year	Temperature (°C)					Germination (%)			
	O	V	F	S	A	La Malva		El Valle	
						Fresh	Stratified	Fresh	Stratified
2008	6.8	7.3	13.5	12.4	9.4	34 $\pm$ 8	98 $\pm$ 1	3 $\pm$ 2	95 $\pm$ 1
2009	3.5	7.7	14.5	14.5	9.1	15 $\pm$ 3	37 $\pm$ 2	11 $\pm$ 3	53 $\pm$ 8
2010	3.9	7.7	14.9	14.1	9.2	0	20 $\pm$ 3	1 $\pm$ 1	33 $\pm$ 5
2011	4.7	9.5	13.2	15.6	10.0	24 $\pm$ 4	83 $\pm$ 3	16 $\pm$ 3	95 $\pm$ 1
2012	5.3	8.1	14.5	14.1	9.7	0	72 $\pm$ 3	9 $\pm$ 3	98 $\pm$ 1



**Figure 1.** Temperature and germination during the study years. Temperature (dashed line) is the monthly average. Germination values of fresh (white bars) and stratified (grey bars) seeds are the averages of the two sites. The beginning of each year in January is marked on the x axis.

(GLMM, binomial distribution, logit link function) with site, year and stratification as fixed factors and Petri dish as random factor. Afterwards, to test the relationship between germination and temperature we used a variable selection procedure (forward/backwards, Akaike’s Information Criterion) to fit a model to germination data (separately for fresh and stratified seeds), with seasonal temperatures and collection site as predictors. We performed statistics with the R-Commander package (v1.5-3) for R (v2.10.1; The R Foundation for Statistical Computing, Vienna, Austria). We also plotted the best predictors for each model using SigmaPlot 12.5 (Systat Software Inc., San José, USA).

**Results**

Dormancy varied among years and sites (Table 1). Germination was significantly lower in fresh ( $6.7 \pm 1.2\%$ ) versus stratified ( $79.2 \pm 2.2\%$ ) seeds ( $\chi^2 = 298.247, P < 0.001$ ). Average germination was

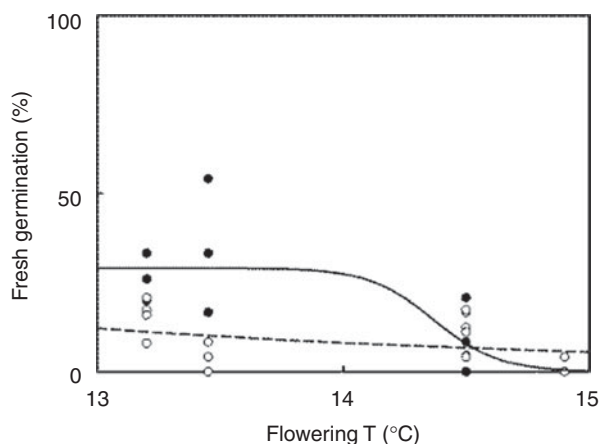
significantly different among years, being higher in 2008 and 2011 ( $63.3 \pm 4.9\%$ ), intermediate in 2009 and 2012 ( $31.5 \pm 4.9\%$ ) and lower in 2010 ( $6.0 \pm 2.0\%$ ) ( $\chi^2 = 28.067, P < 0.001$ ). Conversely, collection site did not have a significant main effect on germination ( $\chi^2 = 1.801, P = 0.185$ ). Stratification had a significant interaction with both year ( $\chi^2 = 17.004, P < 0.001$ ) and site ( $\chi^2 = 6.640, P = 0.012$ ), as dormancy release was stronger in certain years (e.g. 2012) and La Malva seeds were less dormant when fresh but more dormant after stratification than El Valle seeds. Interaction was also significant between year and site ( $\chi^2 = 7.113, P < 0.001$ ), especially as a consequence of the results of year 2008, when fresh germination was the highest of all years at La Malva but one of the lowest at El Valle. The three-way interaction was not significant ( $\chi^2 = 0.492, P = 0.742$ ). Overall, sizeable fresh germination occurred in 2008, 2009 and 2011; whereas a high alleviation of dormancy by cold stratification occurred in 2008, 2011 and 2012 (Fig. 1). Highest germination thus took place in 2008 and 2011, years with warmer winters (Table 1).

For fresh seeds, the variable selection procedure fitted a model with flowering temperature and collection site as explanatory variables (Table 2a). For stratified seeds, the model dropped collection site and included temperatures of overwintering and flowering (Table 2b). The explained deviance of the stratified model (36%) was higher than that of the fresh model (6%), suggesting a stronger effect of the selected predictors. On one hand, flowering temperature was positively related to dormancy levels of fresh (Fig. 2) and stratified seeds (Fig. 3), producing lower germination with increasing mean temperatures from June and July. However, in fresh seeds this effect was not significant in one of the populations (El Valle,  $\chi^2 = 3.154, P = 0.076$ ). On the other hand, overwintering temperature was negatively related to the dormancy levels of stratified seeds (Fig. 3), resulting in higher germination rates with increasing winter year temperatures.

**Table 2.** Generalized Linear Models (GLM, binomial distribution, logit link) fitted to germination data. Effects appear in the order they were included in the models by the variable selection procedure [forwards/backwards, Akaike’s Information Criterion AIC]. Flowering T = average temperature during flowering season (June–July); overwintering T = average temperature during overwintering season (November–February); %V = percentage of variance explained by the model after inclusion of the effect

Model	Effect	Coefficient	SE	$\chi^2$	P	%V
(a) Fresh AIC = 622.4	Intercept	13.441	2.332	33.224	<0.000	
	Flowering T	-1.091	0.169	41.667	<0.000	4.84
	Site [El Valle]	-0.711	0.218	10.602	<0.000	6.00
(b) Stratified AIC = 838.5	Intercept	13.888	2.822	24.216	<0.000	
	Overwintering T	0.956	0.116	68.525	<0.000	29.35
	Flowering T	-1.221	0.178	46.923	<0.000	35.52





**Figure 2.** Effect of flowering temperature on germination of fresh seeds, separated by site (black circles and solid line = La Malva; open circles and dashed line = El Valle). Each circle represents the germination percentage of the seeds from a Petri dish after 4 weeks of incubation at 22/12°C. Lines are the logistic functions fitted to data.

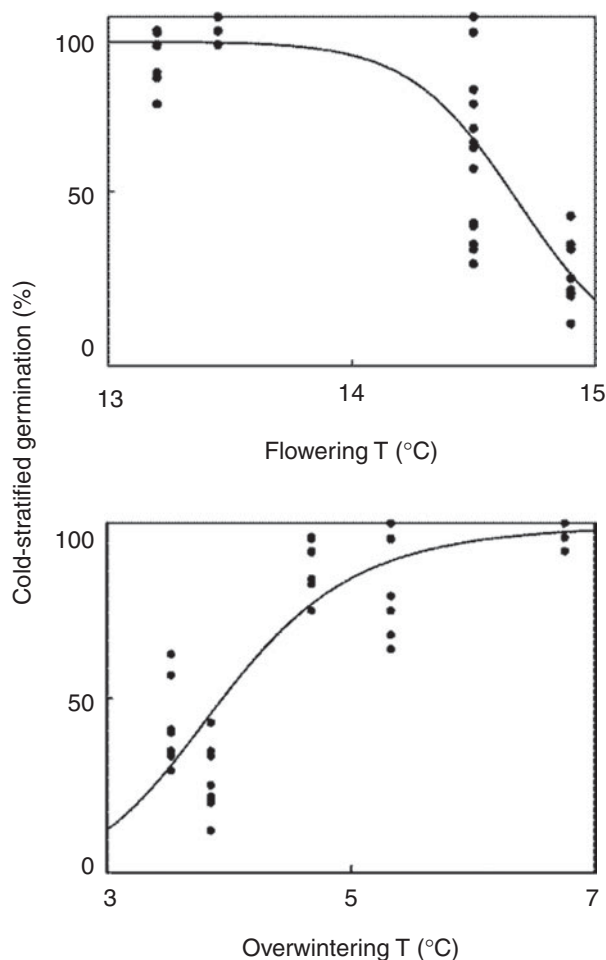
## Discussion

Our study suggests that wild populations of *C. somedanum* show phenotypic plasticity in seed dormancy during successive years. While similar plasticity has been found in other species (Andersson and Milberg, 1998; Schütz and Rave, 2003; Herranz *et al.*, 2010), to our knowledge this is the first time that inter-annual germination plasticity has been related to inter-annual differences in temperature. Influences of local temperatures on dormancy have usually been tested *in space*, i.e. in different populations at a given year (Moyer and Lang, 1976; Wagmann *et al.*, 2012; Fernández-Pascual *et al.*, 2013); but it seems that the same relationship holds in the field *in time*, i.e. in different years in a given population. We also found that plasticity is stronger for the sensitivity to dormancy-breaking factors (in stratified seeds), while dormancy at dispersal (in fresh seeds) is more heritable. This agrees with our previous experiments with *C. somedanum* seeds grown in a common garden, where an altitudinal dormancy cline detected in the field was strongly maintained at dispersal, while the sensitivity to dormancy-breaking factors showed more plasticity in response to the seed maturation environment (Fernández-Pascual *et al.*, 2013).

It must be taken into account that the variation we have detected in this study cannot be attributed exclusively to phenotypic plasticity. Sampled individuals were selected at random each year, and thus the slightly different genetic make-up of each seed collection may be driving some of the differences identified. However, differences do not follow a

random pattern as would be expected if they were influenced by sampling, but are related to annual temperatures. As the study species is not annual but perennial, and does not flower until its second year, a selection of dormancy genotypes by each year's winter is not likely. Thus we may assume that, within the mentioned limitations, the variation we have detected is mainly caused by phenotypic plasticity.

Our analyses at different phenological stages provide interesting outputs concerning the potential effect of environmental temperatures within the same year. In years with warmer winters, *C. somedanum* seeds are more sensitive to dormancy-breaking treatments and their dormancy release is quicker. This follows the traditional rule of dormancy variation: warmer temperatures, be they in the garden or field, equal less dormant seeds (Luzuriaga *et al.*, 2006;



**Figure 3.** Effect of flowering and overwintering temperatures on germination of cold-stratified seeds, averaging germination data for the two populations (with non-significant differences, see Table 2). Each circle represents the germination percentage of the seeds from a Petri dish after 4 weeks of incubation at 22/12°C. Lines are the logistic functions fitted to data.

Qaderi *et al.*, 2006; Hoyle *et al.*, 2008; Chiang *et al.*, 2011; Wagnmann *et al.*, 2012). Surprisingly, we found the opposing effect for the temperature during flowering, as warmer temperatures during that season determined higher dormancy levels. This contrasts with the mentioned rule, especially with those experiments of controlled seed maturation where warmer maturation environments produce less dormant seeds (Fenner, 1991; Fenner and Thompson, 2005). It must be taken into account that, in the five studied years, overwintering and flowering temperatures were negatively related. Thus, the negative influence of flowering temperatures on dormancy could actually be an indirect detection of overwintering temperatures. The critical point, then, is to evaluate which one of the two temperature regimes (or both) has a causal effect on dormancy variation. In our previous experiments in a common garden (Fernández-Pascual *et al.*, 2013) we detected a negative effect of flowering temperatures in seeds produced in an environment that was warmer all year through: while the sensitivity to dormancy-breaking factors was higher in the common garden than in the field, dormancy at dispersal was also higher, showing the response to a warmer flowering season that our model predicts with seeds collected in the field.

We note that the models left a percentage of unexplained variation, showing that temperature is only one of the factors determining phenotypic plasticity in dormancy. Water availability was related to subsequent dormancy levels in controlled garden experiments (Wright *et al.*, 1999; Allen and Meyer, 2002; Luzuriaga *et al.*, 2006; Hoyle *et al.*, 2008), while long-term differences in rainfall influenced dormancy in our study species (Fernández-Pascual *et al.*, 2013). Similarly, daylength during seed maturation has been found to have an effect on dormancy (Fenner, 1991; Fenner and Thompson, 2005). This could explain why La Malva population, situated at the bottom of a shadowy gorge, consistently showed deeper dormancy than sunnier El Valle, despite being at a lower altitude. This suggests that potential seed responses to global warming through phenotypic plasticity might be mediated by more factors than the change in temperature.

Nonetheless, the combined effect of overwintering and flowering temperatures has a direct ecological relevance in our study species. *C. somedanum* occurs from low valleys with oceanic influence to high altitude and continental sites on the southern side of the Cantabrian Mountains (Fernández-Pascual *et al.*, 2013; Jiménez-Alfaro *et al.*, 2013). Shallower dormancy would be beneficial in the milder winters of the northern valleys, as it could favour earlier germination without the associated risks of winter mortality (Donohue *et al.*, 2010). Colder summers and warmer winters (i.e. low seasonality), which we have found

to correlate with shallower dormancy, are characteristic of oceanic low valleys. This plasticity could allow individuals dispersing between continental and oceanic areas to quickly adjust their dormancy phenotype to the more favourable one. Additionally, it might help them to cope with some of the expected effects of climate change (Nicotra *et al.*, 2010; Reed *et al.*, 2011; Walck *et al.*, 2011), acclimatizing dormancy loss to new thermal scenarios (Orrù *et al.*, 2012) and avoiding changes in emergence timing (Mondoni *et al.*, 2012).

Despite uncertainties about the causal effect of overwintering and flowering temperatures on the observed patterns, our results highlight that the rule of thumb 'the colder the weather, the deeper seed dormancy' should consider intra-annual variations in temperature. Accurate predictions of climate change effects on plant regeneration by seeds will therefore require quantifying how temperatures during plant development are translated into subsequent dormancy levels. Moreover, differential effects of temperature depending on the plant activity season highlight the importance of incorporating more information than average temperatures to assess trait plasticity under climate change.

### Acknowledgements

C. Álvarez, R. Álvarez, Á. Bueno, V. García, A.I. García, E. Moratón and J.I. Sanzo contributed to seed collection, which was authorized by the Government of Asturias. Two anonymous reviewers improved the manuscript with their comments. The Spanish National Meteorology Agency sold us temperature data.

### Financial support

This work was supported by the Spanish Ministry of the Environment (Fundación Biodiversidad project 2008-bis-ASTERCENT). E.F.P. was supported by the Government of Asturias (Grant BP09-107, Programa de Ayudas Predoctorales 'Severo Ochoa', Plan de Ciencia, Tecnología e Innovación del Principado de Asturias); B.J.-A. by the project 'Employment of Best Young Scientists for International Cooperation Empowerment' (CZ.1.07/2.3.00/30.0037) co-financed by the European Social Fund and the state budget of the Czech Republic.

### Conflicts of interest

None.

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