



UNIVERSIDAD DE OVIEDO

DEPARTAMENTO DE BIOLOGÍA DE ORGANISMOS Y SISTEMAS

Programa de Doctorado *Biología aplicada a la sostenibilidad de recursos naturales*

# THE ROLE OF TEMPERATURE IN THE SEED GERMINATION ECOLOGY OF MOUNTAIN SPRINGS AND FENS

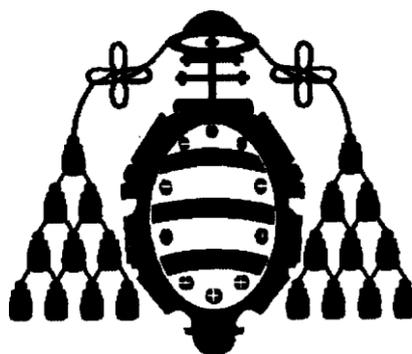


MEMORIA DE TESIS DOCTORAL  
EDUARDO FERNÁNDEZ PASCUAL, 2013









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Departamento de Biología de Organismos y Sistemas

Programa de Doctorado:

“Biología aplicada a la sostenibilidad de recursos naturales (Mención de calidad)”

**“THE ROLE OF TEMPERATURE IN THE SEED GERMINATION ECOLOGY OF  
MOUNTAIN SPRINGS AND FENS”**

**“EL PAPEL DE LA TEMPERATURA EN LA ECOLOGÍA DE LA GERMINACIÓN DE  
SEMILLAS DE FUENTES Y TREMEDALES DE MONTAÑA”**

**TESIS DOCTORAL**

Eduardo Fernández Pascual

**Oviedo 2013**

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*Front cover Swertia perennis.* La Vega Liordes, 43° 9' 11'' N 4° 50' 28'' W, 6 August 2010.

*p11 Fen at the base of the Pena Ubina (2411 m).* Valle Riotuertu, 43° 0' 72'' N 5° 56' 54'' W, 30 June 2010.

*p24 Calcareous spring fen with Pinguicula grandiflora.* Valle Riotuertu, 43° 0' 72'' N 5° 56' 54'' W, 30 June 2010.

*p49 Fen with Eriophorum latifolium.* Anzo, Sierra Cebolleda, 43° 7' 0'' N 4° 56' 3'' W, 5 August 2010.

*p71 Centaurium somedanum* flowering at the Jardín Botánico Atlántico. Xixón, 43° 31' 21'' N 5° 36' 54'' W, 11 August 2012.

*p91 Waterfall at La Malva,* largest known population of *Centaurium somedanum*. Somiedu, 43° 6' 47'' N 6° 15' 16'' W, 24 September 2009.

*p117 Small fen patch with Eriophorum latifolium* in the vicinity of the Picu Valdecoro (1816 m). Igüedri, 43° 8' 8'' N 4° 46' 44'' W, 1 July 2010.

*Back cover Blysmus compressus* at the Jardín Botánico Atlántico. Xixón, 43° 31' 21'' N 5° 36' 54'' W, 11 August 2012.



## RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: El papel de la temperatura en la ecología de la germinación de semillas de fuentes y tremedales de montaña	Inglés: The Role of Temperature in the Seed Germination Ecology of Mountain Springs and Fens

### RESUMEN (en español)

Los tremedales de fuente calcáreos son humedales florísticamente diversos que son alimentados por aguas subterráneas ricas en carbonatos. Esta tesis estudia su ecología de semillas, centrándose en la interacción entre la temperatura ambiental y la germinación. Con este objetivo realizamos experimentos de germinación en laboratorio con semillas de especies de tremedales de fuente calcáreos, para caracterizar la dimensión temperatura de su nicho de germinación e identificar estrategias de germinación a nivel de especie. Exploramos las especiales características térmicas de los suelos de tremedal y sus consecuencias en la germinación. Usando al endemismo cantábrico *Centaurium somedanum* como especie de estudio, investigamos la ecología de la germinación de especialistas de fuentes y llevamos a cabo un experimento de common garden apoyado en un análisis de marcadores ISSR para determinar las influencias genéticas y ambientales en la variación en dormición de semillas. Basándonos en nuestros resultados, proponemos dos estrategias de germinación. Las especies de tremedales siguen una estrategia cálida, caracterizada por una respuesta al calor capaz de saltarse la dormición durante episodios de calor extremo. Las especies de fuentes carecen de esta respuesta al calor y germinan sólo a temperaturas frías. Los suelos de los tremedales tienen un efecto tampón que hace que su temperatura sea menos extrema de lo esperado, especialmente durante la noche y en invierno. Sin embargo, la amplitud del nicho de germinación muestra plasticidad y es menor en regiones con inviernos más duros. La ecología de la germinación de la especialista de fuentes *C. somedanum* difiere de las características comunes en su género, mostrando dormición morfofisiológica y germinación a temperaturas bajas. La dormición de esta especie presenta una clina local relacionada con la composición genética de las poblaciones y con el clima, pero que también responde en el corto plazo al ambiente de maduración de las semillas. Estos resultados muestran el potencial de la germinación para reaccionar frente a cambios climáticos a diferentes escalas espaciales y temporales.

### RESUMEN (en Inglés)

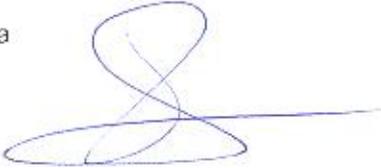
Mountain calcareous spring fens are floristically diverse wetlands fed by carbonate-rich groundwater. This thesis studies their seed ecology, focusing on the interaction between environmental temperature and germination. To this end we conduct laboratory germination experiments with calcareous spring fen species to characterize the temperature dimension of the germination niche and identify specific germination strategies. We explore the special thermal characteristics of fen soils and their consequences on germination. Using the Cantabrian endemic *Centaurium somedanum* as a study species, we investigate the germination ecology of spring specialists and perform a common garden experiment supported by ISSR fingerprinting to determine the genetic and environmental influences on seed dormancy variation. Based on our results, we propose two germination strategies. Fen species generally follow a warm strategy, characterized by a heat response capable of overriding dormancy during episodes of unusual heat. Spring species lack the heat response and germinate only at cool temperatures. Fen soils have a buffer effect that makes their temperature less extreme than predicted, especially during the night and in winter. However, the germination niche breadth of fen species shows plasticity and is narrower in regions with harsher winters. The germination ecology of the spring specialist *C. somedanum* departs from the general germination patterns of its genus, showing morphophysiological dormancy and cool germination. This species has a local dormancy cline that is related to the genetic composition of the populations and to site climate, but that also responds in the short term to the seed maturation environment. These results highlight the potential of germination traits to react to climate changes at different spatial and temporal scales.



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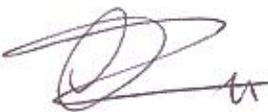


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# CHAPTER 1

## GENERAL INTRODUCTION





## BACKGROUND

Calcareous spring fens are permanent wetlands characterized by a dominant supply of carbonate-rich groundwater. They hold a high diversity of plants and mosses (Kooijman *et al.* 1994; Cantonati *et al.* 2006; Virtanen *et al.* 2009) but face a number of present and future threats. A long history of human drainage has resulted in widespread habitat destruction (ŠeffEROVÁ StanOVÁ *et al.* 2008), while recent changes in land management have caused local extinctions of specialist species (Lienert *et al.* 2002; Bergamini *et al.* 2009). Moreover, these habitats are expected to be particularly sensitive to the alterations of temperature and precipitation predicted in the context of global change (IPCC 2007; Essl *et al.* 2012). They are consequently protected in the European Union by the Habitats Directive (Council Directive 92/43/EEC, Annex I, codes 7220 - ‘petrifying springs with tufa formation’ and 7230 - ‘alkaline fens’).

The Central European mountains are the distribution centre of calcareous spring fen vegetation in the continent, where it spreads through most biogeographical regions with the notable exception of the Mediterranean areas (Jiménez-Alfaro *et al.* 2013b). In transitional mountain ranges with a Mediterranean influence, relict calcareous spring fen communities survive in sub-optimal refugia as is the case in Bulgaria (Hájek *et al.* 2009), Croatia (Topić and Stančić 2006) and Spain (Jiménez-Alfaro *et al.* 2012). It can be expected that these southern outposts will be especially sensitive to global change as a consequence of their border position, isolation and relictualism. In particular, the calcareous spring fens of the Spanish Cantabrian Mountains, which lie at their southwestern distribution limit, hold many threatened taxa that are protected at the regional level (Díaz *et al.* 2003; Llamas *et al.* 2007). Among them, it is worth mentioning the local endemic *Centaureum somedanum* M. Laínz, an indicator species of tufa-forming calcareous springs (Jiménez-Alfaro *et al.* 2011; Jiménez-Alfaro *et al.* 2013a) which is considered a priority taxon by the European Union (Council Directive 92/43/EEC, Annex II) and a top concern species for *ex situ* conservation in the Cantabrian Mountains (Jiménez-Alfaro *et al.* 2010).

Adequate conservation policies must incorporate the study of seed biology, a key element for both *in situ* and *ex situ* conservation programs (Smith *et al.* 2003; Guerrant *et al.* 2004; Bacchetta *et al.* 2008). In the present scenario of climate warming (IPCC 2007), it is essential to understand the interaction between temperature and germination, as it will govern plant responses to climatic alterations (Cochrane *et al.* 2011; Walck *et*

*al.* 2011; Orrù *et al.* 2012). Moreover, good practices of seed banking rely on a sound knowledge about the germination requirements of the stored collections (ENSCONET 2009), necessary to control seed viability, produce new plants and accomplish *in situ* reintroductions. However, despite the conservation value of calcareous spring fens, information on their seed ecology is scarce, in most cases obtained in related lowland fen meadows of Central Europe (Maas 1989; Patzelt *et al.* 2001; Jensen 2004). This is insufficient in the Cantabrian scenario, where the mountain character and the biogeographical position may imply particular vulnerabilities (Topić and Stančić 2006; Parish *et al.* 2008). Hence, the seed ecology of Cantabrian calcareous spring fens becomes a relevant study subject to approach the conservation of these habitats in the mountains of southern Europe.

## SEED GERMINATION ECOLOGY

### *Framework*

Seeds are the next generation of a plant, consisting of an embryo kept in latent life and surrounded by protective tissues (Font i Quer 2000; Fenner and Thompson 2005; Black *et al.* 2006). The transition from seed to seedling is germination, a chain of events that begins with the uptake of water by the seed and ends when the embryonic axis emerges through the surrounding protective tissues (Black *et al.* 2006). Germination is elicited by several germination cues, external signals such as water availability, temperature, light and soil chemistry (Barceló Coll *et al.* 2001; Fenner and Thompson 2005). The resulting combination of germination requirements is the germination niche, a key aspect of the plant regeneration niche (Grubb 1977).

In many species, the germination niche is modulated by seed dormancy. There are different interpretations of this trait, derived from the different study approaches taken by plant physiologists and ecologists. Here we will follow the most widely accepted view, which understands dormancy as *an inner seed characteristic, the degree of which determines what external conditions should be met to make a seed germinate* (Vleeshouwers *et al.* 1995; Finch-Savage and Leubner-Metzger 2006). Thus, a non-dormant seed germinates over its widest possible germination niche; dormant seeds have a reduced or null sensitivity to germination cues. According to this view, the block to germination imposed by external unfavourable conditions that is sometimes classified as dormancy is understood as an intrinsically different process called quiescence (Baskin and Baskin 2004). Although there is an ongoing debate about whether factors alleviating dormancy are fundamentally different from those eliciting germination (Thompson and Ooi 2010; Finch-Savage and Footitt 2012; Thompson and Ooi 2013), in this work we will treat them separately for practical reasons.

The most common type of dormancy in temperate regions is physiological (Baskin and Baskin 1998; Baskin and Baskin 2004). Physiological dormancy is not an all-or-nothing property, but a quantitative trait that subtly modulates seed sensitivity to the external conditions that elicit germination (Batlla and Benech-Arnold 2003; Batlla *et al.* 2003; Batlla and Benech-Arnold 2004; 2005) and whose levels fluctuate throughout the year giving rise to cyclic changes in the germination requirements (Baskin and Baskin 1985; Batlla and Benech-Arnold 2007). Annual changes in physiological dormancy

combine with changes in the environment to determine when the seed germination niche matches the external conditions, i.e. the time of germination in the field (Vleeshouwers *et al.* 1995). Understanding how this timing is controlled in nature, and its ecological and evolutionary origins and consequences, is the broad objective of seed germination ecology (Baskin and Baskin 1998).

### ***The role of temperature in seed germination***

In seasonal climates, environmental temperature plays a paramount role in determining germination timing. Its direct effects are dual, being a primary germination cue but also regulating the annual cycles in physiological dormancy (Vleeshouwers *et al.* 1995; Fenner and Thompson 2005). Seeds germinate over a particular range of temperatures which tends to be species-specific, with base and ceiling thresholds beyond which no germination occurs (Barceló Coll *et al.* 2001). Between these thresholds, the germination rate increases linearly as temperature approaches its optimal value (García-Huidobro *et al.* 1982). At the same time, environmental temperature regulates physiological dormancy, which in turn modifies the germination temperature thresholds (Baskin and Baskin 1998; Batlla and Benech-Arnold 2003; Steadman and Pritchard 2004; Orrù *et al.* 2012).

The annual variation in physiological dormancy in response to the temperature cycles has been termed the dormancy continuum (Baskin and Baskin 1985). The cycle begins with primary dormancy at dispersal, which can be total (i.e., no germination at any temperature) or conditional (i.e., germination in a restricted range of temperatures, which rarely matches the natural temperatures at the time of dispersal) (Baskin and Baskin 1998). Subsequent dormancy loss occurs if the seeds meet adequate dormancy-breaking temperatures, usually quite different from those that elicit germination (Vleeshouwers *et al.* 1995). As the seeds gradually lose dormancy, their germination temperature thresholds change (Batlla and Benech-Arnold 2003; Steadman and Pritchard 2004; Orrù *et al.* 2012). The most common responses are an increase of the ceiling threshold or a decrease of the base threshold, respectively characteristic of winter and summer germinators (Baskin and Baskin 1985). Less frequently, the two thresholds change and the germination temperature range increases in both directions (Baskin and Baskin 2004). Eventually, seeds become non-dormant and are able to germinate over their entire temperature range. However, it is still possible that seeds do

not germinate either because environmental temperatures do not match their germination niche, or because other necessary germination cues such as light are not present. In this case, further annual changes in temperature may induce secondary dormancy, narrowing again the temperature thresholds for germination (Brändel 2005; Kępczyński *et al.* 2006; Leymarie *et al.* 2008).

In the context of climate change, it is expected that these interactions between the temperature cycles and germination timing will be disrupted (Walck *et al.* 2011). New environmental temperatures may no longer match the germination ranges of the species (Cochrane *et al.* 2011), or be inadequate to fulfil their dormancy-breaking requirements (Orrù *et al.* 2012). Such effects can modify the recruitment from the soil seed bank (Ooi *et al.* 2009; Ooi 2012) and shift the germination timing (Mondoni *et al.* 2012), ultimately leading to alterations in community composition and compromising species survival.

There is however an additional complication that obscures the prediction of climate change effects on germination. The germination niche and seed dormancy are not fixed traits, but respond to environmental changes through local adaptation (Donohue *et al.* 2010) and phenotypical plasticity (Fenner 1991). For example, it has been shown in some species that populations from colder regions show higher levels of seed dormancy (Meyer and Monsen 1991), or that colder temperatures during the seed maturation season can result in more dormant seeds (Figuroa *et al.* 2010). Therefore, environmental temperature has a third effect on germination timing, as it modulates the expression of the traits that will later determine the interaction between germination and temperature. Studying this plasticity of seed traits in response to temperature is necessary to accurately assess the effects that climate change will have on germination timing.

## DESCRIPTION OF THE STUDY HABITAT

Defining calcareous spring fens is not a straightforward task, given the traditional unclearness of wetland terminology (Wheeler and Proctor 2000; García-Rodeja and Fraga 2009). A *fen* is widely described as a type of mire that is fed by groundwater (in opposition to a *bog*, fed by precipitation). This is the source of much confusion, because *mire* is a general name for a tract of waterlogged ground occupied by herb and moss communities, but there is not an agreement on its precise usage. Some authors restrict its meaning to peat-forming wetlands, making it synonymous to *peatland* (van Diggelen *et al.* 2006); while others prefer a broader concept including similar habitats that lack peat accumulation (Wheeler and Proctor 2000). Spring fens are precisely in this fuzzy area, as when the groundwater that feeds them has a high enough calcium concentration, peat accumulation stops and is substituted by petrifying calcium carbonate deposits known as *tufa*. To add to the confusion, the line between peat- and tufa- forming fens is difficult to draw and the dominant plant layer is relatively similar in both, and so only some authors clearly differentiate the two types (Hájek *et al.* 2006).

In this thesis we will use the term *calcareous spring fens* to refer to permanent wetlands consisting of a water source rich in calcium carbonate (the *spring*) and the surrounding soils that this water soaks (the *fen*). The main feature of these habitats is that their formation and maintenance is completely dependent on the hydrogeological regime of the spring, which is the main supplier of soil water (Grootjans *et al.* 2006). Due to this groundwater supply, calcareous spring fens are characterized by a minimal fluctuation of the water table in comparison to other wetlands (ŠeffEROVÁ StanOVÁ *et al.* 2008). They are restricted to areas with significant calcareous geological deposits, e.g. limestone, where soil water acquires particular chemical properties through its contact with the carbonate-rich bedrock. These chemical properties include a high concentration of base cations (especially calcium) and an extremely low availability of nutrients (nitrogen and especially phosphorus, the limiting factor in most fens) (Hájek *et al.* 2006; ŠeffEROVÁ StanOVÁ *et al.* 2008). Such conditions prevent the development of the competitive *Sphagnum* species that dominate other fens and lead to their substitution by brown mosses. As we have said, in some cases calcium carbonate reaches a concentration where it starts to precipitate and form tufa, a turning point that determines extreme nutrient deficiencies and subhalophytic conditions that prevent the occurrence of all but the most calcicole specialists (Hájek *et al.* 2006).

Calcareous spring fens are composed by a mosaic of microhabitats known as the *spring-flush-fen gradient* (Wheeler and Proctor 2000). This gradient corresponds with vegetation differences, although many species are shared (Jiménez-Alfaro *et al.* 2011; Jiménez-Alfaro *et al.* 2013a). We will use the term *spring* to refer specifically to the habitat formed at the point and the immediate surroundings of the point where carbonate-rich groundwater intersects the topographic surface and flows into the open air. These surroundings show the most extreme calcareous conditions and the highest tufa petrification. Depending on the local topography, the spring setting can adopt different morphologies (Jiménez-Alfaro *et al.* 2013a). Slow spring flows in vertical walls give rise to tufa concretions known as *travertines*, dominated by the phytosociological alliance *Adiantion*; while springs in rock avalanches produce *calcareous flushes* dominated by *Pinguiculion*. These spring habitats correspond to the EUNIS vegetation type 7220 – ‘petrifying springs with tufa formation’, defined as *hard water springs with active formation of travertine or tufa [...] generally small (point or linear formations) and dominated by bryophytes* (EUNIS 2007).

Similarly, we will use the term *fen* specifically for the habitat formed in the broader surroundings and defined as a permanent groundwater-fed wetland, generally flat or with a soft slope, that hosts productive nutrient-limited vegetation dominated by brown mosses and small sedges (alliance *Caricion davallianae*), and that accumulates peat or tufa (Hájek *et al.* 2002). These fens correspond to the habitats defined as base-rich fens (Jiménez-Alfaro *et al.* 2013b), alkaline fens (Šefferoová Stanová *et al.* 2008), small-sedge fens (Wheeler and Proctor 2000) or extremely-rich (peat-forming) and calcareous (tufa-forming) fens (Hájek *et al.* 2006). They basically match the EUNIS vegetation type 7230 – ‘alkaline fens’, defined as *wetlands mostly or largely occupied by peat- or tufa-producing small sedge and brown moss communities developed on soils permanently waterlogged, with a soligenous or topogenous base-rich, often calcareous water supply, and with the water table at, or slightly above or below, the substratum* (EUNIS 2007).

## AIMS AND STRUCTURE OF THE WORK

This work is funded by the Government of Asturias and has been conducted in the seed bank of the Jardín Botánico Atlántico (Instituto de Recursos Naturales y Ordenación del Territorio, Universidad de Oviedo, Xixón, Spain) in collaboration with the Jardín Botánico Canario “Viera y Clavijo” – Unidad Asociada CSIC (Cabildo de Gran Canaria, Las Palmas de Gran Canaria, Spain), the Masaryk University (Brno, Czech Republic) and the Millennium Seed Bank Partnership (Royal Botanic Gardens, Kew, Wakehurst, UK). As such, it is part of the broader conservation strategy of the involved institutions, especially regarding the *ex situ* preservation of biological collections (Jiménez-Alfaro *et al.* 2006). Its ultimate goal is to improve the understanding of seed ecology in mountain calcareous spring fens and, at the same time, collect seeds and other plant materials for their *ex situ* conservation.

More specifically, we investigate the role of environmental temperature in the regulation of germination in these habitats. We approach the study at two levels. First, we examine the interaction between environmental temperature and germination at the habitat level (**chapters 2 and 3**), analysing the germination patterns of several characteristic species from Cantabrian and Central European calcareous spring fens. Then we focus on *C. somedanum* at the species level (**chapters 4 and 5**), given its relevance for conservation and its particular ecological characteristics as a strict specialist of tufa-forming calcareous springs.

In **chapter 2**, we characterize the temperature dimension of the seed germination niche in calcareous spring fens. Using seeds of 15 species collected from 18 Cantabrian sites during two consecutive years, we conduct laboratory germination experiments using field-inspired thermoperiods for dormancy break and germination. The research questions are: How widespread is seed dormancy at dispersal in calcareous spring fen species and what role does cold temperature play during dormancy break? What is the germination response of calcareous spring fen species to spring/autumn, summer and unusually high thermoperiods? Does the germination niche of calcareous spring fen species follow a common pattern, or may different strategies be identified?

In **chapter 3**, we analyse the potential consequences of the special thermal characteristics of fen soils. These soils are permanently saturated by cold waters that come from underground aquifers. This may buffer soil temperature, allowing fen species to occupy a wider thermal range than they could otherwise and alleviating

climate warming effects. We record soil temperatures *in situ* and collect seeds from fens of two contrasting European regions, the Cantabrian and the Western Carpathian Mountains, to address the following research questions: Is the temperature of fen soils buffered by soil water? If this buffer exists, what is its consistency in time and space? How is this special thermal regime related to the germination niche breadth of fen species at the regional and local levels?

In **chapter 4**, we investigate the germination ecology of *C. somedanum* as a representative of spring specialists, to improve our understanding of how this narrow endemic adapted to a habitat unusual in its genus. We perform laboratory germination experiments with this species and review the literature to compare its germination patterns to those of other *Centaureium* species. We focus on these research questions: What are the temperature and light requirements for *C. somedanum* germination? What type of seed dormancy does this species have, if any? Does this dormancy show a pattern of interpopulation variability?

In **chapter 5**, we use the pattern of seed dormancy shown by the wild populations of *C. somedanum* to study the influence of environmental and genetic variability on seed dormancy variation. We carry out a common garden experiment of seed maturation, characterize the population genetics of *C. somedanum* using Inter Simple Sequence Repeat markers and gather climatic data derived from models. Relying on this combined approach we concentrate on the following research questions: Is the field pattern of dormancy variation maintained or altered when the plants and seeds mature in a common garden, far from their original locations? Is it related to the genetic composition of the populations? Can it be explained by the local climatic differences?

Finally, in **chapter 6** we discuss the results, give an overview of the collected plant materials and summarize the main conclusions.

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A photograph of a fen wetland, showing a dense carpet of green moss and small purple flowers. The scene is captured from a slightly elevated angle, showing the texture of the vegetation and the way light filters through. The overall color palette is dominated by various shades of green, with the purple flowers providing a striking contrast.

## CHAPTER 2

### THE TEMPERATURE DIMENSION OF THE SEED GERMINATION NICHE IN FEN WETLANDS

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## ABSTRACT

**Background and Aims** The seed germination niche partly determines adaptation, ecological breadth and geographic range in plant species. In temperate wetlands, environmental temperature is the chief regulator of germination timing, but the ecological significance of high and low temperatures during dormancy break and germination is still poorly understood. Our aim is to characterize the temperature dimension of the germination niche in mountain calcareous spring fens, determining (1) the effect of different temperatures on dormancy break and germination, and (2) whether different germination strategies may be identified at the species level.

**Methods** We conducted laboratory germination experiments with seeds of 15 species from these habitats, collected in 18 calcareous spring fen sites in the Cantabrian Mountains (Spain) for two consecutive years.

**Key Results** In all the species, the seeds were totally or conditionally dormant at dispersal and stratification produced a significant increase of germination. In most cases, there was not an obligatory requirement for cold temperatures during dormancy break, since warm stratification promoted germination as well. Although the optimal germination thermoperiod was generally high (30/20 °C), most species could also germinate at lower temperatures after cold stratification. We also identified a group of species associated to cold-water springs that germinated only at low temperatures.

**Conclusions** Our results demonstrate that dormancy break in mountain calcareous spring fens does not obligatorily depend on cold temperatures during overwintering. Furthermore, germination at cool temperatures may be more widespread in wetland habitats than previously thought. The existence of two distinctive germination strategies, warm and cool, can potentially give rise to divergent species responses to climate change.

## **RESUMEN EN CASTELLANO**

**Antecedentes y objetivos** El nicho de germinación de semillas determina parcialmente la adaptación, la amplitud ecológica y la distribución de las especies vegetales. En los humedales de zonas templadas, la temperatura ambiental es el principal regulador del momento de la germinación, pero se desconocen las implicaciones ecológicas de temperaturas altas o bajas durante la rotura de la dormición y la germinación. Nuestro objetivo es caracterizar la dimensión temperatura del nicho de germinación de semillas en tremedales de fuente calcáreos, determinando (1) el efecto de diferentes temperaturas en la rotura de la dormición y la germinación, y (2) si es posible identificar diferentes estrategias de germinación al nivel de especie.

**Métodos** Realizamos experimentos de germinación con semillas de 15 especies de estos hábitats, recolectadas en 18 tremedales de fuente calcáreos de la Cordillera Cantábrica durante dos años consecutivos.

**Resultados clave** En todas las especies, las semillas estaban total o condicionalmente dormidas en el momento de la dispersión, y la estratificación produjo un incremento significativo de la germinación. En la mayoría de casos, no existía un requerimiento obligatorio de temperaturas bajas para la rotura de la dormición, ya que la estratificación cálida también aumentó la germinación. Aunque el termoperiodo óptimo para la germinación fue generalmente alto (30/20 °C), la mayoría de especies también pudieron germinar a temperaturas bajas después de la estratificación fría. Identificamos un grupo de especies, asociadas a fuentes de aguas frías, que germinaron sólo a temperaturas bajas.

**Conclusiones** Nuestros resultados demuestran que la rotura de la dormición en tremedales de fuente calcáreos no depende exclusivamente de temperaturas frías durante el invierno. Además, la germinación a temperaturas frías puede ser más común en humedales de lo que se pensaba. La existencia de dos estrategias de germinación, cálida y fría, puede dar lugar a respuestas divergentes de las especies ante el cambio climático.

## INTRODUCTION

The range of conditions under which a seed may germinate is its germination niche, a key aspect of the plant regeneration niche (Grubb 1977) that determines adaptation, ecological breadth and geographic range in plant species (Donohue *et al.* 2010). Among the various dimensions or factors that make up the germination niche, environmental temperature is of paramount importance, influencing both seed dormancy and germination (Fenner and Thompson 2005). In general terms, seeds of a given species may germinate over a particular range of temperatures, with upper and lower thresholds beyond which no germination occurs. In many species, however, this seed germination range is in turn regulated by the environment through physiological dormancy (Baskin and Baskin 1998), a seed characteristic that modifies the germination temperature thresholds (Baskin and Baskin 1998) and defines the environmental conditions that must be met before germination can occur (Finch-Savage and Leubner-Metzger 2006). By means of these processes, environmental temperature controls germination timing and the environment experienced by subsequent life stages (Donohue 2005). Germination timing is especially important in seasonal climates, where it is subjected to natural selection and also determines the action of natural selection in other life history traits (Donohue *et al.* 2005a; 2005b; 2005c). Understanding the relationships between the germination niche and the environment is therefore crucial for assessing the adaptation of plants to their habitats, as well as the effect of the projected rise of world temperatures, which could potentially disrupt such relationships (Cochrane *et al.* 2011; Mondoni *et al.* 2012).

In wetland habitats, the germination niche of emergent species (i.e., those rooted in water at least temporarily, but with stems and leaves permanently above the water table) is generally characterized by physiological dormancy and optimal germination at high temperatures ( $> 25\text{ }^{\circ}\text{C}$ ) while being prevented by low ones ( $< 15\text{ }^{\circ}\text{C}$ ) (reviewed in Baskin and Baskin 1998). This ‘warm germination’ pattern was first described in the comparative studies of Grime *et al.* (1981) and Thompson and Grime (1983), who noted a widespread requirement for fluctuating or unusually high temperatures in wetland species and explained it as a mechanism which detects the fall of the water table in spring, marking the beginning of the growing season. More recently, research has focused in wetland *Carex* L. species from Central Europe (Schütz 1997; Schütz and

Rave 1999; Schütz 2000; Schütz and Rave 2003) and North America (Budelsky and Galatowitsch 1999; Kettenring *et al.* 2006; Kettenring and Galatowitsch 2007a; 2007b) confirming that they follow and exemplify the ‘traditional’ wetland germination pattern: conditional physiological dormancy at dispersal, a requirement for light and fluctuating temperatures, and a preference for high or unusually high (c. 30 °C) germination temperatures. According to Baskin and Baskin (1998), these characteristics would allow the seeds to lose their dormancy during the flooded winter season and germinate as soon as the water recedes. Such interpretation is mainly based on species living on habitats with seasonal hydrological regimes where the water table changes periodically (such as temporary ponds and lake margins) but there are less studies focused on other wetlands. It is still necessary to conduct multi-species research over the main plant communities that may be recognized in different wetland habitats.

Here, we study the germination niche in calcareous spring fens, a particular type of wetlands which occur in the temperate regions of the world (Hájek *et al.* 2006). The water level in these habitats depends only indirectly on precipitation and the soils remain water saturated throughout the year. Despite their high conservation concern (van Diggelen *et al.* 2006; Bergamini *et al.* 2009), the germination patterns of calcareous spring fen plant communities have been seldom studied, especially in mountainous regions where they are expected to be highly sensitive to global warming (Essl *et al.* 2012). To our knowledge, community-level assessments of germination have been exclusively conducted in lowland fen grasslands, semi-natural meadows created from natural fens as a consequence of traditional agriculture (Maas 1989; Patzelt *et al.* 2001; Jensen 2004). Almost all the species tested in these studies had physiological dormancy, with a germination promoting effect of cold stratification. There is a caveat, however, to the interpretation of cold effects in dormancy loss: since only fresh versus cold-stratified seeds were tested, it is not possible to determine whether the dormancy loss required cold temperatures, or was produced simply by the longer wet incubation in comparison with the fresh seeds. In light of the importance it may have in determining the effect of climate change on plant regeneration (Walck *et al.* 2011; Ooi 2012), the role of cold in dormancy loss should be more clearly assessed. Regarding germination temperature, Maas (1989), and Patzelt *et al.* (2001) compared constant versus alternating thermoperiods and found an inhibitory effect of constant temperatures in

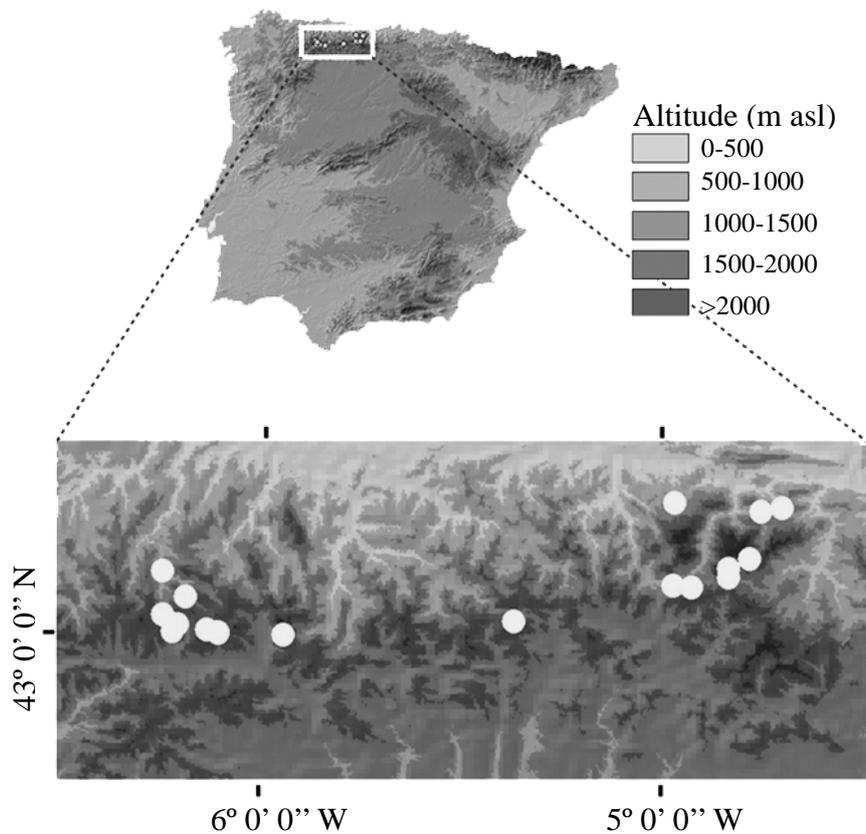
germination. However, only cool to moderate thermoperiods (15/5, 22/12, 25/10 and 25/15 °C) were tested, and therefore they did not address the effect of unusually high and alternating temperatures, which have been found to be optimal in most wetland species. Some species such as *Primula farinosa* L. (Patzelt *et al.* 2001) and *Triglochin palustris* L. (Jensen 2004) germinated well under relatively cool thermoperiods (15/5 °C) but it remains to be seen if they can also germinate under the ‘warm germination’ pattern recognized in wetland habitats (and thus share a broader but overlapping germination niche) or they do not (and follow an alternative germination strategy). Comparing the germination response to a broad temperature range in different calcareous spring fen species is necessary to fully characterize the germination niche in these habitats.

The main aim of this work is to investigate the temperature dimension of the germination niche in mountain calcareous spring fens. We perform laboratory experiments with seeds of 15 species from these habitats to address the following specific questions: (1) How widespread is seed dormancy at dispersal and what role does cold temperature play during dormancy break? (2) What is the germination response to spring/autumn, summer and unusually warm thermoperiods? (3) Is the germination niche in calcareous spring fens characterized by a common pattern, or may different germination strategies be identified at the species level?

## MATERIALS AND METHODS

### *Study system*

The study took place in the Cantabrian Mountains of north-western Spain ( $43^{\circ} 12' N$   $5^{\circ} 27' W$ ), where calcareous spring fens are relatively widespread and support most of the European specialist species of these habitats (Jiménez-Alfaro *et al.* 2012). We selected 18 sites (Figure 2.1) among those commonly referred in the literature as well-developed calcareous spring fen communities, encompassing their whole altitudinal gradient in the study area (710–1,870 m above sea level) and distributed along 140 km from west to east. To record soil temperature during the year, in June 2010 we placed three dataloggers (M-Log5W, GeoPrecision GmbH, Ettlingen, Germany) in three sites covering the entire altitude gradient, burying them 5 cm in the soil in a flat and central area. In the period from September 2010 to August 2011, day/night soil temperature ( $^{\circ}C$ ) ranged from 16/13 at the lowest site to 8/6 at the highest in autumn, 9/7–4/3 in winter, 13/11–9/6 in spring and 19/16–13/10 in summer.



**Figure 2.1** Location of the study area in the Cantabrian Mountains of northern Spain. The white circles indicate the 18 calcareous spring fen sites selected for seed sampling.

The experiments included 15 species mainly restricted to calcareous spring fens and related habitats (mires with pH > 6.5) in the study area (Table 2.1), including some of the plant specialists recognized in similar communities of other European mountains (Bergamini *et al.* 2009). We collected seeds of the study species during the dispersal seasons (June–September) of 2010 and 2011. To take account of possible within-species variation, we carried out repeated collections of the same species in different sites and years (Table 2.1), although in some cases one or both factors could not be replicated as a result of inadequate seed availability. Seed sampling regularly covered the entire area of occupancy of the species at a given site and included all the individuals bearing ripe seeds (i.e., fully matured fruits that could be easily detached from the mother plant). After collection, the fruits spent three weeks in our laboratory (c. 22 °C, 50 % RH) to ensure homogeneous maturation; subsequently we cleaned the seeds and began the germination experiments.

**Table 2.1** Species included in this study. N is the number of repeated collections per species (considering both years and sites). The range of fen water pH (ten measures per site, MM 40 Portable Multimeter, Crison Instruments S.A., Alella, Spain) and altitude in which each species was collected is also indicated.

Species	Family	N	pH	Altitude(m)
<i>Blysmus compressus</i> (L.) Panz. ex Link	Cyperaceae	6	6.6 - 8.2	710 - 1870
<i>Carex davalliana</i> Sm.	Cyperaceae	4	7.3 - 8.0	1530 - 1860
<i>Carex diandra</i> Schrank	Cyperaceae	3	7.0 - 7.2	1110 - 1780
<i>Carex echinata</i> Murray	Cyperaceae	4	6.6 - 7.6	1440 - 1860
<i>Carex lepidocarpa</i> Tausch	Cyperaceae	8	7.3 - 8.2	710 - 1870
<i>Carex pulicaris</i> L.	Cyperaceae	2	6.7 - 8.0	1420
<i>Centaurium somedanum</i> M. Laínz	Gentianaceae	1	8.0 - 8.4	1280
<i>Eriophorum latifolium</i> Hoppe	Cyperaceae	6	6.6 - 8.4	1460 - 1600
<i>Juncus alpino-articulatus</i> Chaix	Juncaceae	2	7.3 - 8.0	1530 - 1860
<i>Parnassia palustris</i> L.	Saxifragaceae	2	7.4 - 7.5	1540 - 1610
<i>Pedicularis mixta</i> Gren.	Scrophulariaceae	2	8.0 - 8.5	1530 - 1670
<i>Pinguicula grandiflora</i> Lam.	Lentibulariaceae	2	8.2 - 8.6	710
<i>Primula farinosa</i> L.	Primulaceae	2	8.5 - 8.6	710 - 1200
<i>Swertia perennis</i> L.	Gentianaceae	5	7.3 - 7.7	1610 - 1860
<i>Triglochin palustris</i> L.	Juncaginaceae	2	7.4 - 8.1	1420

### ***Germination experiments***

We conducted laboratory germination experiments on 1 % distilled water agar, held in Petri dishes sealed with Parafilm to prevent desiccation (four dishes with 25 seeds each per experimental treatment). The experiments had a fully factorial design with two factors: pre-treatment and germination thermoperiod. To assess seed dormancy, the dishes were subjected to one of the following pre-treatments: (1) fresh; where seeds were incubated at the germination thermoperiods immediately after sowing; and (2) cold stratification; where after sowing the seeds were kept at 3 °C in darkness for 12 weeks prior to incubation at the germination thermoperiods. Additionally, in order to ascertain the role of cold during dormancy break, the seeds of the fresh pre-treatment that did not germinate during their first incubation at the germination thermoperiods were subjected to (3) warm stratification at 25 °C in darkness for eight weeks; and then incubated for a second time at the same germination thermoperiods (the results for this pre-treatment included germination during the first incubation). To determine the germination response to temperature, we incubated the dishes for four weeks under three germination thermoperiods: 14/4 °C (spring/autumn), 22/12 °C (summer) and 30/20 °C (unusual heat). The thermoperiods were programmed in growth chambers (Grow-S 360, Ing. Climas, Barcelona, Spain) and coupled 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). During the incubations we counted and discarded germinated seeds weekly (radicle emergence was the criterion for germination) and after the experiments we opened the non-germinated seeds with a scalpel, classifying them as normal, empty and fungus infected. We excluded the empty and infected seeds from the calculation of germination percentages and the statistical analyses. Some treatments that lacked variance (i.e., germination = 0 or 100 %) had to be transformed (to germination = 1 or 99 %) so they could be included in the statistical tests.

### ***Data analysis***

We analysed the effect of the experimental treatments on germination (separately for each species) by fitting Generalized Linear Mixed Models (GLMM, binomial error distribution, logit link function) with the experimental treatments as fixed factors and

seed collection as a random factor. First, we fitted main effects GLMM with the three levels of thermoperiod and two levels of pre-treatment to establish if there were significant differences in seed germination between (1) fresh and cold-stratified seeds; (2) fresh and warm-stratified seeds; (3) cold- and warm-stratified seeds. Afterwards, we performed pairwise GLMM comparisons within pre-treatments to determine (4) which germination thermoperiods produced significantly different results in the fresh and (5) in the cold-stratified seeds. We fitted the GLMMs using SPSS (version 20.0, IBM, Armonk, USA). Finally, to assess germination niche variability among the study species, we carried out a Principal Component Analysis (PCA) using germination per collection as multivariate variables (mean germination values for fresh and cold-stratified seeds in the three germination thermoperiods). We computed the PCA using the correlation coefficient as implemented in the PAST software (Hammer *et al.* 2001), and used the two main PCA axes to identify similar germination responses in the ordination space.

## RESULTS

### *Pre-treatments*

The range of tested conditions was generally successful in promoting germination, as in 14 out of 15 species more than 85 % of the seeds germinated in at least one collection and treatment, the exception being *C. somedanum*. In *C. somedanum*, *P. mixta* and *P. grandiflora* the germination of the fresh seeds was zero or almost zero, while in the rest of the species significant fresh germination occurred in at least one of the germination thermoperiods (Figure 2.2). The GLMM (Table 2.2) confirmed that, taking into account the variation among seed collections, cold stratification had a significant effect on seed germination in all the species, producing a significant increase from fresh seed germination in at least one of the germination thermoperiods (Figure 2.2). Similarly, warm stratification produced a significant increase from fresh germination in most species, except for *B. compressus*, *P. mixta* and *P. farinosa* where it had no effect (Figure 2.2). Even so, in most of the species where both stratifications had a significant effect, this effect was not the same (Figure 2.2). In *C. davalliana*, *C. diandra*, *C. echinata*, *C. lepidocarpa*, *P. palustris*, *P. grandiflora* and *S. perennis* cold stratification produced significantly better results, with higher germination percentages and germination at more thermoperiods. Conversely, warm stratification produced significantly better results in *C. pulicaris* and *J. alpino-articulatus*. The effect of the two stratification types was not significantly different in *C. somedanum* and *T. palustris*. In *E. latifolium* the two stratifications were as well not significantly different, but in this case it was due to exceptionally high dark germination during warm stratification of the seeds from the 14/4 °C thermoperiod, probably produced by the transfer to warmer temperatures (25 °C). Excluding this artefact, cold stratification produced significantly ( $F = 311.556$ ,  $p < 0.001$ ) better results in *E. latifolium*.

**Table 2.2** Results of the GLMM comparing the effect of different pre-treatments in seed germination across three germination thermoperiods. The models included seed collection as a random blocking factor.

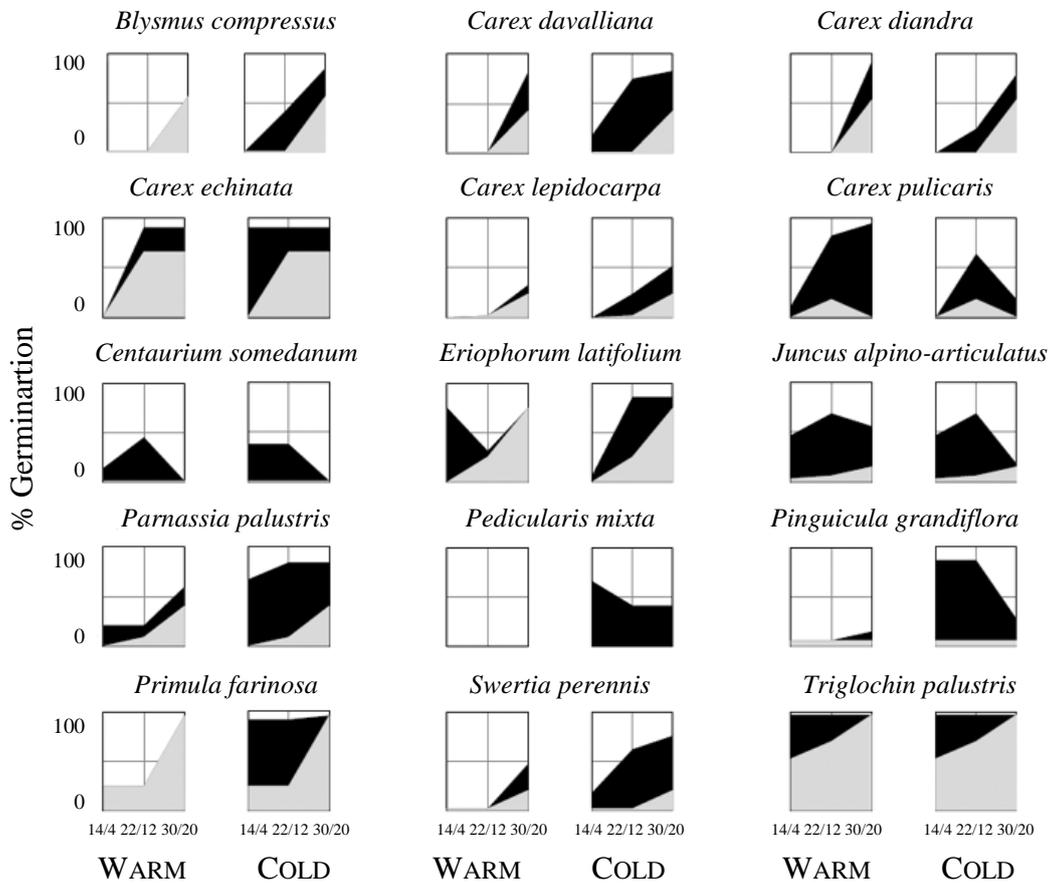
Species	Fresh vs. Cold-stratified				Fresh vs. Warm-stratified				Cold vs. Warm-stratified			
	Pre-treatment		Thermoperiod		Pre-treatment		Thermoperiod		Pre-treatment		Thermoperiod	
	F	p	F	p	F	p	F	p	F	p	F	p
<i>B. compressus</i>	154.768	<0.001	222.689	<0.001	0.057	0.812	188.706	<0.001	151.072	<0.001	224.805	<0.001
<i>C. davalliana</i>	352.815	<0.001	172.515	<0.001	122.992	<0.001	198.045	<0.001	183.457	<0.001	248.675	<0.001
<i>C. diandra</i>	94.426	<0.001	176.461	<0.001	73.619	<0.001	176.856	<0.001	9.982	0.002	206.924	<0.001
<i>C. echinata</i>	405.464	<0.001	121.858	<0.001	151.770	<0.001	193.166	<0.001	218.048	<0.001	217.333	<0.001
<i>C. lepidocarpa</i>	269.857	<0.001	198.257	<0.001	17.693	<0.001	162.557	<0.001	185.633	<0.001	233.003	<0.001
<i>C. pulicaris</i>	103.020	<0.001	77.717	<0.001	343.027	<0.001	114.533	<0.001	178.468	<0.001	139.628	<0.001
<i>C. somedanum</i>	48.585	<0.001	10.661	0.001	39.348	<0.001	22.221	<0.001	1.098	0.307	38.637	<0.001
<i>E. latifolium</i>	309.088	<0.001	355.041	<0.001	246.668	<0.001	211.575	<0.001	0.320	0.573	152.195	<0.001
<i>J. alpino-articulatus</i>	188.855	<0.001	20.426	<0.001	276.913	<0.001	18.153	<0.001	37.860	<0.001	49.964	<0.001
<i>P. palustris</i>	316.655	<0.001	44.433	<0.001	49.131	<0.001	83.928	<0.001	207.100	<0.001	37.737	<0.001
<i>P. mixta</i>	166.979	<0.001	20.884	<0.001	0.185	0.669	0.842	0.437	183.608	<0.001	19.588	<0.001
<i>P. grandiflora</i>	321.661	<0.001	66.298	<0.001	8.332	0.006	6.479	0.003	319.418	<0.001	51.227	<0.001
<i>P. farinosa</i>	275.400	<0.001	56.297	<0.001	2.396	0.129	91.382	<0.001	248.881	<0.001	52.077	<0.001
<i>S. perennis</i>	547.588	<0.001	185.893	<0.001	88.584	<0.001	162.258	<0.001	361.119	<0.001	235.179	<0.001
<i>T. palustris</i>	59.741	<0.001	17.060	<0.001	58.085	<0.001	15.753	<0.001	0.243	0.624	0.318	0.729

**Table 2.3** Results of the GLMM pairwise comparisons analysing significant differences in germination between germination thermoperiods within fresh and cold-stratified seeds. The models included seed collection as a random blocking factor.

Species	Fresh seeds						Cold-stratified seeds					
	30/20 vs. 22/12		30/20 vs. 14/4		22/12 vs. 14/4		30/20 vs. 22/12		30/20 vs. 14/4		22/12 vs. 14/4	
	F	p	F	p	F	p	F	p	F	p	F	p
<i>B. compressus</i>	124.186	<0.001	101.105	<0.001	0.661	0.421	145.930	<0.001	173.406	<0.001	67.749	<0.001
<i>C. davalliana</i>	62.045	<0.001	60.730	<0.001	0.001	0.977	5.647	0.024	214.267	<0.001	174.516	<0.001
<i>C. diandra</i>	87.593	<0.001	69.501	<0.001	0.425	0.521	124.277	<0.001	110.765	<0.001	37.428	<0.001
<i>C. echinata</i>	3.551	0.069	100.018	<0.001	109.519	<0.001	1.880	0.180	0.228	0.636	0.688	0.413
<i>C. lepidocarpa</i>	74.327	<0.001	77.804	<0.001	4.145	0.046	90.817	<0.001	237.900	<0.001	110.952	<0.001
<i>C. pulicaris</i>	17.836	0.001	0.000	0.993	17.956	0.001	77.197	<0.001	17.643	0.001	50.395	<0.001
<i>C. somedanum</i>	0.394	0.553	0.000	0.993	0.382	0.559	32.133	0.001	27.845	0.002	0.628	0.458
<i>E. latifolium</i>	201.290	<0.001	165.610	<0.001	61.305	<0.001	0.790	0.379	415.118	<0.001	405.110	<0.001
<i>J. alpino-articulatus</i>	7.075	0.190	13.689	0.002	2.068	0.172	65.576	<0.001	44.480	<0.001	14.451	0.002
<i>P. palustris</i>	43.093	<0.001	33.014	<0.001	8.414	0.012	0.391	0.542	15.919	0.001	11.176	0.004
<i>P. mixta</i>	0.170	0.686	0.196	0.665	0.001	0.978	2.397	0.144	36.889	<0.001	27.169	<0.001
<i>P. grandiflora</i>	4.533	0.051	0.284	0.602	2.927	0.109	119.752	<0.001	101.208	<0.001	4.209	0.059
<i>P. farinosa</i>	83.077	<0.001	83.374	<0.001	0.054	0.820	5.274	0.038	11.536	0.004	3.920	0.068
<i>S. perennis</i>	50.763	<0.001	50.952	<0.001	0.001	0.977	28.140	<0.001	263.498	<0.001	167.064	<0.001
<i>T. palustris</i>	16.322	0.001	34.256	<0.001	7.972	0.014	0.119	0.736	0.101	0.755	0.001	0.979

### Germination thermoperiods

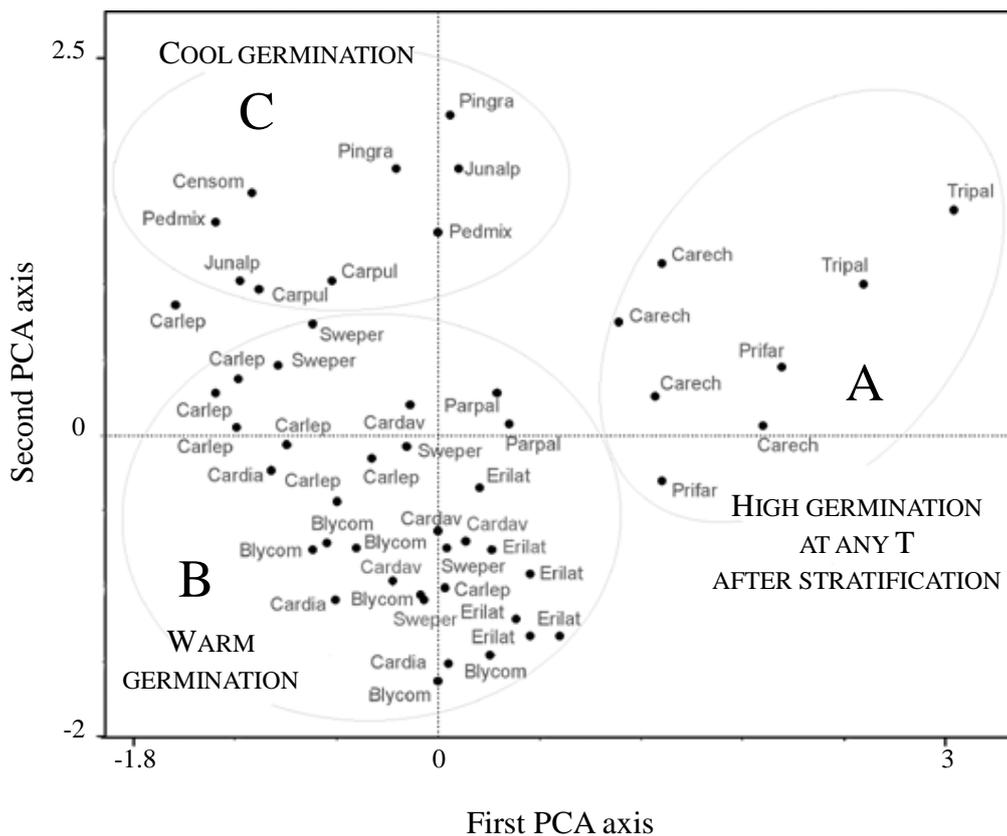
The germination thermoperiod that produced significantly (Table 2.3) better results was 30/20 °C in most species (Figure 2.2); although in *C. echinata* optimal germination extended also to 22/12 °C. In *C. pulicaris* and *J. alpino-articulatus* the best results occurred at 22/12 °C; and only in *P. mixta* was 14/4 °C the optimal germination thermoperiod. *P. grandiflora* and *C. somedanum* germinated equally well at 22/12 °C and 14/4 °C, although in the latter species germination was relatively low (51 % ± 7) indicating that either dormancy was not fully broken or that the optimal germination temperature was even lower.



**Figure 2.2** Effect of stratification on seed germination and dormancy loss across the range of germination thermoperiods (14/4 °C, 22/12 °C, 30/20 °C). The grey areas represent the germination percentages (from 0 to 100 %) of fresh seeds, the black areas the significant increases (GLMM,  $p < 0.05$ ) of germination percentages at each thermoperiod produced by warm (left blocks) and cold (right blocks) stratification. The germination percentages were calculated averaging treatments that were not significantly different ( $p > 0.05$ ).

### Species ordination

The first two PCA axes (Figure 2.3) accounted for 72 % of the total variance. The first axis (51 % of explained variation) showed a high positive correlation ( $r > 0.6$ ) with all the germination variables, and clearly separated the collections of three species (Group A) characterized by high germination ( $> 85\%$ ) at the three thermoperiods after cold stratification (Table 2.4). On the other hand, differences along the second axis (21 % of explained variation) were mainly correlated with the germination of stratified seeds at  $14/4\text{ }^{\circ}\text{C}$  ( $r = 0.6$ ) and  $30/20\text{ }^{\circ}\text{C}$  ( $r = -0.65$ ). Such differences separate a main group of species having similar germination responses to high temperatures (Group B) from another five species (Group C) in which optimal germination occurred at cool or moderate temperatures (Table 2.4). In general, the collections from a same species showed a congruent distribution along the ordination axes, although two species (*S. perennis* and *C. lepidocarpa*) showed a relative higher variation.



**Figure 2.3** Principal Component Analysis (PCA) representing the main two axis of variation on the germination patterns. Only the results of fresh and cold-stratified seeds were analysed. Each spot represents a collection and its species is indicated by the three first letters in the genus names and the specific epithet. The collections are clustered in three main groups according to their correlation with the variables and the germination behaviour of the species.

## DISCUSSION

The germination niche in most of the species analysed in this study is characterized by dormancy at dispersal and optimal germination at unusually high temperatures, as is generally the case in wetland species (Grime *et al.* 1981; Thompson and Grime 1983; Baskin and Baskin 1998; Schütz 2000). Nonetheless, two caveats must be made. First, contrary to what is frequently assumed (Schütz and Rave 1999; Kettenring and Galatowitsch 2007b) the requirement for cold temperatures during dormancy-breaking stratification is not obligatory but facultative in most of the cases (i.e. although cold improves germination percentages and broadens the germination temperature range, dormancy break occurs as well at warm temperatures, albeit at a slower rate). Second, unlike in a majority of wetland species (Baskin and Baskin 1998), germination in mountain calcareous spring fens may also occur at relatively cool temperatures, which are more realistic in the permanently water-saturated soils of these habitats.

### *Seed dormancy*

Since stratification improved seed germination in all our species, we can conclude that all of them have dormancy at dispersal, as has been found in most fen grassland species (Maas 1989; Patzelt *et al.* 2001; Jensen 2004). Still, the degree and characteristics of dormancy vary greatly among them. In *C. somedanum*, *P. mixta* and *P. grandiflora* dormancy at dispersal is total as fresh germination is marginal across the entire germination temperature range. More frequently, the species show conditional dormancy (i.e., some fresh germination occurs in part of the germination temperature range; Baskin and Baskin 1998).

In all species, dormancy break produces both an increase in germination percentages and a broadening of the temperature range where significant germination takes place. In *B. compressus*, *P. farinosa* and *P. mixta* dormancy break occurs exclusively during cold stratification, and thus they have an obligatory requirement for cold *per se*. In most of the study species, however, the cold requirement is facultative because they also lose dormancy during warm stratification but the effect of cold is more intense. Our study shows that in these cases the main role of cold temperatures during dormancy break is to allow germination at autumn/spring temperatures, which would not produce germination had the temperature during stratification been warm. On the other hand, some species respond equally to warm and cold (*T. palustris*, *C. somedanum*) or even

better to warm (*C. pulicaris*, *J. alpino-articulatus*) stratification and consequently they have not a requirement for cold *per se*.

Judging by the positive effect of stratification, the most probable type of seed dormancy in all of our species is physiological (*sensu* Baskin and Baskin 2004). Nonetheless, as no embryo measurements were made, we cannot exclude a morphological component of dormancy (Baskin and Baskin 2005). For example, Fernández-Pascual *et al.* (2012) found morphophysiological dormancy in *C. somedanum*, and it may be that more of our species share this kind of germination delay, especially the ones where warm stratification has the same or a better effect than cold.

### ***Warm germination***

In the majority of the study species, optimal germination occurs at unusually high temperatures (30/20 °C). This ‘warm germination’ is characteristic of wetland species (Grime *et al.* 1981; Thompson and Grime 1983) and especially of the genus *Carex* (Schütz 2000). However, according to the data collected by our dataloggers, such thermoperiod may be rather unusual in the soil of mountain calcareous spring fens. At least in the 2010–2011 season, soil temperature never attained so high values: 25 °C were only exceeded for two days in the medium altitude site, while the temperature was always less than 22 °C in the lowest and 18 °C in the highest sites. Although temperature in the immediate surface can be potentially warmer in bright sunlight, the top layers of the fen are made up by bryophytes and the water flows drain the seeds into the deeper layers, permanently saturated by cold mountain spring waters which stabilize soil temperatures and keep them cool.

That being the case, values around 30 °C will only be reached in extraordinary situations when parts of the fen become dried. In such a scenario, mortality of existing vegetation may follow, and thus gaps may appear for new individuals. As the 30/20 °C temperature regime not only produces optimal germination in non dormant seeds (i.e., after stratification), but also triggers fresh seed germination in most of our species, it may serve as a gap detecting mechanism which quickly overrides seed dormancy and produces ‘short term’ germinative responses in certain extraordinary situations where high recruitment becomes favourable.

Even so, in regular conditions, soil temperatures will be more similar to our 22/12 °C and especially 14/4 °C thermoperiods. In this scenario, when no gaps occur as a consequence of episodes of soil drying, seeds will follow a ‘long term’ germinative path: after dispersal in autumn germination will be prevented by dormancy; the cold winter season will produce dormancy break; and seeds will germinate as temperatures become warmer in spring and summer. Patzelt *et al.* (2001) and Jensen (2004) already found germination at 15/5 °C in some fen species, and it seems that the capacity to germinate at such thermoperiods is relatively widespread in mountain calcareous spring fen species once dormancy is broken.

### ***Specific germination strategies***

The short term ‘warm germination’ response, however, is not uniformly shared by all calcareous spring fen species. The species we included under groups A and B follow the traditional wetland ‘warm germination’ strategy. Although in both groups fresh germination occurs at 30/20 °C and cold stratification produces a broadening of the germination niche towards cooler temperatures, in group A this broadening is significantly greater and so is the capacity to germinate at 14/4 °C. As a consequence, the majority of seedling emergence in group A species is expected to take place shortly after dispersal or during the next spring, while most group B species would wait until the summer or even delay germination until the next year. Such differences may be related to the broader habitat specialization in group A species, since *T. palustris* can be found in both coastal and mountain marsh areas and *C. echinata* has a wider ecological range along the pH gradient of mountain fens (Hájek *et al.* 2006). *P. farinosa*, on the other hand, is a plant specialist closely related to calcareous spring fens but has been characterized as having a restricted ability to colonize new sites (Hájek *et al.* 2011). The broad germination niche we found in this species suggests that this restriction is not related to the germination stage of the regeneration cycle.

Species assigned to Group C clearly depart from the traditional wetland ‘warm germination’ pattern as their germination is reduced or even prevented by unusually high temperatures. These species also show differences in seed dormancy, as it includes species that either have total instead of conditional dormancy at dispersal, or which respond better to warm stratification. In the Iberian Peninsula, *C. somedanum*, *J. alpino-articulatus* and *P. grandiflora* are usually associated to mountain springs, which differ

from flat fens in that they are more intensely influenced by the spring water and have steeper slopes (Jiménez-Alfaro *et al.* 2011). In these particular spring settings episodes of soil drying and heating are not expected; quite on the contrary, their temperature is kept stable and cool by the spring waters. In a previous work, we already defined *C. somedanum* as following a ‘mountain spring’ germination strategy (Fernández-Pascual *et al.* 2012), and it would seem that this type of germination behaviour is not uncommon in mountain calcareous springs.

**Table 2.4** Summary of the germination traits of the study species. The species are classified according to the results of the PCA. The time of dispersal was calculated averaging all the collection dates. The predicted period when at least 50 % germination would occur in the field was estimated from the germination results assuming that 22/12 °C (fresh seeds) represented the weather at dispersal in summer, 14/4 °C (fresh seeds) dispersal in autumn, 14/4 °C (cold-stratified seeds) next spring, 22/12 °C (cold-stratified seeds) next summer.

Species	Dispersal	Dormancy at dispersal	Cold requirement	> 50 % Germination	Optimal T (°C)
Group A					
<i>C. echinata</i>	Jul.	Conditional	Facultative	Dispersal	30/20 - 22/12
<i>P. farinosa</i>	Jul.	Conditional	Obligatory	Next spring	30/20
<i>T. palustris</i>	Sep.	Conditional	None	Dispersal	30/20
Group B					
<i>B. compressus</i>	Aug.	Conditional	Obligatory	2 <sup>nd</sup> Year	30/20
<i>C. davalliana</i>	Jul.	Conditional	Facultative	Next summer	30/20
<i>C. diandra</i>	Aug.	Conditional	Facultative	2 <sup>nd</sup> Year	30/20
<i>C. lepidocarpa</i>	Jul.	Conditional	Facultative	2 <sup>nd</sup> Year	30/20
<i>E. latifolium</i>	Jul.	Conditional	Facultative	Next summer	30/20
<i>P. palustris</i>	Sep.	Conditional	Facultative	Next spring	30/20
<i>S. perennis</i>	Sep.	Conditional	Facultative	Next summer	30/20
Group C					
<i>C. pulicaris</i>	Sep.	Conditional	Warm req.	Next summer	22/12
<i>C. somedanum</i>	Sep.	Total	None	2 <sup>nd</sup> Year	14/4 - 22/12
<i>J. alpino-articulatus</i>	Sep.	Conditional	Warm req.	Next summer	22/12
<i>P. mixta</i>	Aug.	Total	Obligatory	Next spring	14/4
<i>P. grandiflora</i>	Jul.	Total	Facultative	Next spring	14/4 - 22/12

### ***Conclusions***

The temperature dimension of the germination niche in mountain calcareous spring fens may embody two general strategies. The traditional wetland ‘warm germination’ strategy is characterized by a dual germinative path. ‘Short-term’ responses to high temperature override dormancy and may allow the species to detect gaps produced by episodes of soil drying. In the absence of such drying events, the ‘long-term’ path follows through conditional dormancy at dispersal, dormancy break during overwintering and spring/summer germination at lower temperatures. An alternative germination strategy is followed by a group of ‘cool germination’ species, mostly related to springs. These species lack the ‘short-term’ response to soil heat and may germinate only at low temperatures.

In any case, the strategies here described are based on laboratory germination experiments and *in situ* measured temperature, and therefore their direct application to field conditions must be interpreted with care. It will be necessary to conduct field experiments to corroborate if the predicted emergence patterns are observed in nature. Additionally, it would be interesting to assess the potential effects of global warming on these germination strategies. Climate change is expected to alter the rate of dormancy loss and germination (Walck *et al.* 2011). In the case of calcareous spring fens, warming could differentially affect the regeneration of warm and cool germinators, producing an alteration of the vegetation composition and a subsequent detrimental effect on fen survival and conservation.

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## CHAPTER 3

### POTENTIAL PERSISTENCE OF MOUNTAIN FENS UNDER CLIMATE WARMING: SOIL TEMPERATURE BUFFER AND GERMINATION NICHE PLASTICITY

This chapter is unpublished.



**ABSTRACT**

**Background and Aims** Soils in base-rich fens are permanently saturated by cold waters flowing from underground aquifers. This might buffer their temperature, allowing fen vegetation to occupy a wider thermal range than it could otherwise and alleviating climate warming effects. In this work we analyse the extent of such buffering, its consistency in time and space, and its relation with a relevant plant trait, the seed germination niche breadth (GNB).

**Methods** We studied six fens from two mountain regions (the Cantabrian Mountains, Spain, and the Western Carpathians, Slovakia), covering the local altitudinal gradients. In each fen we recorded soil temperature and compared it with the WorldClim predictions. We also collected seeds from five Cyperaceae species to conduct laboratory germination experiments and to characterize their GNB estimated as Pielou's evenness index. We tested the correlation between GNB and site temperature.

**Key Results** Recorded soil temperatures differed from the predicted ones in their narrower thermal amplitude and warmer minimum and winter values. The most remarkable differences in soil temperature and GNB were those between mountain regions, the Slovakian winter being colder and the Spanish seeds having a broader niche. There was a significant positive correlation between the GNB of the collections and the winter minimum temperatures.

**Conclusions** The temperature of fen soils shows a buffer effect, being less variable and less cold than predicted by WorldClim. The effect is stronger during the night and in winter. Despite the buffer, soil temperature differs among sites and regions, and the studied species produce seeds with a narrower germination niche in the region with the harsher winters. These results suggest fen persistence under climate warming. The buffer is nonetheless sensitive to soil drying, and thus changes in precipitation and evapotranspiration seem the main threat to the future persistence of these habitats.

## RESUMEN EN CASTELLANO

**Antecedentes y objetivos** Los suelos de los tremedales ricos en bases están permanentemente saturados de aguas frías procedentes de acuíferos subterráneos. Esto puede tamponar su temperatura, permitiendo a la vegetación de tremedal ocupar un rango termal más amplio y aliviando los efectos del calentamiento climático. En este trabajo analizamos la extensión de dicho tampón, su consistencia en el tiempo y en el espacio, y su relación con un carácter vegetal de relevancia, la amplitud del nicho de germinación de semillas.

**Métodos** Estudiamos seis tremedales en dos regiones montañosas (la Cordillera Cantábrica, España, y los Cárpatos Occidentales, Eslovaquia), cubriendo los gradientes locales de altitud. En cada tremedal medimos la temperatura del suelo y la comparamos con las predicciones de WorldClim. Además, recolectamos semillas de cinco especies de ciperáceas para llevar a cabo experimentos de germinación en laboratorio y caracterizar su amplitud de nicho de germinación, estimada según el índice de Pielou. Comprobamos la correlación entre la amplitud del nicho de germinación y la temperatura de cada localidad.

**Resultados clave** Las temperaturas medidas en el suelo difirieron de las predichas en su menor amplitud termal y en sus valores mínimos y del invierno, más cálidos. Las diferencias más notables en temperatura del suelo y amplitud del nicho de germinación se dieron entre las dos regiones montañosas, siendo los inviernos eslovacos más fríos y el nicho de germinación de las semillas españolas más amplio. Hubo una correlación positiva significativa entre la amplitud del nicho de germinación y las temperaturas mínimas del invierno.

**Conclusiones** Las temperaturas de los suelos de tremedales muestran un efecto tampón, siendo menos variables y menos frías de lo predicho por WorldClim. El efecto es más fuerte durante la noche y en invierno. A pesar de este efecto, la temperatura del suelo varía entre localidades y regiones, y las especies estudiadas producen semillas con un nicho de germinación más amplio en la región con el invierno más frío. Estos resultados sugieren la permanencia de los tremedales frente al calentamiento climático. El tampón es en cualquier caso sensible al secado de los suelos, y por tanto los cambios en precipitación y evapotranspiración parecen ser las principales amenazas para la futura permanencia de estos hábitats.

## INTRODUCTION

Mountain mires are crucial in the context of global change because of their role in the carbon cycle (Bellamy *et al.* 2005; Joosten 2010). They face the general climate warming effects predicted for mountain ecosystems (Theurillat and Guisan 2001; Walther *et al.* 2005; IPCC 2007; Nogués-Bravo *et al.* 2007), but their dependence on fastidious conditions of climate and hydrogeology makes them particularly vulnerable (Parish *et al.* 2008; Essl *et al.* 2012). Mires fed by carbonate-rich groundwater are called base-rich (Jiménez-Alfaro *et al.* 2013b), small-sedge (Wheeler and Proctor 2000) or extremely-rich and calcareous fens (Hájek *et al.* 2006). Their formation and maintenance is governed by the hydrological regime of mountain calcareous springs (Jones and Renault 2010; Jiménez-Alfaro *et al.* 2013a), the suppliers of fen groundwater (Amon *et al.* 2002; Grootjans *et al.* 2006). Fen plant communities are rich in vascular and bryophyte specialists, but historical human drainage and recent land-use changes have had deleterious effects (Lienert *et al.* 2002; Šeffero­vá Stanová *et al.* 2008; Bergamini *et al.* 2009). For this reason they are protected in the European Union by the Habitats Directive (Council Directive 92/43/EEC, Annex I, code 7230 ‘alkaline fens’). However, for all their biological interest and threatened status, they have been understudied in global change literature in comparison with other habitats such as forests (Felton *et al.* 2009).

European fens spread from Atlantic Iberia to boreal Fennoscandia and from low valleys to the alpine belt (Jiménez-Alfaro *et al.* 2013b). Throughout this climatically-varied area, their constituent species are relatively homogeneous (Jiménez-Alfaro *et al.* 2013b) suggesting a climatic tolerance that is further stressed by their relictualism in their distribution limits (Amon *et al.* 2002; Topić and Stančić 2006; Hájek *et al.* 2009; Jiménez-Alfaro *et al.* 2012). This tolerance might be a result of the buffer effect of fen groundwater on soil temperature. The cold waters that saturate fens flow endlessly from underground aquifers (Grootjans *et al.* 2006; Jiménez-Alfaro *et al.* 2013a) and may stabilize soil temperatures throughout the year. As root-zone temperature is known to affect plant ecophysiology and vegetation patterns (Körner and Paulsen 2004), such a buffer might allow fen species to occupy a wider thermal range than they could otherwise. More importantly, it could favour fen persistence under climate change, alleviating those effects described in other mountain plant communities such as alterations of recruitment (Mondoni *et al.* 2012; Orrù *et al.* 2012), upward range shifts

(Chen *et al.* 2011; Pauli *et al.* 2012), loss of suitable habitats (Engler *et al.* 2011; Dullinger *et al.* 2012) and vegetation changes (Vittoz *et al.* 2008; Gottfried *et al.* 2012). However, to our knowledge, this hypothetical buffer effect has never been studied directly and its actual field characteristics are not known. Most climate warming studies rely on mathematical models of temperature (Felton *et al.* 2009; Essl *et al.* 2012), making an *in situ* investigation of soil temperatures much needed. Among the questions that need to be addressed are the extent of the buffer, its persistence throughout the year, its consistency at different altitudes and mountain regions, and its relation with relevant plant processes.

One such relevant process is plant regeneration and particularly seed germination. Germination is essentially governed by environmental temperature (García-Huidobro *et al.* 1982; Batlla and Benech-Arnold 2003; Steadman and Pritchard 2004) and so it will be affected by climate warming (Ooi *et al.* 2009; Cochrane *et al.* 2011; Mondoni *et al.* 2012; Ooi 2012; Orrù *et al.* 2012). Plasticity of germination traits, which allows plants to find suitable habitats amidst environmental changes (Donohue 2005), is expected to support species persistence under global change (Fenner and Thompson 2005; Walck *et al.* 2011). Among those traits showing plasticity is the breadth of the germination niche, i.e. the range of external conditions that elicit germination. Broad germination niches permit broader ecological ranges and are favoured by fecundity selection, but also increase the risks of germinating into suboptimal or lethal environments (Donohue *et al.* 2010). Environmental variation, especially when it is temporal (e.g. seasonal climates) rather than spatial (e.g. patchy landscapes), makes narrower niches adaptive because postponing germination may increase the chance of finding adequate environments (Donohue *et al.* 2010). In the case of fens, their patchy distribution and the potential buffer effect should generally favour broad germination niches. Nevertheless, plasticity may arise as a consequence of climatic differences, and it can be hypothesized that the germination niche breadth will decrease as temperature becomes more extreme, either at local or regional scales. A correct understanding of this relationship between germination niche breadth and soil temperature is necessary to assess the sensitivity of fen vegetation to temperature-related changes such as climate warming.

In this work we analyse the soil temperatures and the seed germination niche of base-rich fens in two contrasting regions of their European distribution, the centre in Central Europe and the south-western limit in the Iberian Peninsula. In this study system we

check the existence of a buffer effect, its consistency in time and space, and its relation with the plasticity of the germination niche. Specifically, we test two predictions, that (1) temperatures recorded in the soil should be less extreme than temperatures predicted by WorldClim, and (2) the germination niche breadth should be correlated to site temperatures.

## MATERIALS AND METHODS

### *Study system and temperature data*

The study took place in the Cantabrian Mountains (Spain) and the Western Carpathians (Slovakia). Within each region, we selected three sites covering the local altitudinal gradients of fen vegetation (Table 3.1). In June 2010 we placed temperature dataloggers (M-Log5W, GeoPrecision, Ettlingen, Germany) in each site, buried 5 cm in the soil in a flat and central area. It is at this depth that the true soil begins to develop, under the porous upper layers made up of live mosses. After one year, we analysed the variability among regions and altitudes in the recorded temperatures using ANOVA. At the same time, we downloaded the data available at WorldClim (Hijmans *et al.* 2005) and, from both data sources, we calculated several parameters: (1) the monthly averages of daily means, (2) the monthly averages of daily maximums or day temperatures; (3) the monthly averages of daily minimums or night temperatures; (4) the temperature limits in winter ( $W_{\min}$ , December-February average of the daily minimums) and (5) in summer ( $S_{\max}$ , June-August average of the daily maximums); (6) the number of months with mean temperatures under 1 °C; and (7) the number of months with minimum temperatures under zero. We compared the parameters obtained in the soil to those derived from WorldClim using Pearson's correlation and Generalized Linear Mixed Models (GLMM, linear or binary logistic depending on the nature of the data, with country and altitude gradient nested within country as random factors).

**Table 3.1** Description of the study sites. The temperature limits for each site ( $W_{\min}$  = average of the winter minimums,  $S_{\max}$  = average of the summer maximums) are given as calculated from the dataloggers (DL) and WorldClim (WC).

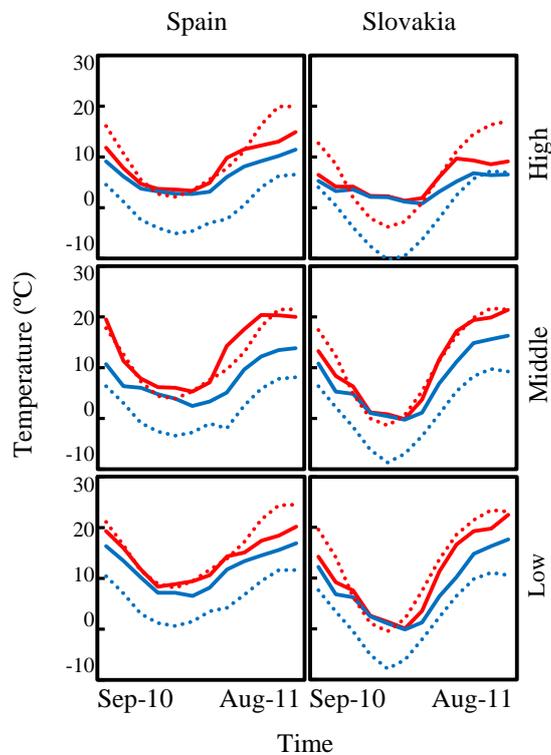
Region	Site	Coordinates	Altitude (m)	$W_{\min}$ (°C)		$S_{\max}$ (°C)	
				DL	WC	DL	WC
Spain (ES)	Low	43°07'09"N 06°15'10"W	710	6.9	1.1	18.7	23.5
	Middle	43°01'28"N 06°12'37"W	1530	3.7	-2.9	20.2	20.3
	High	43°00'38"N 05°56'49"W	1860	2.9	-4.6	13.4	18.7
Slovakia (SK)	Low	49°08'51"N 19°09'35"E	440	1.2	-6.4	20.5	22.7
	Middle	48°56'22"N 19°19'23"E	890	0.4	-7.3	20.2	21.0
	High	49°16'57"N 19°17'02"E	1310	1.8	-9.1	8.9	15.9

### ***Germination experiments***

Detecting across-species patterns in the germination niche requires an ecologically and taxonomically homogeneous study group (Luna and Moreno 2012). Here we chose Cyperaceae Juss., the dominant plant family in base-rich fens, whose members share a common germination pattern: conditional dormancy at dispersal, fresh germination at warm temperatures and germination at cooler temperatures once dormancy is broken (Schütz 2000; Fernández-Pascual *et al.* 2013b). During the 2011 dispersal season (July-September) we collected seeds from five Cyperaceae commonly found in the study habitats: *Blysmus compressus* (L.) Panz. ex Link, *Carex davalliana* Sm., *Carex echinata* Murray, *Carex lepidocarpa* Tausch and *Eriophorum latifolium* Hoppe. Seeds spent three weeks in our laboratory (c. 22 °C, 50 % RH) before being used in germination experiments to characterize the germination niche of each collection. For each collection and treatment we sowed four Petri dishes (holding 1 % distilled water agar and sealed with Parafilm to prevent desiccation) with 25 seeds each. The experimental factors were (1) pre-treatment (fresh = no pre-treatment; cold-stratified = 12 weeks in 1 % agar at 3 °C in darkness) and (2) germination thermoperiod (12/12 h incubation regimes of 30/20, 22/12 and 14/4 °C). These treatments encompass the germination response to temperature shown by the study species (Fernández-Pascual *et al.* 2013b). The incubations took place in growth chambers (Grow-S 360, Ing. Climas, Barcelona, Spain) and were coupled 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). We counted and discarded germinated seeds weekly (2 mm radicle emergence was the germination criterion) and after four weeks we opened the remaining seeds with a scalpel, classifying them as normal, empty and infected. We excluded the empty and infected seeds from further analyses.

We estimated the germination niche breadth (GNB) of each collection as Pielou's evenness index (Brändle *et al.* 2003; Cerabolini *et al.* 2003; Luna and Moreno 2012), using the average percentage germination at each of the six experimental treatments. This index takes values from zero (high sensitivity to one treatment, narrower GNB) to one (germination evenly distributed across treatments, broader GNB). The GNB had a normal distribution. We analysed the variability in GNB among species, regions and sites using GLMM (normal distribution, identity link, species as a random factor). Subsequently, we analysed the correlation between the GNB and site temperature by

fitting GLMMs to the GNB data with  $W_{\min}$  and  $S_{\max}$  as fixed predictors. We chose these two variables to represent the temperature limits or more extreme temperatures at each site. We fitted two models, using the variables calculated from the dataloggers and from WorldClim. The two temperature variables were not significantly correlated, independently of the data source that was used for their calculation (dataloggers, Pearson's  $R = 0.075$ ,  $p = 0.888$ ; WorldClim, Pearson's  $R = 0.635$ ,  $p = 0.175$ ). The model residuals were not spatially autocorrelated (in both cases  $p > 0.05$ , Moran's I test computed using ArcGis v9.3, Esri, Redlands, USA). We performed the statistics with SPSS (v20.0, IBM, Armonk, USA).



**Figure 3.1** Soil temperatures at each site from September 2010 to August 2011. The solid lines are the monthly averages of the maximum and minimum temperatures as recorded by the dataloggers. The dashed lines are the WorldClim predicted values.

## RESULTS

### *Temperature*

Soil temperatures (Figure 3.1) were significantly colder in Slovakia (on average 7.5 °C vs. 10.0 °C in Spain;  $F = 700.615$ ,  $p < 0.001$ ) and, within regions, in higher sites (in Slovakia,  $F = 1455.483$ ,  $p < 0.001$ ; in Spain,  $F = 650.601$ ,  $p < 0.001$ ). The air temperatures predicted by the WorldClim model (Figure 3.1) were significantly correlated to those recorded in the soil, considering the mean (Pearson's  $R = 0.934$ ,  $p < 0.001$ ), day (Pearson's  $R = 0.925$ ,  $p < 0.001$ ) and night (Pearson's  $R = 0.926$ ,  $p < 0.001$ ) values, as well as the temperature limits (Table 3.1)  $W_{\min}$  (Pearson's  $R = 0.921$ ,  $p = 0.009$ ) and  $S_{\max}$  (Pearson's  $R = 0.867$ ,  $p = 0.025$ ). Nonetheless, the absolute values were different. Mean temperatures were slightly colder in the WorldClim predictions (6.4 vs. 8.7 °C,  $F = 12.966$ ,  $p = 0.005$ ), although there was seasonal variation as the differences were significant in winter (-1.4 vs. 3.1 °C,  $F = 18.823$ ,  $p = 0.001$ ) but not in summer (14.2 vs. 14.7 °C,  $F = 0.207$ ,  $p = 0.659$ ). Differences were marked in night temperatures, predicted to be much colder (1.7 vs. 7.5 °C,  $F = 151.191$ ,  $p < 0.001$ ), while day temperatures were not significantly different (11.3 vs. 10.3 °C,  $F = 1.342$ ,  $p = 0.274$ ). The temperature limits were significantly different in both seasons, as WorldClim predicted hotter summer maximums (20.4 vs. 17.0 °C,  $F = 6.641$ ,  $p = 0.028$ ) and colder winters minimums (-4.9 vs. 2.8 °C,  $F = 66.486$ ,  $p < 0.001$ ). In a similar vein, WorldClim predicted more months with mean temperatures under 1 °C, indicating snow cover (2.8 vs. 0.5,  $F = 13.140$ ,  $p < 0.001$ ); and more months with negative minimum temperatures, suggesting soil freezing (4.7 vs. 0.3,  $F = 22.391$ ,  $p < 0.001$ ).

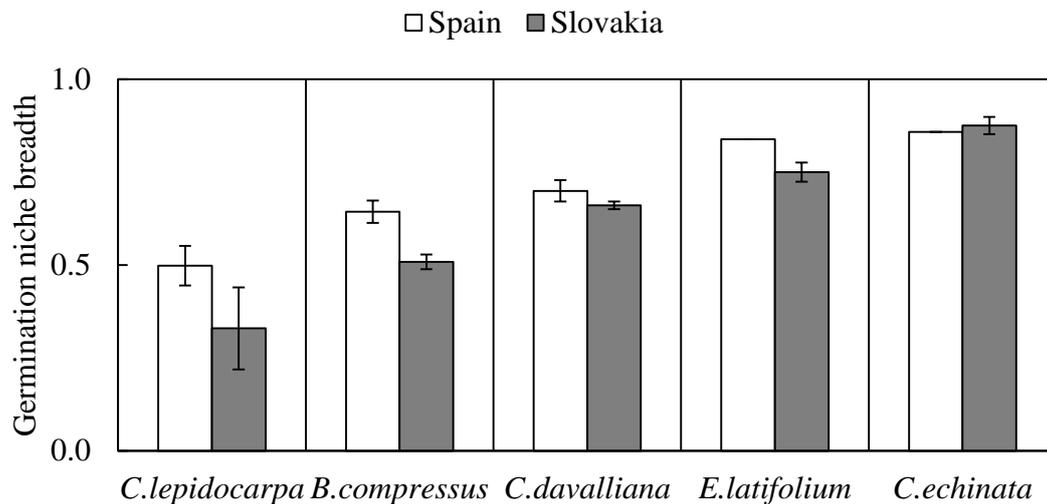
### Germination

The germination of all the species (Table 3.2) shared a common pattern: fresh germination only at warm temperatures; germination at cooler temperatures after stratification. All germinated at 30/20 °C without previous cold stratification, although percentages were usually low. Fresh germination also occurred at 22/12 °C in *B. compressus*, *E. latifolium* and especially *C. echinata*. Cold stratification increased germination in all the species at 30/20 and 22/12 °C, and also at 14/4 °C in *C. davalliana*, *C. echinata* and *E. latifolium*.

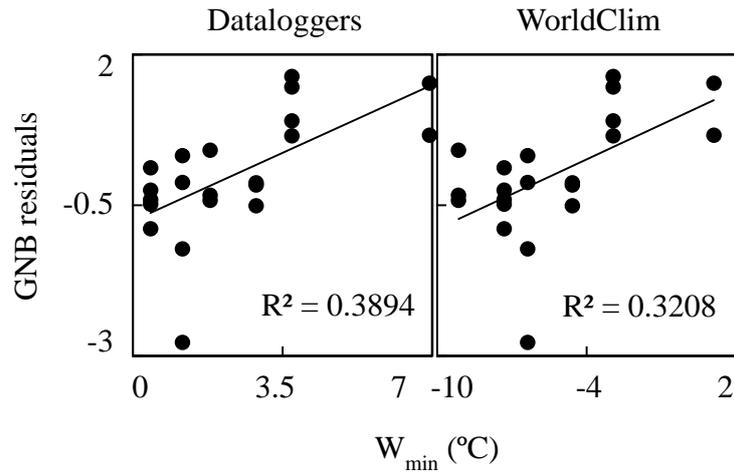
**Table 3.2** Results of the germination experiments. Germination percentages per collection of fresh and cold-stratified seeds after four weeks of incubation under three germination thermoperiods. The percentages are the average  $\pm$  SE of four dishes. GNB is the germination niche breadth of the collections calculated from the germination results using Pielou's evenness index.

Species	Site	Fresh seeds			Cold-stratified seeds			GNB
		14/4	22/12	30/20	14/4	22/12	30/20	
<i>B. compressus</i>	SK High	0	0	22 $\pm$ 08	0	36 $\pm$ 13	83 $\pm$ 06	0.53
	SK Middle	0	05 $\pm$ 03	83 $\pm$ 10	0	08 $\pm$ 08	92 $\pm$ 05	0.53
	SK Low	0	0	64 $\pm$ 22	0	08 $\pm$ 08	91 $\pm$ 05	0.47
	ES Middle	0	11 $\pm$ 11	100	0	68 $\pm$ 04	100	0.67
	ES Low	0	03 $\pm$ 03	82 $\pm$ 03	0	42 $\pm$ 17	100	0.61
<i>C. davalliana</i>	SK Middle	0	0	16 $\pm$ 04	04 $\pm$ 02	29 $\pm$ 10	42 $\pm$ 06	0.65
	SK Low	0	0	21 $\pm$ 07	10 $\pm$ 10	44 $\pm$ 10	62 $\pm$ 04	0.67
	ES High	0	0	15 $\pm$ 01	30 $\pm$ 05	89 $\pm$ 02	72 $\pm$ 04	0.67
	ES Middle	0	0	56 $\pm$ 05	27 $\pm$ 07	80 $\pm$ 05	90 $\pm$ 02	0.73
<i>C. echinata</i>	SK High	0	91 $\pm$ 03	96 $\pm$ 02	97 $\pm$ 01	100	97 $\pm$ 02	0.90
	SK Middle	0	40 $\pm$ 11	59 $\pm$ 03	17 $\pm$ 08	26 $\pm$ 05	49 $\pm$ 12	0.85
	ES High	0	71 $\pm$ 06	28 $\pm$ 03	100	100	90 $\pm$ 05	0.86
<i>C. lepidocarpa</i>	SK Middle	0	0	06 $\pm$ 03	0	16 $\pm$ 06	51 $\pm$ 05	0.44
	SK Low	0	0	0	0	04 $\pm$ 04	26 $\pm$ 11	0.22
	ES High	0	0	01 $\pm$ 01	0	03 $\pm$ 02	12 $\pm$ 03	0.39
	ES Middle	0	0	08 $\pm$ 05	01 $\pm$ 01	19 $\pm$ 06	38 $\pm$ 07	0.56
	ES Low	0	0	16 $\pm$ 07	0	32 $\pm$ 06	57 $\pm$ 09	0.55
<i>E. latifolium</i>	SK High	0	06 $\pm$ 03	17 $\pm$ 04	11 $\pm$ 02	57 $\pm$ 03	39 $\pm$ 09	0.75
	SK Middle	0	07 $\pm$ 02	39 $\pm$ 02	07 $\pm$ 02	89 $\pm$ 03	71 $\pm$ 04	0.71
	SK Low	0	25 $\pm$ 01	73 $\pm$ 08	16 $\pm$ 05	96 $\pm$ 01	98 $\pm$ 02	0.80
	ES Middle	0	20 $\pm$ 04	63 $\pm$ 03	28 $\pm$ 07	71 $\pm$ 06	72 $\pm$ 06	0.84

The GNB (Figure 3.2) was significantly different among species ( $F = 31.987$ ;  $p < 0.001$ ) and on average it was estimated at  $0.43 \pm 0.06$  in *C. lepidocarpa*,  $0.56 \pm 0.04$  in *B. compressus*,  $0.68 \pm 0.02$  in *C. davalliana*,  $0.77 \pm 0.03$  in *E. latifolium* and  $0.87 \pm 0.01$  in *C. echinata*. The GNB was significantly broader in the Spanish collections ( $F = 9.153$ ;  $p = 0.009$ ) but showed no significant differences among the sites nested within the regions ( $F = 1.990$ ;  $p = 0.148$ ). Within each region there was not a correlation between the GNB and site altitude ( $F = 0.050$ ;  $p = 0.816$ ). Regarding site temperatures, the GNB was significantly correlated to the  $W_{\min}$  at each site (dataloggers  $F = 10.232$ ,  $p = 0.005$ ; WorldClim  $F = 12.017$ ,  $p = 0.003$ ) but not to the  $S_{\max}$  (dataloggers  $F = 0.300$ ,  $p = 0.590$ ; WorldClim  $F = 2.899$ ,  $p = 0.106$ ). After ruling out the differences among species, a significant positive correlation existed between the GNB residuals of the collections and the  $W_{\min}$  at each site (Figure 3.3).



**Figure 3.2** Germination niche breadth (GNB) with respect to temperature per species and region. Each bar represents the average GNB  $\pm$  SE of a species in a region (white bars = Spain, grey = Slovakia).



**Figure 3.3** Linear regression between the germination niche breadth (GNB) residuals and the average of the winter minimum temperatures ( $W_{\min}$ ) calculated from the dataloggers (left panel) and WorldClim (right panel). The residuals are the studentized residuals of a Generalized Linear Model (normal distribution, identity link) fitted to the GNB using species as predictor. Each black dot is the residual of a collection.

## DISCUSSION

### *Soil temperature buffer*

The temperature of fen soils shows a buffer effect, being less variable and less cold than forecast by the WorldClim models (Hijmans *et al.* 2005), but the magnitude of the effect varies temporally. During the day and in summer the soils seem relatively sensitive to air temperature, as the mean values are the same as predicted. During these periods the soils can be expected to be relatively drier as a consequence of a warmer air and direct sun, and this likely reduces the buffer effect. In contrast, during the night and in winter the temperatures never get as cold as in the models, and so the buffer effect seems stronger over the cold end of the thermal gradient. This suggests that the main consequence of the buffer is to protect the soils from extreme cold, allowing fen species to occupy colder sites than they could otherwise, rather than warmer sites. Interestingly, fens in Western Siberia are especially rich in relictual thermophilic species, contrasting with rain-fed bogs which host relicts from colder periods (Lapshina 2004). If night and winter cold is indeed the limiting factor that soil water buffering is masking, the direct risks of climate warming are lessened, but confirming this will require to study in detail this potential thermophilic nature of fen species.

Despite the buffer, soil temperature varies among altitudes and regions, although not as pronouncedly as predicted. The soils are generally colder in Slovakia and in higher sites, following the classical gradients of latitude and altitude, but again different patterns can be described in winter and in summer. In winter, Slovakian soils are much colder and temperatures close to zero indicate episodes of snow cover that do not occur in Spain, but altitudinal differences are relatively small. On the other hand, summer soil temperatures are relatively similar in both regions and differences seem in this case more driven by altitude, with a sharp drop in the high sites. In any case, it is important to note that the pattern of variation among sites is in general terms correlated to the predicted one, even though the absolute values differ. This validates the use of model-derived temperatures when what matters are the relative differences among sites (Montesinos-Navarro *et al.* 2011; Essl *et al.* 2012).

Further interpretation of the fen buffer effect will require comparative studies with other soils. WorldClim predicts air temperatures, and a certain degree of buffering is expected in any kind of soil (Farouki 1986). However, dry soils under the predicted air

temperatures would suffer frequent episodes of freezing, which rarely occur in the recorded data. In moist soils, when temperature drops to zero the transition of water to ice releases latent heat that stops further cooling until all groundwater freezes, a process called the zero-curtain effect (Outcalt *et al.* 1990; McKenzie *et al.* 2007; Boike *et al.* 2008). In fens, the continuous input of groundwater might greatly prolong this effect, determining fundamental differences with other moist soils with a limited water supply, such as bogs.

### ***Germination niche plasticity***

The germination patterns in response to temperature are homogeneous in all the studied species, suggesting a general adaptation to the fen habitat. The germination niche is generally broad and responds to an ample thermal range. Nevertheless, there is plasticity in the germination niche breadth correlated with strong regional differences in the winter minimum temperatures. This agrees with the initial hypothesis, whereby in more extreme sites seeds with restricted germination conditions should be produced as a consequence of the higher risks of germinating at the wrong time (Donohue *et al.* 2010). Restricted germination through seed dormancy has already been identified as a bet-hedging strategy conferring fitness advantages in variable environments (Gremer *et al.* 2012), and it seems that the germination niche breadth further attunes germination timing to environmental variability. The broad nature of the germination niche, coupled with its plasticity, can contribute to the persistence of fen species under climate warming (Fenner and Thompson 2005; Walck *et al.* 2011). However, this plasticity seems to operate only at regional scales. Within regions, altitudinal differences were not significant, contrasting with the altitudinal variation that has been reported for other traits such as seed/fruit weight (Wirth *et al.* 2010; Montesinos-Navarro *et al.* 2011; Duivenvoorden and Cuello 2012; Gulias and Traveset 2012) or seed dormancy (Cavieres and Arroyo 2000; Giménez-Benavides *et al.* 2005; Fernández-Pascual *et al.* 2013a). This coincides with the observation that, in this habitat, the buffer effect reduces altitudinal differences in winter temperatures, precisely those related to the germination niche breadth.

Our study proves a correlation between a seed trait and winter temperatures, but analysing if there is causality and adaptive value will require of future field experiments involving reciprocal transplants (Hufford and Mazer 2003). Although a similar

correlation has been established with seed dormancy (Figuroa *et al.* 2010; Wagmann *et al.* 2012), to our knowledge this is the first time that it has been explicitly confirmed with the germination niche breadth, a more comprehensive concept that includes the spreading of germination over a wider or narrower range of conditions. Germination niche breadth is known to correlate with species distribution and abundance (Brändle *et al.* 2003; Luna and Moreno 2010), and our study further highlights its essential role in plant ecology.

### ***Conclusions***

This study explored two mechanisms that might play a role in the response of base-rich fens to environmental changes, the buffer effect of soil water and the germination plasticity of fen species. Our results point to the potential persistence of these habitats under rising global temperatures, explaining as well their relictualism in several European regions (Topić and Stančić 2006; Hájek *et al.* 2009; Hájek *et al.* 2011; Jiménez-Alfaro *et al.* 2012). However, all wetlands depend on the quantity and quality of their water supply (Erwin 2009), and fens may be extremely sensitive to the predicted changes in precipitation and evapotranspiration (IPCC 2007; Essl *et al.* 2012), which could compromise the buffer effect. In fact, present fens are restricted to non-Mediterranean regions (Jiménez-Alfaro *et al.* 2013b) and threatened in the transitional areas (Topić and Stančić 2006), and therefore mediterrization might be the main threat to their future persistence.

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## CHAPTER 4

# GERMINATION ECOLOGY OF THE PERENNIAL *CENTAURIUM SOMEDANUM*, A SPECIALIST SPECIES OF MOUNTAIN SPRINGS

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**ABSTRACT**

**Background and Aims** To improve the understanding of how a rare endemic species of *Centaurium* adapts to a specialized ecological niche, we study the germination ecology of the mountain spring specialist, *C. somedanum*, a perennial species restricted to an unusual habitat for this genus.

**Methods** We conducted laboratory experiments with fresh seeds collected from two populations for three consecutive years, to investigate: (1) the effect of temperature and light on germination; (2) the existence of seed dormancy; and (3) interpopulation and interannual variation in germinability.

**Key Results** Germination occurred only in the light and at relatively low temperatures (15–22 °C) with no differences between constant and alternating regimes, and a significant decrease at high temperatures (25 °C and 30 °C). We found non-deep simple morphophysiological dormancy and variation in seed germinability depending on the year of seed collection.

**Conclusions** *Centaurium somedanum* diverges from the common germination characteristics of the genus in: (1) its germination at lower temperatures, which contrasts with what is generally expected in wetland species but could be adaptive in the spring habitat; and (2) its morphophysiological dormancy, which we report here for the first time in the genus and which could be an adaptation to its mountain habitat.

## RESUMEN EN CASTELLANO

**Antecedentes y objetivos** Para conocer cómo una especie rara y endémica de *Centaureum* se adapta a un nicho ecológico especializado, estudiamos la ecología de la germinación de la planta especialista de fuentes de montaña *C. somedanum*, una especie perenne restringida a un hábitat inusual en el género.

**Métodos** Llevamos a cabo experimentos de laboratorio con semillas frescas recolectadas en dos poblaciones durante tres años consecutivos, para investigar: (1) el efecto de la temperatura y de la luz en la germinación; (2) la existencia de dormición de semillas; y (3) la variación en germinabilidad entre años y poblaciones.

**Resultados clave** La germinación sólo se produjo con luz y a temperaturas relativamente bajas (15-22 °C), sin diferencias entre termoperiodos constantes o alternos, y con una marcada disminución a temperaturas altas (25 °C y 30 °C). Encontramos dormición morfofisiológica simple no profunda y variación en la germinabilidad dependiendo del año de recolección.

**Conclusiones** *Centaureum somedanum* difiere de las características de germinación comunes en su género en: (1) su germinación a temperaturas bajas, que contrasta con lo que se espera por regla general en especies de humedales pero que podría ser adaptativa en el hábitat de fuente; y (2) su dormición morfofisiológica, que describimos aquí por primera vez en su género y que podría ser una adaptación al hábitat de montaña.

## INTRODUCTION

*Centaurium* Hill (Gentianaceae), as defined by Mansion (2004), includes c. 27 species of Mediterranean origin and Old World distribution. It is comprised of annual, biennial and, rarely, perennial herbs occurring in different habitats of the Mediterranean basin and surrounding areas (Mansion *et al.* 2005). Because of its wide distribution and pharmacological importance (Jensen and Schripsema 2002; Sefi *et al.* 2011), certain aspects of *Centaurium* germination ecology have been studied in detail. The comparative germination study of Grime *et al.* (1981) reported germination of the biennial *C. erythraea* Rafn. over a wide range of temperatures (13–29 °C) and only in light. Thompson and Grime (1983) confirmed the light requirement for the same species and reported that germination was insensitive to temperature fluctuations, while Schat (1983) found very similar germination characteristics in another biennial, *C. littorale* (D. Turner) Gilmour. More recently, a series of studies focusing on the effects of salinity, manganese and plant growth regulators reported successful germination at 24–25 °C in light (Mijajlovic *et al.* 2005; Zivkovic *et al.* 2007; Todorovic *et al.* 2008; Mistic *et al.* 2009; Todorovic *et al.* 2009) for the same two species as well as for *C. maritimum* (L.) Fritsch, *C. pulchellum* (Swartz) Druce, *C. spicatum* (L.) Fritsch and *C. tenuiflorum* (Hoffmanns. & Link) Fritsch, all of them annual or biennial. All these reports also indicated that germination takes place without previous dormancy-breaking treatments, in contrast with the requirement for cold stratification found in other genera of Gentianaceae (Favarger 1953) and the morphophysiological dormancy reported in *Frasera caroliniensis* Walt. (Threadgill *et al.* 1981) and *Sabatia angularis* (L.) Pursh (Baskin and Baskin 2005). However, investigating dormancy was not the aim of any of the aforementioned *Centaurium* references, and they only studied lowland annual/biennial generalist species of broad geographical distribution. It remains to be seen if the same germination patterns apply in perennial *Centaurium* species, especially in those living in particular habitats departing from those commonly inhabited by the genus.

The rare *C. somedanum* M. Laínz shows several divergences from the general *Centaurium* traits, as it is a perennial chamaephyte and a specialist species of mountain calcareous springs (Jiménez-Alfaro *et al.* 2005). It is also a narrow endemic species, confined to a small geographical area (210 km<sup>2</sup>) located in a transitional region between Mediterranean and Oceanic climatic zones in the Cantabrian Mountains of north-west

Spain (Jiménez-Alfaro *et al.* 2010). Because of the ecological and geographical uniqueness of *C. somedanum*, we may expect divergences from the general germination patterns of the annual/biennial, generalist and widely distributed *Centaureum* species. For example, temperate wetland species show a preference for relatively high (c. 30 °C) or alternating germination temperatures (Grime *et al.* 1981; Thompson and Grime 1983; Schütz 2000). Another possible adaptation of *C. somedanum* could be seed dormancy to avoid the risks of winter germination in a mountain environment (Baskin and Baskin 1998), although this dormancy could vary among years and populations, as is usually the case in wild species (Andersson and Milberg 1998; Giménez-Benavides *et al.* 2005).

In the present study, we investigate the germination ecology of *C. somedanum* as a contribution to understanding germination in *Centaureum* and the adaptation of this rare endemic species to an ecological niche unusual in the genus. Specifically, we want to determine: (1) the temperature and light requirements for its germination; (2) the possible existence of physiological and/or morphological dormancy; and (3) the existence of interpopulation and interannual variation in seed germinability.

## MATERIALS AND METHODS

### *Plant material*

Discrete populations of *C. somedanum* occur from 600 to 1700 m above sea level (asl) at the edges of calcareous springs. In these habitats, carbonate-rich spring waters (pH > 7.7) flow from aquifers continuously through the year and are relatively cold even in summer (mean day temperature in late summer =  $14.1 \pm 0.2$  °C; data from 160 measurements covering the entire altitudinal gradient of the species). Depending on the slope and species composition of the spring edges, two main habitat types harbour the populations of *C. somedanum*: vertical travertines and flat fens (Jiménez-Alfaro *et al.* 2005).

Although little information is available regarding its reproductive biology, *C. somedanum* is assumed to be a facultative outcrosser, like other species in the genus (Brys and Jacquemyn 2011). Flowering begins in early July, proceeds during summer and ripe seeds are dispersed in September and October (Jiménez-Alfaro *et al.* 2010). We observed abundant seedlings in the field in August, so we assume that emergence occurs in summer (mean summer temperature = 15 °C, min. = 10 °C, max. = 22 °C), after the cold season has ended (mean winter temperature = 4 °C, min. = 0 °C, max. = 8 °C) (data extrapolated from neighbouring climate stations, Spanish National Meteorological Agency). Seed set is relatively high in wild populations, with c. 140 seeds per fruit. The seeds are roughly spherical, extremely small (c. 390 µm wide) and show a reticulate pattern which is common in the *Centaurium* genus (Bouman *et al.* 2002). Seed mass (c. 20 µg) is also similar to that of other *Centaurium* species (Royal Botanic Gardens Kew 2008). The embryo belongs to Martin's dwarf category (Martin 1946).

For three consecutive years, we harvested fruits from the two largest populations of *C. somedanum*, which are representative of the two associated habitat types and the altitude gradient of the species (Table 4.1). In both sites, we sampled all individuals bearing ripe fruits (dry and brownish). The fruits spent a 3-week period in our laboratory (c. 22 °C, 50 % relative humidity) to ensure a homogeneous maturation state. Afterwards, we removed the seeds from the fruits; cleaned them using sieves and by hand sorting, and used them immediately in the experiments.

**Table 4.1** Description of the two populations included in this study.

Collection site	Altitude (m asl)	Habitat, mean annual temperature	Coordinates (N, W)	Collection dates
La Malva	600	Travertine, 10.4 °C	43° 07' 05'' 06° 15' 05''	17/09/2008 24/09/2009 02/09/2010
El Valle	1280	Flat fen, 8.3 °C	43° 04' 19'' 06° 11' 49''	17/09/2008 09/10/2009 02/09/2010

### *Embryo measurements*

We sowed seeds from 2009, from both populations, on 1 % distilled water agar in Petri dishes (diameter 6 cm) sealed with Parafilm to prevent desiccation (four dishes with 25 seeds each per population) and kept them in continuous darkness for 24 h at 20 °C. Afterwards, we excised and measured embryos from 15 seeds per population using a dissecting microscope (MZ6, Leica Microsystems GmbH, Wetzlar, Germany) equipped with a micrometer. The remaining seeds were cold-stratified for 12 weeks (3 °C, darkness) and then 15 embryos per population were measured. After cold stratification, seeds were incubated in a growth chamber (Grow-S 360, Ing. Climas, Barcelona, Spain) with a 12/12 h photoperiod (c. 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by six Philips TLD30W/54-765 cool fluorescent tubes) at 22/12 °C, the summer temperature expected to be optimal. We examined them daily and when the seed coat began to split, i.e. when the embryo had reached its critical length for radicle emergence, we measured another 15 embryos per population. To analyse the embryo measurements, we performed a main effects analysis of variance (ANOVA) with stratification/incubation stage and population as fixed effects using SPSS for Windows 15.0.1 (SPSS Inc., Chicago, USA).

### *Germination experiments*

We carried out laboratory germination experiments on 1 % distilled water agar in Petri dishes sealed with Parafilm. For each treatment, we sowed four dishes with 25 seeds each. To assess the effect of incubation temperature on germination, we incubated seeds from 2009, from both populations, in growth chambers under a 12/12 h photoperiod at 22/12, 15, 20, 25 and 30 °C. To take into account the interannual variation on seed germinability, we also incubated seeds from 2008 and 2010 at 22/12 °C. Finally, to assess the effect of light on germination, we incubated seeds from 2008 at 22/12 °C in continuous darkness (achieved by wrapping the dishes in two layers of

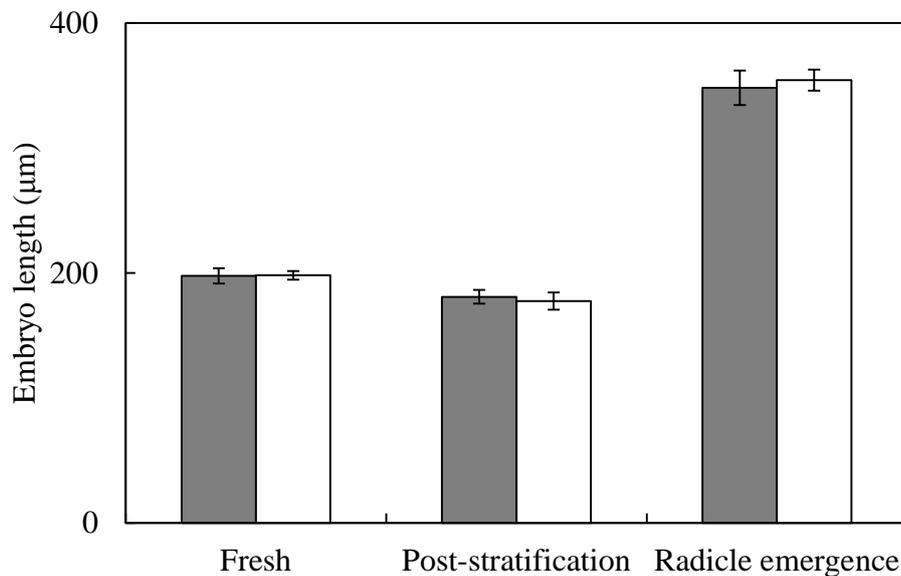
aluminium foil). In all cases, we incubated seeds both after 0 (= fresh, i.e. after three weeks of after-ripening in the laboratory) and 12 (= stratified) weeks of cold stratification in 1 % agar at 3 °C in darkness, to check for the existence of physiological dormancy. We did not consider warm stratification as it would be ecologically meaningless according to available knowledge on the habitat and dispersion timing of the species.

We counted and discarded germinated seeds three times a week (with the exception of the dark-incubated seeds, which we only checked at the end of the experiment). Radicle emergence was the criterion for germination. After four weeks, we terminated all germination tests and opened the non-germinated seeds with a scalpel, classifying them as normal, empty or fungus infected. We excluded the empty and infected seeds from the statistical analyses and the calculation of germination percentages (pooling all the dishes, empty seeds = 3 %  $\pm$  1; infected seeds = 3 %  $\pm$  1). We analysed the results by fitting main effects Generalized Linear Models (GLM, binomial error distribution, logit link function) with the test conditions as fixed factors, using SPSS.

## RESULTS

### *Embryo growth*

We found significant differences in embryo length depending on the stage of the stratification/incubation process ( $F = 279.592$ ;  $p < 0.001$ ). While after cold stratification embryo length was similar to that of fresh embryos, at the point of radicle emergence embryos had undergone an increase of 86 % in their length (Figure 4.1). We did not detect significant differences in embryo growth between the populations ( $F = 0.029$ ;  $p = 0.866$ ).

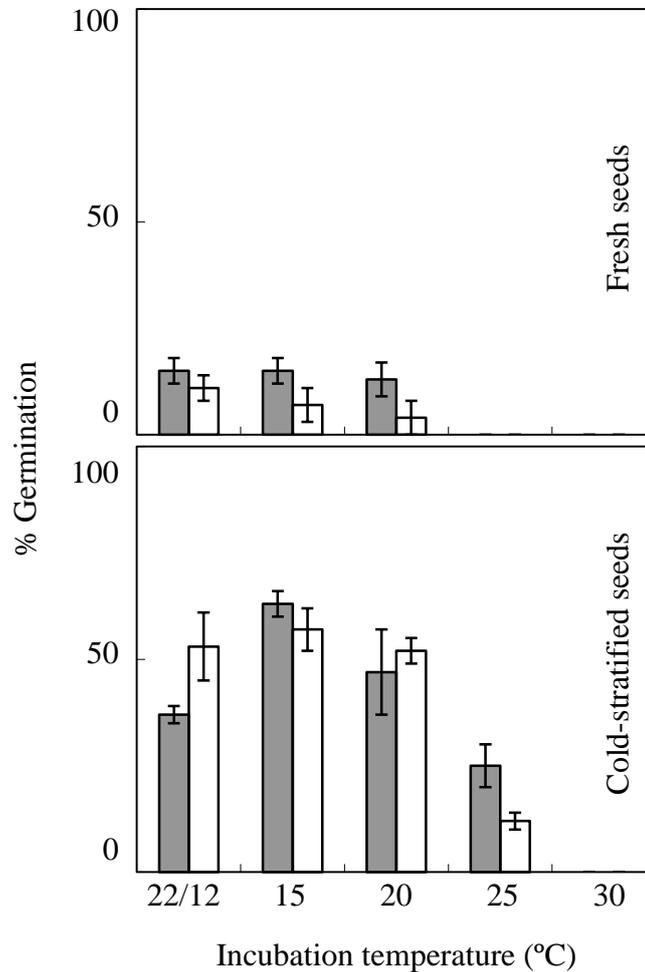


**Figure 4.1** Embryo length ( $\pm$  SE) in two populations (La Malva, grey bars; El Valle, white bars) at three different stages of the seed stratification–incubation process.

### *Germination temperature*

Higher seed germination occurred at lower temperatures (15–22 °C) with a marked decrease at warmer temperatures (Figure 4.2). No seeds germinated at 30 °C and only cold-stratified seeds germinated to a low percentage at 25 °C. These two temperature regimes were qualitatively different and thus we did not include them in the statistical analysis, as their lack of variance would alter the GLM procedure. Analysing the results for the other three temperatures, we detected a significant positive effect of cold stratification on germination ( $\chi^2 = 186.720$ ;  $p < 0.001$ ) but neither an effect of incubation temperature ( $\chi^2 = 5.195$ ;  $p = 0.074$ ) nor of population ( $\chi^2 = 0.352$ ;  $p = 0.553$ ). Thus, in the range most favourable for seed germination, we did not find differences

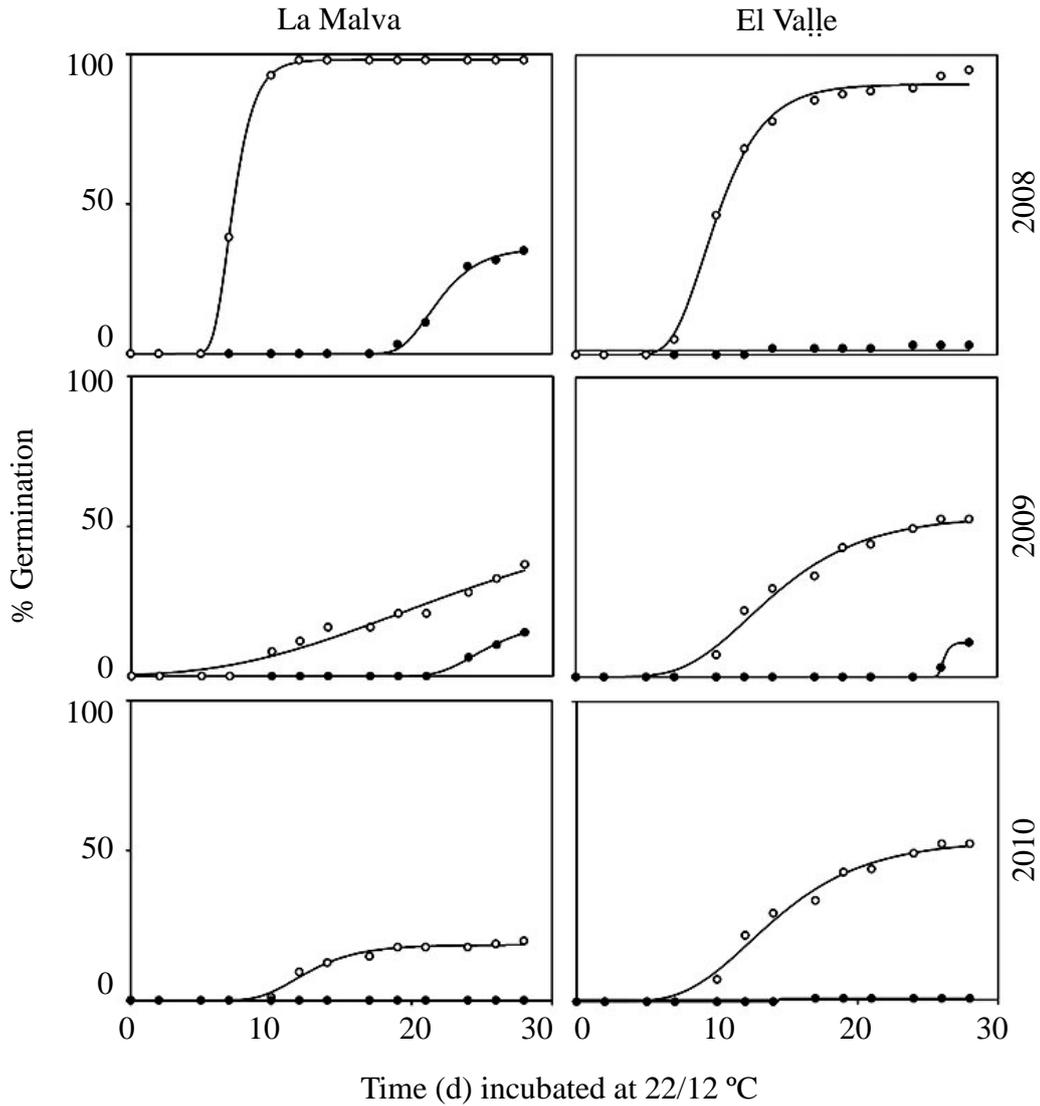
between constant (15, 20 °C) and alternating (22/12 °C) regimes and no differences between the germination temperature range of seeds from the two populations.



**Figure 4.2** Final germination percentages of fresh (top panel) and stratified (bottom panel) seeds from two populations (La Malva, grey bars; El Valle, white bars) after four weeks of incubation at different temperatures. Percentages are the mean  $\pm$  SE of four dishes.

### *Germinability variation*

We found differences in seed germinability among our collections (Figure 4.3). Cold stratification produced a significant germination increase ( $\chi^2 = 222.293$ ;  $p < 0.001$ ) across years and populations, but we only obtained almost complete germination in 2008. In the other two years, a percentage (47–80 %) of the seeds did not germinate after 12 weeks of cold stratification. Analysing together all years and populations, we found a significant effect of year ( $\chi^2 = 173.515$ ;  $p < 0.001$ ) but not of population ( $\chi^2 = 0.647$ ;  $p = 0.421$ ).



**Figure 4.3** Cumulative germination of fresh (black circles) and stratified (open circles) seeds from each population and year of collection after four weeks of incubation at 22/12 °C. The lines represent the Gompertz function fitted to the germination data using SigmaPlot 11.0 (Systat Software Inc., San José, USA).

### *Effect of light*

Darkness had a clear negative effect on germination. When incubated in darkness, the fresh seeds did not germinate at all, and only 1 %  $\pm$  1 cold-stratified seeds from one population (La Malva) did germinate. This clear-cut effect of darkness made it impossible, as well as unnecessary, to apply any statistical test.

**DISCUSSION**

*C. somedanum* shows two significant divergences from the previously reported *Centaureium* germination patterns: (1) a lower germination temperature range; and (2) seed dormancy. Furthermore, its germination requirements are in contrast to the typical responses of wetland species (Grime *et al.* 1981; Thompson and Grime 1983; Schütz 2000) as germination not only occurred at low temperatures, but also showed no increase in response to alternating temperatures. Among the tested conditions, highest germination occurred between 15 and 22 °C. As we did not test colder temperatures, it is not possible to establish the lower temperature limit of the species. In addition, the low germination percentages achieved at 22/12 °C in 2009 and 2010 could indicate that these are suboptimal conditions and that the optimal germination temperature is even lower. However, since the germination of the 2008 stratified seeds was almost complete at 22/12 °C, the lower germination found in the following years seems more related to a deeper dormancy in those years, suggesting that the optimal germination temperature should not be too far from the 15–22 °C range.

In any case, the upper germination temperature limit of *C. somedanum* is below what was expected. Germination at low temperatures is a common trait of lowland Mediterranean species (Escudero *et al.* 1997; Doussi and Thanos 2002), and consequently the temperature requirement for *C. somedanum* germination would seem to be related to the Mediterranean origin of the genus. However, lower germination temperature ranges are usually interpreted as an adaptation to the seasonality of Mediterranean climates that prevents germination during the dry season (Doussi and Thanos 2002), and this is obviously not the case in the extremely wet environment of *C. somedanum*. The traditional understanding of germination in wetland habitats, as proposed by Thompson and Grime (1983), indicates that the fall of the water table during the spring season produces rising soil temperatures and an increase in diurnal temperature fluctuations. Wetland seeds perceive these signals as marking the optimal season for germination, but the conditions may be very different in mountain spring habitats where water flow is continuous, and even more intense during the spring season when snowmelt recharges aquifers. In our study system, seed germination and seedling establishment in *C. somedanum* take place in the soil of spring edges, where the constantly cold running waters heavily influence the temperature. According to data obtained by a soil data-logger in La Malva population (M-Log5W, GeoPrecision

GmbH, Ettlingen, Germany; data from September 2010 to September 2011), the diurnal thermal amplitude in the soil of the spring edges is relatively low throughout the year (winter =  $1.9 \pm 0.1$  °C, summer =  $3.0 \pm 0.1$  °C) and the summer temperature is considerably less variable than expected (mean = 16.9 °C, min. = 15.7 °C, max. = 18.8 °C). In this environment, germination at low and constant temperatures is probably the only option for the species. However, since we could not establish the lower germination temperature limit, it cannot be excluded that seed germination begins earlier in the year, as from April onwards the soil mean temperature exceeds 10 °C.

The inability of *C. somedanum* to germinate at 25 °C and above also differs from the successful germination achieved at 25 °C (Mijajlovic *et al.* 2005; Zivkovic *et al.* 2007; Todorovic *et al.* 2008; Todorovic *et al.* 2009) and the 29 °C upper germination limit (Grime *et al.* 1981) found in generalist *Centaureum* species of broad European distribution. It is still necessary to determine whether the lower range in *C. somedanum* is an ancestral Mediterranean character of the genus, which was conserved in this rare endemic and allowed it to colonize the spring habitat; or if it is rather a recent adaptation acquired in the course of such colonization. Although the phylogenetic origin of *C. somedanum* is unclear, the molecular study of Mansion *et al.* (2005) suggests an allopolyploid origin from the perennial *C. scilloides* (L. fil.) Samp and the biennial Spanish endemic *C. gypsicola* (Boiss & Reut.) Ronniger. *C. scilloides* is a generalist species widely distributed over the Atlantic coasts of Europe, while *C. gypsicola* is a specialist species of Mediterranean semi-arid gypsum communities of the central Iberian Peninsula. Thus, investigating the germination response of these species would shed some light on whether the low germination temperature trait of *C. somedanum* has a phylogenetic origin or is a new trait.

The second divergence from the *Centaureum* germination pattern regards the seed dormancy we found in the three years analysed, contrasting with the non-dormant behaviour reported in the literature for other *Centaureum* species. Our results show that *C. somedanum* seeds have non-deep simple morphophysiological dormancy (MPD) according to the classification system of Baskin and Baskin (2004) and that the embryo belongs to the underdeveloped linear embryo category proposed by Baskin and Baskin (2007). Fresh seeds have a non-deep physiological dormancy (PD) which prevents embryo growth and which is overcome by a period of cold stratification. As we did not test warm stratification, it is not possible to determine if cold is a requirement *per se*,

but in nature this dormancy release takes place during the cold winter season. Once the PD is broken, embryo growth must take place before the seed germinates (morphological dormancy, MD). Since embryo growth did not occur during cold stratification, but did occur at warmer temperatures after the seeds were moved to 22/12 °C, they have non-deep simple MPD. To our knowledge, this is the first time that MPD has been found in the *Centaurium* genus, although it has been reported in New World Gentianaceae genera (Threadgill *et al.* 1981; Baskin and Baskin 2005). As we have already explained, the thermal regime in the spring habitat is stable during the year in comparison to other, more seasonal, wetland environments. In this context, seed dormancy becomes of foremost importance to ensure that germination takes place in the correct period of the year, i.e. after the colder months of winter.

It is interesting to note that, contrary to what happened with the temperature and light requirements, we found a relatively large variation among seed collections in their germinability. Such variation, depending on year and population, is a well-known phenomenon (Andersson and Milberg 1998; Giménez-Benavides *et al.* 2005), which has been usually related to genetic adaptation to local conditions or to the parental environment effect on seed maturation (Fenner 1991; Baskin and Baskin 1998). In our case study, the significant effect of year rather than location suggests that it is the environment in each maturation season that determines seed germinability. Nevertheless, further studies controlling either the seed maturation environment (common garden studies) or the genetic background of the populations are necessary to elucidate the relative contribution of the genotype versus the environment.

Besides the divergences commented on above, *C. somedanum* germination agrees with previous *Centaurium* studies regarding the lack of response to alternating temperatures (Thompson and Grime 1983) and the light requirement for germination (Grime *et al.* 1981; Schat 1983). The incapacity to germinate in darkness is a trait generally associated with the ability to form a soil seed bank (Pons 1991; Milberg 1994) especially in small seeds (Milberg *et al.* 2000). Other traits of *C. somedanum* seeds tend to be related to persistence in the soil bank, namely their small size and rounded shape (Thompson *et al.* 1993; Cerabolini *et al.* 2003). Thus, it is possible that *C. somedanum* forms a persistent soil seed bank, as has been reported for other *Centaurium* species (Thompson *et al.* 1997).

In conclusion, our study shows that a rare perennial species of *Centaureum* living in an unusual habitat presents divergences from the general germination patterns of the genus. The lower germination temperature range in *C. somedanum* could be of adaptive significance in the very particular conditions of mountain calcareous springs, where water flow is continuous and relatively cold throughout the year. It would be of great interest to study the germination temperature ranges of those species phylogenetically linked to *C. somedanum*, i.e. *C. scilloides* and *C. gypsicola*, to improve understanding of the evolutionary history of this trait in the genus. Additionally, it remains to be seen if MPD, a possible adaptation to mountain spring habitats, is shared by more *Centaureum* species, especially those with a similar ecology, such as *C. littorale* subsp. *uliginosum* (Waldst. & Kit.) Melderis, which lives in calcareous spring fens of central Europe. Our findings also suggest that the germination traits of species living in temperate mountain springs could differ from those living in other wetland types, although more studies focused on spring specialists are needed to clarify germination ecology in these habitats.

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## CHAPTER 5

# A LOCAL DORMANCY CLINE IS RELATED TO THE SEED MATURATION ENVIRONMENT, POPULATION GENETIC COMPOSITION AND CLIMATE

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## ABSTRACT

**Background and Aims** Seed dormancy varies within species in response to climate, both in the long term (through ecotypes or clines) and in the short term (through the influence of the seed maturation environment). Disentangling both processes is crucial to understand plant adaptation to environmental changes. Here we investigate the local patterns of seed dormancy in a narrow endemic species, *Centaurium somedanum*, to determine the influence of the seed maturation environment, population genetic composition and climate.

**Methods** We performed laboratory germination experiments to measure dormancy in (1) seeds collected from different wild populations along a local altitudinal gradient and (2) seeds of a subsequent generation produced in a common garden. We characterized the genetic composition of the original populations using Inter-Simple Sequence Repeat PCR and PCoA, and analysed its correlation to the dormancy patterns of both generations. We also modelled the effect of the local climate on dormancy.

**Key Results** We found an altitudinal dormancy cline in the wild populations, which was maintained by the plants grown in the common garden. However, seeds from the common garden responded better to stratification, and their release from dormancy was more intense. The patterns of dormancy variation were correlated with genetic composition, whereas lower temperature and summer precipitation at the population sites predicted higher dormancy in the seeds of both generations.

**Conclusions** The dormancy cline in *C. somedanum* is related to a local climatic gradient and also corresponds with genetic differentiation among populations. This cline is further affected by the weather conditions during seed maturation, which influence the receptiveness to dormancy-breaking factors. These results show that dormancy is influenced by both long and short term climatic variation. Such processes at such a reduced spatial scale highlight the potential of plants to adapt to fast environmental changes.

## RESUMEN EN CASTELLANO

**Antecedentes y objetivos** La dormición de semillas varía intraespecíficamente en respuesta al clima, tanto en el largo plazo (a través de ecotipos o clinas) como en el corto (mediante la influencia del ambiente de maduración de las semillas). Desenmarañar ambos procesos es crucial para entender la adaptación de las plantas a los cambios ambientales. En este trabajo investigamos los patrones locales de dormición en un endemismo de distribución reducida, *Centaureum somedanum*, para determinar la influencia que sobre ellos tienen el ambiente de maduración de las semillas, la composición genética de las poblaciones y el clima.

**Métodos** Llevamos a cabo experimentos de germinación en laboratorio para medir la dormición de (1) semillas recolectadas en diferentes poblaciones silvestres a lo largo de un gradiente altitudinal local y (2) semillas de una generación posterior producidas en un experimento de common garden. Caracterizamos la composición genética de las poblaciones mediante Inter-Simple Sequence Repeat PCR y PCoA, y analizamos su correlación con los patrones de dormición de ambas generaciones. Además, modelamos el efecto del clima local en la germinación.

**Resultados clave** Encontramos que las poblaciones silvestres presentaban una clina altitudinal en dormición, clina que era mantenida por las plantas cultivadas en el common garden. Sin embargo, las semillas del common garden respondieron mejor a la estratificación, y su salida de la dormición fue más intensa. Los patrones de variación en dormición estuvieron correlacionados con la composición genética de las poblaciones; mientras que valores más bajos de temperatura y precipitación estival en las localidades originales predijeron mayores niveles de dormición en las semillas de ambas generaciones.

**Conclusiones** La clina de dormición en *C. somedanum* está relacionada con el gradiente climático local y con la diferenciación genética de las poblaciones. La clina es además modulada por las condiciones climáticas concretas durante la maduración de las semillas, las cuales afectan a la sensibilidad a los factores de rotura de la dormición. Estos resultados muestran que la dormición está influenciada por la variación climática tanto a largo como a corto plazo. Tales procesos en una escala espacial tan reducida dan muestra del potencial de las plantas para adaptarse a cambios ambientales rápidos.

## INTRODUCTION

Dormancy is a quantitative seed trait that defines the range of environmental conditions that must be met before germination can occur (Vleeshouwers *et al.* 1995; Finch-Savage and Leubner-Metzger 2006). A seed able to germinate over the widest possible range of conditions is non-dormant; any narrowing of these conditions is by definition an increase in the level of seed dormancy. Dormancy levels vary temporally in any individual seed, most markedly in the case of physiological dormancy (Baskin and Baskin 2004). Upon dispersal, these seeds show primary dormancy, either total (i.e., no germination under any conditions) or conditional (i.e., germination under a restricted set of conditions) (Baskin and Baskin 1998; Fenner and Thompson 2005). This dormancy can be alleviated by certain environmental signals, the dormancy-breaking factors (Batlla and Benech-Arnold 2003; Batlla *et al.* 2003; Batlla and Benech-Arnold 2004; 2005). In response to these factors, the seeds gradually lose dormancy and their range of germination conditions broadens (Steadman and Pritchard 2004; Orrù *et al.* 2012) until eventually they become non-dormant. In some cases, the seeds that do not meet adequate germination conditions enter secondary dormancy as a response to further environmental cues (Brändel 2005; Kępczyński *et al.* 2006; Leymarie *et al.* 2008). These dormancy cycles (Batlla and Benech-Arnold 2007) go on throughout the year, matching the annual temperature cycles, and have been termed the dormancy continuum (Baskin and Baskin 1985).

At any given time, spatial variation in seed dormancy can be observed among individuals and populations of the same species, as a consequence of different stages in the dormancy continuum. Variation becomes apparent not only in the dormancy levels, but also in the receptiveness to dormancy-breaking factors (Black *et al.* 2006). Many studies have found dormancy variation among seed collections from different sites and years (Moyer and Lang 1976; Andersson and Milberg 1998; Schütz and Rave 2003; Koutecká and Leps 2009; Herranz *et al.* 2010). These investigations have usually considered broad geographical scales, detecting changes in dormancy associated with latitudinal differences (Ren and Abbott 1991; Skordilis and Thanos 1995; Wagmann *et al.* 2012). Similarly, a positive correlation between dormancy and population altitude has often been established (Vickery 1983; Beardsell and Mullet 1984; Holm 1994; Cavieres and Arroyo 2000), but exceptions to this pattern have also been found (Barclay and Crawford 1984; Giménez-Benavides *et al.* 2005). Further dormancy variation has

been detected among seed collections from different environments. For example, in *Artemisia tridentata* Nutt., a negative effect of the winter temperature on seed dormancy was described on a wide gradient along western North America (Meyer and Monsen 1991). *Thymelaea hirsuta* (L.) Endl. showed differences in the germinability of seeds collected in six different desert habitats, with lower germination in seeds from the more extreme sites (El-Keblawy *et al.* 1996). Regional differences in climatic conditions, which vary with latitude/altitude and among habitats, are probably the cause of some of these correlations.

Field studies, however, cannot disentangle the long and the short term effects of climate. Long term effects arise from sustained climatic differences among sites, which may result in inheritable dormancy differences through ecotypes (Hufford and Mazer 2003) and clinal variation (Montague *et al.* 2008). On the other hand, short term effects are produced by the specific weather during the seed maturation season, and are usually termed the parental or maternal environment effect (Fenner 1991; Donohue 2009). This issue is partially addressed by common garden experiments, where seeds with controlled genetic origin are matured under controlled conditions. Such an approach has confirmed that low temperatures and water availability during seed maturation result in more dormant seeds (Wright *et al.* 1999; Allen and Meyer 2002; Luzuriaga *et al.* 2006; Qaderi *et al.* 2006; Hoyle *et al.* 2008; Figueroa *et al.* 2010). At the same time, work has been done to unravel the genetic basis of dormancy variation, understood as a complex quantitative trait controlled by a large number of genes, plant hormones and maternal factors (Koornneef *et al.* 2002). In the model species *Arabidopsis thaliana* (L.) Heynh., variation in dormancy related genes (Bentsink *et al.* 2006; Bentsink *et al.* 2010) has been identified in relation to maturation environment, seed provenance (Chiang *et al.* 2011) and local adaptation (Kronholm *et al.* 2012). Therefore, both inheritable genetic differences and the seed maturation environment seem to determine dormancy variation in the field, but understanding the respective roles of these two sources of variation is not trivial. In seasonal climates germination traits allow a precise timing of seed germination and seedling establishment, are subjected to natural selection, and determine the action of natural selection in subsequent life history traits (Donohue 2005; Donohue *et al.* 2005a; 2005b; 2005c). Thus, ascertaining how seed dormancy responds to local climate in the short and the long term is crucial to understand how germination

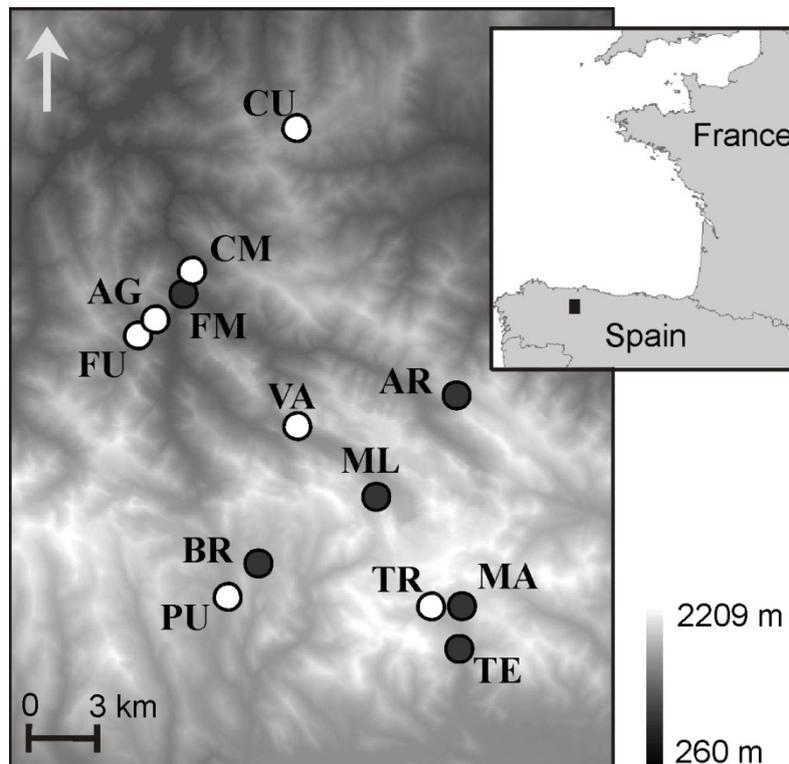
timing and plant regeneration will be affected by environmental change, especially climate warming (Walck *et al.* 2011; Ooi 2012).

The interpretation of local adaptation and clinal variation in *A. thaliana* and other widespread species, however, is often obscured because only part of the species distribution is studied; as a consequence the influence of biogeographic and historical events (e.g., different colonization events by already adapted ecotypes) cannot be entirely assessed (Montesinos-Navarro *et al.* 2011). One clear advantage of studying narrow endemic species is that biogeographical and historical influences are not substantial, and local adaptation can be assumed to have taken place *in situ* along the environmental gradient. Furthermore, the results obtained in plants with widespread distributions should not be directly extrapolated to the reduced geographical scales where some endemic species occur. One such species is the mountain calcareous spring specialist *Centaureum somedanum* M. Laínz (Gentianaceae), an endemic perennial herb of the Cantabrian Mountains of north-western Spain. The seeds of this species have non-deep simple morphophysiological dormancy; and the physiological component of this dormancy shows within-species variation at a local scale (Fernández-Pascual *et al.* 2012). In this work, we separate the effects of environmental and genetic variability on the observed physiological dormancy variation of this species to test whether (1) the patterns of intraspecific variation in seed dormancy levels at dispersal and receptiveness to dormancy-breaking factors, shown by the populations in nature, are maintained or altered when the plants and the seeds mature in a common garden environment; (2) such patterns are correlated to the genetic composition of the original wild populations; and (3) such patterns may be explained by the local climate at the population sites.

## MATERIALS AND METHODS

### *Plant material*

*Centaurium somedanum* is confined to a 210 km<sup>2</sup> mountain area (Jiménez-Alfaro *et al.* 2010) where discrete populations occur in close association with calcareous springs (Jiménez-Alfaro *et al.* 2013). The regional climate is transitional between Oceanic and Continental-Mediterranean zones, and the local climatic differences depend mainly on altitude (600-1700 m above sea level) but also on the distance to the sea and the exposure of the mountain range (Rodríguez 1985). The reproductive output in nature is high, usually two-four fruits per individual with c. 140 seeds each, although the number of fruits can reach up to 15 (Jiménez-Alfaro *et al.* 2005). The proportion of viable seeds is usually over 95 % (Fernández-Pascual *et al.* 2012). The species is assumed to be a facultative outcrosser, as is common in the genus (Rich 2005; Brys and Jacquemyn 2011). Flowering begins in early July, proceeds during summer, and ripe seeds are dispersed in September and October (Jiménez-Alfaro *et al.* 2010).



**Figure 5.1** Map of the study area showing the 13 studied population sites. The open circles indicate the seven populations that were also included in the common garden and F<sub>2</sub> germination experiments.

In September-October 2009, we visited the 16 known populations of *C. somedanum* and took a census by direct counting of all the reproductive adults (i.e., individuals with flowers or fruits) living there in that moment (the initial parent generation, P) (Table 5.1). We collected leaf tissue for genetic analysis from these P individuals, sampling a number of adults proportional to the local population size and occupation area (Table 5.1), and evenly distributed across each site. Immediately after collection, we dried the leaves over silica gel in sealed containers and kept them there until further use. From 13 populations covering the entire distribution area of the species (Figure 5.1), we collected seeds (the first seed generation, F<sub>1</sub>) sampling all P individuals with ripe fruits. The seed collections spent a three week period at moderate humidity (c. 22 °C, 50 % RH) in our laboratory to ensure a homogeneous maturation state before being cleaned and used in the germination experiments.

**Table 5.1** Description of the study sites. JBA is the common garden. Alt = altitude (m above sea level); T = mean annual temperature (°C); P = summer precipitation (mm); N<sub>t</sub> = population size (reproductive individuals); N<sub>s</sub> = sample size for the genetic analysis; G = position of the populations in the first PCoA axis obtained from the ISSR matrix.

Site	Coordinates (N, W)	Alt	T	P	N <sub>t</sub>	N <sub>s</sub>	G
AG	43°06'35" 06°15'22"	830	10.1	154	12	12	0.75
AR	43°05'13" 06°06'43"	1470	7.0	214	108	32	-1.20
BR	43°01'28" 06°12'36"	1540	6.5	139	46	16	0.66
CM	43°07'05" 06°15'05"	600	11.1	143	233	42	0.62
CU	43°10'53" 06°11'59"	1350	7.5	203	11	10	0.02
FM	43°07'11" 06°15'08"	710	10.7	148	38	18	0.73
FU	43°06'19" 06°16'26"	870	9.9	158	65	16	1.37
MA	43°00'35" 06°06'30"	1670	5.7	147	62	15	-2.99
ML	43°02'36" 06°09'21"	1590	6.5	231	142	34	0.66
PU	43°00'37" 06°13'29"	1420	7.2	130	163	21	0.38
TE	42°59'40" 06°06'31"	1580	6.3	140	99	33	-1.84
TR	43°00'33" 06°07'11"	1630	6.0	143	48	10	-1.99
VA	43°04'19" 06°11'49"	1280	7.9	193	283	44	0.83
JBA	43°31'15" 05°36'49"	25	14.0	175	-	-	-

### ***Germination and common garden experiments***

We sowed seeds from all F<sub>1</sub> populations on 1 % distilled water agar held in 6-cm-diameter Petri dishes (eight dishes with 25 seeds each per population), sealed with Parafilm to prevent desiccation. Half of the dishes (the fresh seeds) went immediately into the incubation conditions for germination to check dormancy at dispersal; the other half (the stratified seeds) were exposed to wet-cold stratification (12 weeks, 1 % agar, 3 °C, darkness) before being placed in the germination conditions, to assess their receptiveness to dormancy-breaking factors. The germination incubations were conducted in a growth chamber (Grow-S 360, Ing. Climas, Barcelona, Spain) programmed with a 12h-light/12h-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 thermoperiod. These conditions promote *C. somedanum* germination once dormancy is broken (Fernández-Pascual *et al.* 2012; Fernández-Pascual *et al.* 2013). Apart from the population-level germination, we analysed the germination of seeds produced by the same individual plant in four F<sub>1</sub> populations (CM, BR, PU and VA; three individuals per population), using the same germination protocol. During the incubations, we counted and discarded germinated seeds three times a week. Radicle protrusion was the criterion for germination. We terminated the experiments after four weeks and opened the non germinated seeds with a scalpel, classifying them as normal, empty or fungus infected. We excluded the empty (1.3 %  $\pm$  0.3) and infected (1.0 %  $\pm$  0.2) seeds from the statistical analyses and the calculation of germination percentages.

At the end of the F<sub>1</sub> germination experiment we chose a reduced sample of seven populations (covering the entire distribution area, Figure 5.1) and grew their seedlings in a common garden experiment to produce a second seed generation (the F<sub>2</sub>). To prevent an artificial selection of less dormant genotypes, we left the F<sub>1</sub> stratified seeds for ten additional weeks in the germination incubations, until at least 80 % of the seeds had germinated. We planted the seedlings in 5 cm<sup>3</sup> plastic pots containing standard growth medium (Sustrato Universal, Pons Agropecuaria S.L., Madrid, Spain), and kept them saturated in water in a greenhouse at c. 20 °C during their first summer. In September 2010 we randomly selected 20 plants per population, transplanted them to 10 cm<sup>3</sup> plastic pots with the same medium and carried them to an open air garden (Jardín Botánico Atlántico, Xixón, Spain) to meet the vernalization requirement for flowering. This garden is 70 km away from the original populations, at sea level, and its climate is

warmer (Table 5.1), making more evident any possible seed maturation environment effects on dormancy. We placed the pots in floating trays (one tray per population) inside a pool protected from the wind and constantly inundated by rain water to represent the original spring habitat. Periodically we changed the position of the trays in a random way. Shortly before flowering (May 2011) we covered each tray with a white nylon mesh to prevent interpopulation pollination. In July 2011, we collected the ripe  $F_2$  fruits and used them in a new population-level germination experiment with the same conditions as for the  $F_1$ .

We analysed the patterns of dormancy variation in the seven populations represented in the common garden by (1) fitting a linear regression between the  $F_1$  and  $F_2$  germination percentages per population; and (2) fitting a factorial Generalized Linear Mixed Model (GLMM, logit link function, binomial distribution) to the germination data with stratification (yes/no) and seed generation as fixed predictors, and population as a random factor. We performed these tests with the SPSS Statistics 20 software (IBM, Armonk, USA).

### ***Genetic analysis***

For DNA extraction we used the CTAB 2X method as modified by Caujapé-Castells *et al.* (2011). We quantified the extracted DNA concentration using a Biophotometer (Eppendorf, Hamburg, Germany) and deposited aliquots of the DNA extracts in the DNA Bank of the Jardín Botánico Canario "Viera y Clavijo" - Unidad Asociada CSIC, Cabildo de Gran Canaria. Afterwards we conducted an Inter-Simple Sequence Repeat PCR (ISSR) using eight different 3'-anchored universal primers described in Meimberg *et al.* (2006) (Table 5.2), including two primers instead of one in the amplification reactions to increase the number of PCR fragments. Each 25  $\mu$ l reaction volume contained 2.5  $\mu$ L 10x PCR buffer (100 mM Tris-HCl, 15 mM MgCl<sub>2</sub>, 500 mM KCl), 1  $\mu$ L BSA (20 mg/mL), 0.5  $\mu$ L dNTPs (10 mM each), 0.5  $\mu$ L of each primer (10  $\mu$ M), 0.3  $\mu$ L Roche Taq DNA polymerase and 1  $\mu$ L of DNA (20 ng). The amplification reactions were carried out using a C1000 Thermal Cycler (Bio-Rad, Hercules, USA) with the following PCR profile: (1) 94 °C for 2 min; (2) 35 cycles at 94 °C for 1 min, specific annealing temperature for each primer (Table 5.2) for 1 min, 72 °C for 1 min; and (3) final extension at 72 °C for 5 min. Afterwards we electrophoresed 5  $\mu$ l of the PCR products on 1.8 % agarose gels stained with SyBr Safe DNA Gel Stain (Life

Technologies, New York, USA) during 1.5 h at 100 V in 1X TBE buffer. We visualized and photographed the gels using an AlphaImager EP imaging system (Cell BioSciences Inc., Santa Clara, USA). The 100 bp ladder H3 RTU (Nippon Genetics Europe GmbH, Düren, Germany) provided DNA fragment size verification. Amplification fragments on the gels had a variable length between 150 and 1700 bp. We manually constructed a binary 0/1 matrix based on the absence/presence of the DNA bands; the final matrix included 54 diallelic loci and 324 individuals.

**Table 5.2** ISSR primers used in this study. Two primers were included in one PCR, the annealing temperature (T) is shown for this reaction.

Primer	Sequence 5'-3'	T (°C)
1 I-GA8C	GAGAGAGAGAGAGAGAC	53
I-CA9G	CACACACACACACACACAG	
2 I-AC9G	ACACACACACACACACACG	55
I-AC9C	ACACACACACACACACACC	
3 I-ACG5G	ACGACGACGACGACGG	57
I-ACG5C	ACGACGACGACGACGC	
4 I-TCG5G	TCGTCGTCGTCGTCGG	55
I-TCG5C	TCGTCGTCGTCGTCGC	

We performed a Principal Coordinate Analysis (PCoA) of this binary matrix, based on the Euclidean Distance (as implemented in PAST, Hammer *et al.* 2001), to represent the genetic relationships among populations and individuals (hereafter referred to as genetic composition). This technique reduces the complex multidimensional variability of a given matrix in two or more dimensions or ordination axes (Leps and Smilauer 2003). Because of their capability of representing genetic differentiation, ordination axis obtained from a neutral loci matrix can be used to assess correlations between genetic composition and other variables (Lee and Mitchell-Olds 2011; Treier and Müller-Schärer 2011). Here, we used the population and individual values from the first PCoA axis (Table 5.1) to test possible correlations between seed dormancy and genetic composition. We fitted binary logistic regressions between the germination results (separately per generation and fresh/stratified seeds) and the mean value of the P populations in the first PCoA axis. When germination data per individual plant were available, we repeated the binary logistic regression using the individual values in the genetic axis, and including the population of origin as a random factor. We performed these tests with SPSS Statistics 20.

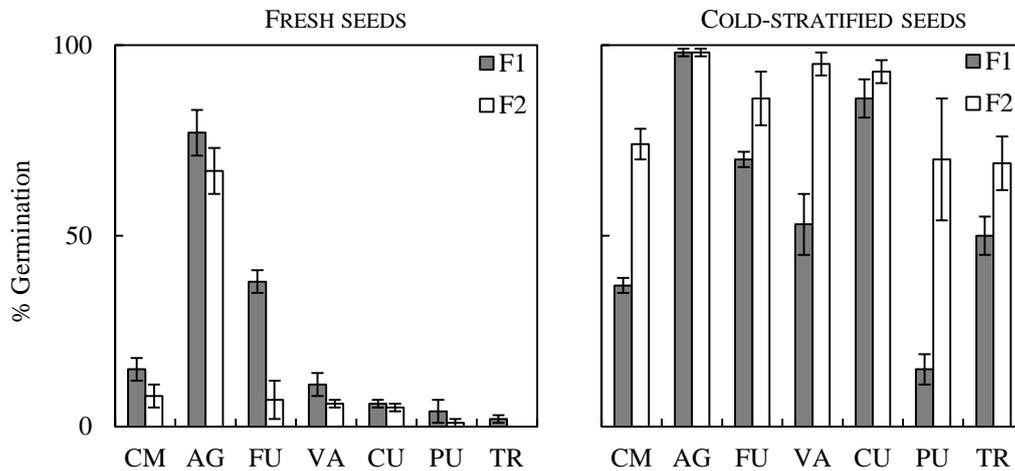
### *Climatic models*

To determine if dormancy variation could be predicted from the local environment of the original wild populations, we fitted main effects GLMs to the germination results (separately per generation and fresh/stratified seeds), using the local values of annual temperature and summer precipitation as predictors. Data from neighbouring climate stations were extrapolated to obtain the local values at each site (Table 5.1; Sánchez Palomares *et al.* 1999). These variables reflected long term differences among sites, as they were obtained averaging 50 years of measures. We also included population size (reproductive adults) in the models to control demographic and sample size effects in germinability. The populations were not spatially autocorrelated for any of the three variables, either considering all the populations or only the ones in the common garden; the same was true for the model residuals (in all cases  $p > 0.05$ , Moran's I test computed using ArcGis v9.3, Esri, Redlands, USA). The three explanatory variables were not significantly correlated according to the Pearson test ( $p > 0.05$ ) and their variance inflation factor was  $< 2$ . We fitted the models using a forward/backwards stepwise variable selection procedure with Akaike's information criterion (AIC) as implemented in the R-Commander package (v1.5-3) for R (v2.10.1, The R Foundation for Statistical Computing).

## RESULTS

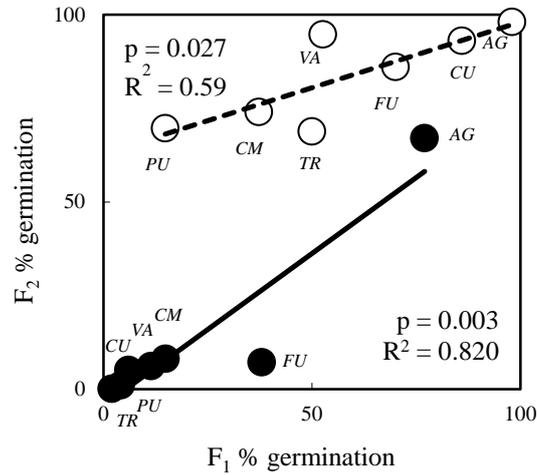
### *Seed dormancy patterns*

When analysing the results for the seven populations represented in the common garden, we found the same pattern of dormancy variation in both seed generations, with seeds from higher altitude populations showing higher dormancy (Figure 5.2). The patterns of the two generations were significantly correlated (Figure 5.3), i.e. the populations that were more dormant in the  $F_1$  were also the more dormant ones in the  $F_2$ . The GLMM detected nevertheless significant differences between the two seed generations ( $F = 6.512$ ,  $p = 0.012$ ), as on average the  $F_2$  was 9 percentage points (pp) more dormant than the  $F_1$  in fresh, but 26 pp less dormant after stratification.



**Figure 5.2** Population germination (mean  $\pm$  SE of four dishes) after four weeks of incubation at 22/12 °C of fresh (left panel) and stratified (right panel) seeds from the two seed generations ( $F_1$  = grey bars;  $F_2$  = white). The populations are placed from lowest (left) to highest (right) altitude of origin.

Stratification produced a significant dormancy release in both generations ( $F = 654.541$ ,  $p < 0.001$ ), but not enough to break dormancy completely in most populations (Figure 5.2). The dormancy-breaking effect of the stratification was significantly higher in the  $F_2$  (70 pp germination increase vs. 37 pp in the  $F_1$ ), as shown by the significant interaction between seed generation and stratification ( $F = 107.348$ ,  $p < 0.001$ ).

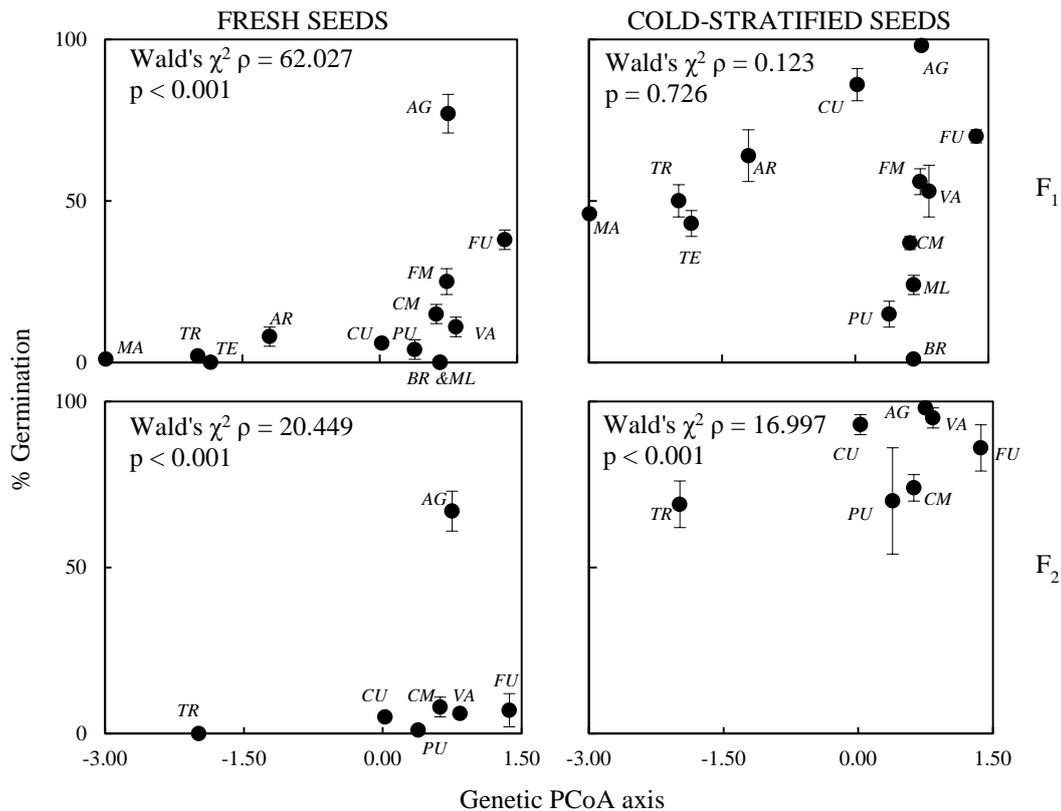


**Figure 5.3** Scatter plot and fitted regression lines between  $F_1$  and  $F_2$  seed germination after four weeks of incubation at 22/12 °C. The circles represent the % germination of a population (mean of four dishes; black circles = fresh seeds; open circles = stratified seeds). Population codes are indicated next to each symbol. The results of the linear regression are given.

**Correlation with the genetic composition**

The population-level dormancy patterns of the seeds were significantly correlated to the genetic composition of the wild populations of origin, except in the case of the stratified F<sub>1</sub> seeds (Figure 5.4). Two population groups could be identified in the scatter plots, especially within F<sub>1</sub> plants: (1) the four south-eastern populations (AR, MA, TE and TR), more genetically divergent from the rest, displayed high dormancy in fresh and a parallel decrease after stratification; and (2) the rest of the populations, which formed a genetically closer group, but showed a higher variation in dormancy, both in fresh and after stratification.

When analyses at the individual level were performed with the F<sub>1</sub> seeds, the genetic composition of the mother plants was also significantly correlated with the dormancy of the seeds they produced, even after ruling out the differences due to the population of collection, both in fresh ( $F = 3.847$ ;  $p < 0.001$ ) and stratified ( $F = 2.990$ ;  $p = 0.004$ ) seeds.



**Figure 5.4** Scatter plots of population germination (mean  $\pm$  SE of four dishes) versus population genetic ordination, represented by the value of the first PCoA axis calculated from the ISSR matrix, in fresh and stratified seeds of the F<sub>1</sub> (13 populations) and F<sub>2</sub> (seven populations) seed generations. Each circle represents a population with its code next to it. The results of the binary logistic regressions are given.

***Effect of the local climate***

The climatic variables significantly explained the dormancy patterns of fresh and stratified seeds from both generations (Table 5.3). The selection procedure included all the variables in the final GLMs, except for summer precipitation in the Fresh-F<sub>2</sub> model, although population size in the Stratified-F<sub>2</sub> model was not significant at the 5 % level ( $p = 0.053$ ). The models explained a greater portion of the variance and were more informative in the fresh seeds of both generations, where annual temperature and population size were the main variables. In the Stratified-F<sub>1</sub> model, all variables shared a similar percentage of explained variance. The Stratified-F<sub>2</sub> model was the less informative and in this case summer precipitation became the main explanatory variable. In all cases, annual temperature and summer precipitation had a negative effect on dormancy, while population size had a positive one.

**Table 5.3** Explanatory models fitted to the germination data by means of binary logistic regression. The effects appear in the order they were included by the variable selection procedure (forwards/backwards, AIC). T = annual temperature; P = summer precipitation; N = population size (reproductive adults); %V = percentage of the variance explained by the model after the inclusion of the effect.

Model	Effect	Coefficient	S.E.	$\chi^2$	p	%V
<b>Fresh F<sub>1</sub></b> 13 populations AIC=789.05	Intercept	-9.74E+00	1.33E+00	53.509	0.000	
	T	7.99E-01	7.41E-02	116.381	0.000	17.22
	N	-7.87E-03	1.17E-03	45.603	0.000	21.41
	P	1.04E-02	4.78E-03	4.748	0.029	21.81
<b>Fresh F<sub>2</sub></b> 7 populations AIC=403.11	Intercept	-9.30E+00	1.17E+00	63.250	0.000	
	T	9.32E-01	1.24E-01	56.475	0.000	9.82
	N	-1.38E-02	1.81E-03	57.988	0.000	22.17
<b>Stratified F<sub>1</sub></b> 13 populations AIC=1584.2	Intercept	-4.05E+00	4.43E-01	83.686	0.000	
	T	3.12E-01	3.43E-02	83.211	0.000	4.59
	N	-6.79E-03	7.89E-04	74.149	0.000	9.30
	P	1.38E-02	1.91E-03	51.811	0.000	13.56
<b>Stratified F<sub>2</sub></b> 7 populations AIC=579.22	Intercept	-3.99E+00	8.72E-01	20.986	0.000	
	P	2.96E-02	5.21E-03	32.228	0.000	6.18
	T	1.56E-01	5.91E-02	6.948	0.008	6.90
	N	-2.35E-03	1.21E-03	3.733	0.053	7.43

## DISCUSSION

Seed dormancy in *C. somedanum* exhibits a cline along a local gradient of altitude and climate, and this cline is related both to genetic composition and the seed maturation environment. Although the general field cline is maintained when the plants and the seeds mature in a common garden, we found certain differences that can be explained by the maturation environment. Being at sea level, the common garden is 6 °C warmer than the original sites as well as frost free for almost all the year, so the plants and seeds would perceive an extraordinarily warm year. The populations of *C. somedanum* show a fixed ‘baseline’ level of dormancy at dispersal, as revealed by the high levels of dormancy found in the common garden fresh seeds, even higher than in the natural populations. However, the same populations react to the environment during seed maturation and their dormancy release after stratification is more pronounced in the common garden, i.e. they are more receptive to dormancy-breaking factors. This behaviour agrees well with what was found by Chiang *et al.* (2011) in *A. thaliana*, where both the population of origin and the temperature during seed maturation influenced the levels of dormancy and the expression of a gene which controls dormancy variation in nature.

The fact that field and common garden seeds exhibited similar patterns of variation in seed dormancy suggests a genetic basis accounting for dormancy in this set of populations. Such possibility is further supported by the correlation between the patterns of variation in the phenotypic trait of interest (seed dormancy), and the pattern of underlying neutral genetic variation shown by the first PCoA axis. However, since our genetic analysis characterized neutral variation, and not variation in adaptive dormancy-related genes, support for a genetic basis of seed dormancy must be considered indirect. The lack of correlation precisely in the F<sub>1</sub> stratified seeds gives supplementary evidence of the influence of the seed maturation environment on the receptiveness to dormancy-breaking factors. While in the stratified seeds of the F<sub>1</sub> the different field maturation environments produced the greatest departures from the fixed dormancy cline, in the F<sub>2</sub> the common environment made the cline more evident. A similar correlation between genetic variation and dormancy-related seed traits along a regional climatic gradient has been found in the widespread species *A. thaliana* (Montesinos-Navarro *et al.* 2012) and *Beta vulgaris* L. (Wagmann *et al.* 2012), and our study suggests that the same adaptive processes operate at reduced geographical scales.

Nevertheless, non-genetic persistent parental effects (Rossiter 1996) coming from the F<sub>1</sub> seed maturation environments in the field cannot be entirely excluded. Alexander and Wulff (1985) reported such behaviour in *Plantago lanceolata* L., where high temperatures during the F<sub>1</sub> seed maturation produced less dormant F<sub>1</sub> seeds but more dormant F<sub>2</sub> seeds, although their study was inconclusive as this only occurred in one of the genetic lines they studied. In any case, it is difficult to see how the possible F<sub>1</sub> parental effects could be so strongly expressed above the homogenising F<sub>2</sub> seed maturation environment without an underlying genetic basis for the cline.

Future work analysing the genetic regulatory pathways for seed dormancy in this species is nonetheless needed to confirm this genetic basis. To this end, new generations must be produced in controlled common garden conditions, in order to minimize undesirable environmental variability and better quantify the effects of the seed maturation environment and the genetic lines. In this work, our goal was to capture phenotypic field variation in conditions close to the natural ones, and as a consequence environmental influences were considerable. Even so, our results generally agree with those obtained in more controlled experimental settings (Chiang *et al.* 2011; Kronholm *et al.* 2012).

The story behind dormancy and genetic variation, however, seems to be more complicated than a linear relationship. Fixed local adaptation is expected when the environmental variation is at a greater spatial scale than gene flow, while the opposite situation would favour plasticity through parental environment effects (Galloway 2005). It is possible that in *C. somedanum* local adaptation explains dormancy differences among genetically isolated groups while the seed maturation environment plays a main role within those groups. The four easternmost populations (AR, MA, TE and TR) are genetically distant from the rest and in the case of MA, TE and TR live at the highest altitudes and at the more continental sites, secluded from the majority of the populations by the highest peaks of the area. The germination of these four populations shows similarities, with total or nearly total dormancy at dispersal and a similar receptiveness to dormancy-breaking factors. Meanwhile, the rest of the populations form a genetically homogeneous group and exhibit the altitudinal cline in dormancy at dispersal, but their receptiveness to dormancy-breaking factors varies from a null response in BR to a large germination increase in CU.

Another departure from a linear relationship is the extraordinary low dormancy in AG, which is also one of the smallest populations with 12 reproductive adults, and may be the consequence of a recent colonization of AG by low dormant genotypes. Indeed, the AG population is largely responsible for the important effect of population size in the predictive models, where bigger population sizes correlate to lower seed germination. This relationship is contrary to what could be expected if the demographic effect was related to a reduction of germinability produced by reduced fitness and higher inbreeding depression in the smaller populations (Heschel and Paige 1995). If the AG data are removed, the population size effect is much reduced in the F<sub>1</sub> models and completely disappears from the F<sub>2</sub> models.

Regardless of population size, the dormancy cline found in *C. somedanum* is related to the local variations in climate. Correlations between higher altitude sites and higher dormancy levels have usually been reported at broader geographical scales (Vickery 1983; Beardsell and Mullet 1984; Holm 1994; Cavieres and Arroyo 2000). In our case, the sites with lower annual temperatures and summer precipitation produce seeds with higher dormancy levels. The effect of temperature is in agreement with the results reported by Fenner (1991) and Fenner and Thompson (2005) in their reviews of seed maturation under controlled conditions experiments, as well as the behaviour found in the field by Meyer and Monsen (1991). In the case of *C. somedanum*, the plants growing at lower altitudes, under a generally milder climate, produce seeds that will germinate earlier, benefiting from a longer growing season. Plants from higher altitudes, where winters are harsher, produce seeds which will not germinate until the unfavourable season is over. This work cannot definitely determine that local adaptation is behind the cline, as that would require reciprocal transplant experiments and fitness measures. Nonetheless, a correlation between an environmental factor and a cline in a trait hypothetically related to fitness in response to that factor may be regarded as a clue of such adaptation (Montesinos-Navarro *et al.* 2011).

The ecological meaning of the summer precipitation effect, on the other hand, is less evident, especially due to its secondary role in comparison to temperature. Most experiments on seed maturation under controlled conditions actually reported a positive correlation between water availability and dormancy (Allen and Meyer 2002; Hoyle *et al.* 2008). Studies performed with the weed *Sinapis arvensis* L. hypothesized that adequate soil moisture during seed maturation results in more dormant seeds because

better developed seeds are produced (Wright *et al.* 1999; Luzuriaga *et al.* 2006), but in the spring habitats occupied by *C. somedanum* water availability should not be a limiting factor. However, precipitation may affect the plant through air humidity or the alteration of the water regime and the courses of the springs to which *C. somedanum* germination is especially adapted (Fernández-Pascual *et al.* 2012).

In conclusion, our study shows that seed dormancy has the capacity to adapt, even at reduced local scales, to long term climatic differences through inheritable clinal variation; but also to adjust itself to the weather during seed maturation especially through the receptiveness to dormancy-breaking factors. The processes behind phenotypical plasticity in dormancy which have been detected at broad scales and in widely distributed species (Chiang *et al.* 2011; Montesinos-Navarro *et al.* 2012; Wagmann *et al.* 2012) seem to operate as well at local scales and in narrowly distributed taxa. Therefore, the risk of plants not being able to match their dormancy-breaking requirements as a consequence of the global increase in temperature (Ooi *et al.* 2009; Orrù *et al.* 2012) is lessened. The very presence of intraspecific variation on such a reduced scale highlights the great potential of physiological dormancy to adapt to fast environmental changes in time and space.

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# CHAPTER 6

## FINAL DISCUSSION AND CONCLUSIONS





**GERMINATION ECOLOGY OF MOUNTAIN SPRINGS AND FENS*****Germination patterns at the habitat level***

We have described two general germination strategies, warm and cool, that characterize the germination niche of calcareous spring fen species. Although exceptions do exist, our results indicate that the warm strategy is followed by species such as *Carex davalliana* Sm., *Eriophorum latifolium* Hoppe, *Parnassia palustris* L., *Primula farinosa* L. and *Triglochin palustris* L., i.e. those typically associated with fens (Jiménez-Alfaro *et al.* 2011; Jiménez-Alfaro *et al.* 2013b). The cool strategy characterizes species like *Centaureum somedanum* M. Laínz and *Pinguicula grandiflora* Lam., characteristic of the spring setting (Jiménez-Alfaro *et al.* 2011; Jiménez-Alfaro *et al.* 2013a).

The main difference between strategies is the response to heat shown by warm germinators. This response is a pervasive trait of species from seasonal wetlands (Baskin and Baskin 1998) and especially those of the genus *Carex* L. (Schütz 2000), although it may be less common in Mediterranean wetlands (Carta *et al.* 2013). It is usually interpreted as a mechanism to detect the fall of the water table at the beginning of the favourable season (Grime *et al.* 1981; Thompson and Grime 1983; Baskin and Baskin 1998), but this interpretation does not seem appropriate in groundwater-fed wetlands without a seasonal water regime. We have proposed that in fens it may work as a gap-detecting mechanism, overriding dormancy during episodes of soil drying when a high mortality of the standing vegetation favours higher recruitment from the soil seed bank. Since our work was limited to laboratory germination tests, future field emergence experiments will be necessary to test this interpretation and to assess how the two described strategies behave in natural conditions.

The two germination strategies may respond differently to climate change. In general terms, global warming is expected to affect the rate of dormancy break in the soil seed bank, resulting in emergence at the wrong time (Ooi *et al.* 2009; Ooi 2012) or compromising dormancy release (Orrù *et al.* 2012). Our study shows that many calcareous spring fen species have a facultative rather than obligate requirement for cold during dormancy break, and therefore their dormancy release should not be completely stopped. However, changes in the rate of the process are to be expected, especially in those species with the heat response. Further changes may occur in the germination rate,

which could respectively decrease and increase in cool and warm germinators. Such alterations in germination-related processes might shift the germination timing (Mondoni *et al.* 2012), change plant recruitment (Walck *et al.* 2011) and, ultimately, modify the performance of plant populations. These potential effects must be evaluated experimenting with field emergence under manipulated conditions.

Nevertheless, our results highlight the potential of germination traits to respond to environmental change. The breadth of the germination niche seems capable of adapting to the particular climate of different mountain regions. Furthermore, seed dormancy responds to climatic variability at a much reduced geographical scale, both in the long and the short term. Climate warming may therefore produce a shift in the germination niche and dormancy conditions of seeds, attuning their germination to the new climatic scenario. An interesting approach to address this topic will be the development of quantitative seed dormancy models (García-Huidobro *et al.* 1982; Steadman and Pritchard 2004; Orrù *et al.* 2012) that consider the role of temperature not only in breaking dormancy and triggering germination, but also in modulating the temperature requirements for dormancy break and germination. The study system we have set up in fens of the Cantabrian and Western Carpathian Mountains, with *in situ* measured soil temperatures and a known germination strategy, shows promise for the development of these models.

### ***Germination of C. somedanum***

We have studied in detail the germination of the calcareous spring indicator *C. somedanum*, a species of top conservation concern in the Cantabrian Mountains (Jiménez-Alfaro *et al.* 2010) that shows a relatively unique ecology. One of the few vascular plants that is a strict specialist of tufa-forming springs, its distribution is linked to these habitats independently of altitude and the dominance of vascular plants or bryophytes at the micro-scale (Jiménez-Alfaro *et al.* 2013a). Our study suggests that its germination is especially adapted to this condition, as it is elicited by the constant and relatively cold temperatures that prevail in the spring setting.

In addition to its conservation value and ecological interest, *C. somedanum* has proved to be a valuable study system to investigate germination-related processes in rare and endemic taxa. Studying the comparative germination ecology of the genus *Centaureium* Hill might shed light into the evolution of germination traits in novel

habitats and into the proposed allopolyploid origin of *C. somedanum* from the Iberian endemic *C. gypsicola* and the widespread *C. scilloides* (Mansion *et al.* 2005; Fernández Prieto *et al.* 2012). Similarly, the established common garden populations could be of great value to address topical questions in seed biology, such as the molecular basis of dormancy variation (Chiang *et al.* 2011; Kronholm *et al.* 2012). Additional work with these populations is needed to fully confirm, with reciprocal transplant experiments, that the detected dormancy cline corresponds to local adaptation. Seed maturation experiments in more controlled environments and with purer genetic lines could also contribute to quantify the effects that maturation temperature has on the subsequent traits of seeds.

### CONSERVATION REMARKS

During this work we visited 40 calcareous spring fens and related mires, located both in Spain and Slovakia (Supplementary table 1). The main goal of these field trips was collecting seeds for the laboratory experiments and for their *ex situ* conservation in the seed bank of the Jardín Botánico Atlántico (Xixón, Spain). The final count reached 119 collections from 19 species (Supplementary table 2). The Cantabrian priority taxon *C. somedanum* is especially well represented, with samples from all the populations known at that moment and repeated collections in several years. Further details about the seed collections are available for authorized users in the database of the Jardín Botánico Atlántico (<http://www.aimjb.net/babilonia/>).

The germination experiments resulted in protocols for the optimal germination of the studied species (Supplementary table 3). This information will be necessary for the future use of the stored collections in plant production and reintroduction. We also checked the viability of the seeds after the conservation process, which involved drying to c. 5 % moisture content and freezing at -13 °C (Supplementary table 3). In *E. latifolium*, *Juncus alpino-articulatus* Chaix, *P. palustris* and *Pedicularis mixta* Gren. we found that this process significantly decreased seed viability. Future work must confirm this effect and develop more adequate conservation protocols.

Additionally, DNA samples of 326 *C. somedanum* individuals from 16 populations were extracted (Supplementary table 4) and are now conserved in the DNA bank of the Jardín Botánico Canario “Viera y Clavijo” (Las Palmas de Gran Canaria, Spain). The ISSR survey showed two clear genetic groups, differentiating the Torre de Babia populations from the rest. These two groups must be taken into account when planning any conservation action on this species. Future analysis of the ISSR data will produce a detailed picture of *C. somedanum* conservation genetics. In any case, from our results it can be concluded that the wild populations of *C. somedanum* show an adequate reproductive fitness, with high seed production and viability.

## CONCLUSIONS

1. All the studied calcareous spring fen species show total or conditional seed dormancy at dispersal. In most cases, dormancy break does not obligatorily depend on cold overwintering.
2. The majority of fen species follow a warm germination strategy characterized by a dual germinative path. Short term responses to heat quickly override seed dormancy and produce immediate germination. In the absence of heat events, a long term path follows through dormancy break during overwintering and subsequent germination at mild to cool temperatures in spring or summer.
3. Spring species follow an alternative cool germination strategy. Lacking the short term response to heat, they germinate only at low temperatures.
4. Fens show a soil temperature buffer effect produced by soil water. This effect, which is especially strong during the night and in winter, reduces the variation in soil temperature among altitudes and mountain regions.
5. The breadth of the germination niche in fen species differs among mountain regions, but not at more reduced scales. This variation is correlated to differences in the minimum winter temperatures.
6. The germination of the rare spring specialist *Centaurium somedanum* shows divergences from the general germination patterns of its genus including cool germination and morphophysiological dormancy.
7. Seed dormancy in *Centaurium somedanum* shows a cline along a local climatic gradient. Through this inheritable cline, dormancy levels are adapted to long term climatic differences. Dormancy also responds in the short term to the weather during seed maturation, especially through the receptiveness to dormancy-breaking factors.

## CONCLUSIONES EN CASTELLANO

1. Todas las especies de tremedales de fuente calcáreos estudiadas presentan dormición total o condicional en el momento de la dispersión. En la mayoría de los casos, la rotura de la dormición no depende del frío durante en invierno.
2. La mayoría de especies de tremedales siguen una estrategia de germinación cálida caracterizada por una doble ruta germinativa. Respuestas a corto plazo al calor pueden saltarse rápidamente la dormición y producir una germinación inmediata. Si no tienen lugar episodios de calor, las semillas siguen una ruta a largo plazo que pasa por la rotura de la dormición durante el invierno y la posterior germinación a temperaturas de moderadas a frías en primavera o verano.
3. Las especies de fuentes siguen una estrategia alternativa de germinación fría. Al carecer de la respuesta a corto plazo al calor, germinan únicamente a temperaturas bajas.
4. Los tremedales muestran un efecto tampón en la temperatura de sus suelos producido por el agua del suelo. Este efecto, que es especialmente fuerte durante la noche y en invierno, reduce las diferencias en la temperatura del suelo entre altitudes y regiones montañosas.
5. La amplitud del nicho de germinación de especies de tremedales difiere entre regiones montañosas, pero no a escalas más reducidas. Esta variación está relacionada con diferencias en las temperaturas mínimas del invierno.
6. La germinación de la rara especialista de fuentes *Centaurium somedanum* muestra divergencias respecto a los patrones generales de germinación en *Centaurium*, incluyendo germinación a temperaturas frías y dormición morfofisiológica.
7. La dormición en *Centaurium somedanum* muestra una clina a lo largo de un gradiente climático local. A través de esta clina, los niveles de dormición se adaptan a diferencias climáticas a largo plazo. La dormición también responde en el corto plazo al ambiente durante la maduración de las semillas, especialmente a través de la sensibilidad a los factores de rotura de la dormición.

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## SUPPLEMENTARY MATERIALS



**Supplementary table 1** Visited sites. The water pH is the average of ten measures taken with a MM40 Portable Multimeter (Crison Instruments S.A., Alella, Spain). When a soil temperature datalogger was installed, the datalogger code is indicated.

Site	Toponym	Municipality	Province	Country	UTM ED50	Altitude (m asl)	pH	Datalogger
AA	Arroyo Anzo	Posada de Valdeón	León	Spain	30T 342700 4775800	1600	6.6	-
AG	Aguinu	Somiedo	Asturias	Spain	29T 722600 4776700	830	8.1	-
AR	Foz de los Arroxos	Somiedo	Asturias	Spain	29T 734900 4774400	1470	8.3	-
BÑ	La Brañueca	Posada de Valdeón	León	Spain	30T 339000 4776200	1440	7.6	-
BO	Bobias	Somiedo	Asturias	Spain	29T 727500 4774200	1060	8.4	-
BR	La Bruxa	Cabrillanes	León	Spain	29T 727300 4767400	1530	8.0	A511BC
CA	Caunéu	Somiedo	Asturias	Spain	29T 724100 4771900	1080	7.9	-
CI	Veiga Cimera	Somiedo	Asturias	Spain	29T 723800 4767400	1570	4.6	A5023A
CM	La Malva (Carretera)	Somiedo	Asturias	Spain	29T 723600 4777600	600	8.3	-
CU	Cueiru	Belmonte de Miranda	Asturias	Spain	29T 727600 4785200	1350	7.1	-
CZ	La Carbaza	Caso	Asturias	Spain	30T 308600 4775400	1590	4.3	-
ER	La Ercina	Cangas de Onís	Asturias	Spain	30T 339300 4792900	1110	7.2	-
FM	La Malva (Área Forestal)	Somiedo	Asturias	Spain	29T 723500 4777800	710	8.2	A511AE
FU	Furáu	Somiedo	Asturias	Spain	29T 721700 4775700	870	8.1	-
GU	Gurugú	Somiedo	Asturias	Spain	29T 723300 4776600	730	8.5	-
KUB	Kubínska Ho'la	Dolný Kubín	Žilina	Slovakia	34T 375200 5460400	1310	-	A5023E
LC	Los Cándanos	Llanes	Asturias	Spain	30T 365900 4805100	220	3.0	-
LI	Liordes	Posada de Valdeón	León	Spain	30T 350200 4779500	1870	7.3	-
LIP	Liptovská Lúžna	Liptovská Lúžna	Žilina	Slovakia	34T 377200 5422200	890	-	A5023C
LR	La Recoleta	Cabrillanes	León	Spain	29T 735400 4766900	1780	7.2	-
LV	Las Verdes	Cabrillanes	León	Spain	29T 733300 4765100	1720	7.0	-
MA	Malconcecho	Cabrillanes	León	Spain	29T 735600 4766000	1670	8.5	-
ML	Murias Llongas	Somiedo	Asturias	Spain	29T 731700 4770000	1590	7.7	-
NI	Nisaléu	Somiedo	Asturias	Spain	29T 724100 4768800	1460	8.4	-
NY	Marimanya	Alt Àneu	Lleida	Spain	31T 338900 4730600	2380	-	-

(Continues)

Supplementary materials

(Continues)

Site	Toponym	Municipality	Province	Country	UTM ED50	Altitude (m asl)	pH	Datalogger
PI	Los Piélagos	Belmonte de Miranda	Asturias	Spain	29T 727200 4791500	1030	7.2	-
PU	El Puertu	Cabrellanes	León	Spain	29T 726100 4765700	1420	7.8	-
ROJ	Rojkov-Stankovany	Stankovany	Žilina	Slovakia	34T 365800 5445600	440	-	A5023F
RS	Resaláu	Camaleño	Cantabria	Spain	30T 354500 4781500	1630	7.7	-
RT	Riotuertu	San Emiliano	León	Spain	30T 259800 4766300	1860	7.3	A511C9
RX	Requexón	Puebla de Lillo	León	Spain	30T 306500 4769000	1610	7.4	-
TE	Veiga Teixera	Cabrellanes	León	Spain	29T 735500 4765700	1580	8.5	-
TH	Tañadura	Peñamellera Alta	Asturias	Spain	30T 361200 4791800	1200	8.5	-
TI	Tielve	Cabrales	Asturias	Spain	30T 356800 4791000	710	8.6	-
TR	Treméu	Cabrellanes	León	Spain	29T 735100 4766000	1630	8.1	-
VA	El Valle	Somiedo	Asturias	Spain	29T 728400 4772600	1280	8.2	-
VB	Valcabao	Posada de Valdeón	León	Spain	30T 350200 4777900	1540	7.5	-
VN	La Venta	Caso	Asturias	Spain	30T 309000 4775300	1540	4.5	-
VO	Valdeloso	Camaleño	Cantabria	Spain	30T 354600 4773000	1600	8.2	-
VV	Veiga Ventana	Somiedo	Asturias	Spain	29T 725200 4768400	1410	8.2	-

**Supplementary table 2** Seed collections conserved in the seed bank of the Jardín Botánico Atlántico (Xixón, Spain). The collection sites are indicated with the two letters codes used in Supplementary table 1. The accession code can be used to access to further details about the collections in the data base of the Jardín Botánico Atlántico (<http://www.aimjb.net/babilonia/>). The first six digits of the accession code indicate the collection date in the format ddmmyy. G = average percentage of initial germination in the optimal conditions; C = average percentage of seeds contaminated during the germination experiments; E = average percentage of empty seeds.

Species	Site	Accession code	Banking date	Weight (g) banked	G	C	E
<i>Blysmus compressus</i>	AA	250810-01	17/02/2011	1.406	92	10	20
	BR	020910-01	17/02/2011	0.462	94	10	54
	BR	130911-01	13/04/2012	0.553	100	0	73
	FM	020810-01	17/02/2011	0.277	89	15	41
	FM	130711-02	13/04/2012	2.517	100	5	21
	KUB	250811-01	13/04/2012	1.595	83	1	30
	KUB	290810-01	17/02/2011	1.383	82	5	17
	LI	140910-01	17/02/2011	3.022	66	1	5
	LIP	020811-07	25/04/2012	0.242	92	4	61
	LIP	290810-02	17/02/2011	0.515	85	14	42
	ROJ	020811-01	25/04/2012	1.163	91	1	54
	ROJ	290810-03	17/02/2011	0.086	-	-	-
	RT	020910-02	17/02/2011	0.247	-	-	-
<i>Carex davalliana</i>	BR	030810-01	17/02/2011	0.999	96	15	13
	BR	100712-01	04/03/2013	4.802	50	0	11
	BR	130711-06	25/04/2012	0.514	91	2	40
	LIP	020811-08	25/04/2012	1.513	57	4	44
	MA	100811-01	25/04/2012	7.525	-	-	-
	NY	020811-12	25/04/2012	2.708	-	-	-
	PU	100712-02	04/03/2013	0.247	64	0	5
	ROJ	020811-02	25/04/2012	0.085	62	2	63
	RT	040810-01	23/09/2010	0.421	94	4	5
	RT	180711-01	25/04/2012	0.023	89	2	5
<i>Carex diandra</i>	ER	140711-02	25/04/2012	7.232	95	0	0
	ER	270712-01	04/03/2013	5.250	100	0	0
	LR	100811-02	26/04/2012	17.918	83	0	21
	LR	250712-05	04/03/2013	2.214	100	6	3
	LV	160910-01	17/02/2011	0.680	100	3	12
	LV	260809-01	14/12/2009	2.627	97	0	0
<i>Carex echinata</i>	AA	050810-01	17/02/2011	4.706	96	2	3
	BÑ	050810-02	17/02/2011	2.167	97	5	15

(Continues)

(Continues)

Species	Site	Accession code	Banking date	Weight (g) banked	G	C	E
	BÑ	200711-01	26/04/2012	3.085	98	1	18
	KUB	250811-02	26/04/2012	1.843	100	1	8
	LIP	020811-09	26/04/2012	1.277	59	3	64
	PI	180712-20	04/03/2013	6.445	100	21	1
	RT	180711-02	26/04/2012	2.395	100	1	2
<i>Carex lepidocarpa</i>	AA	250810-02	23/02/2011	4.157	-	-	-
	BR	030810-02	23/02/2011	21.626	51	14	37
	BR	130711-05	26/04/2012	0.770	38	2	26
	FM	020810-02	17/02/2011	10.227	69	9	21
	FM	130711-01	26/04/2012	4.933	57	0	20
	LI	060810-01	23/02/2011	0.111	76	1	9
	LIP	020811-10	26/04/2012	11.610	51	0	54
	LIP	290810-04	23/02/2011	4.508	56	3	82
	NI	030810-03	23/02/2011	3.709	-	-	-
	ROJ	020811-03	03/05/2012	6.297	26	0	90
	ROJ	290810-05	23/02/2011	6.922	0	1	98
	RT	040810-02	23/02/2011	4.422	44	2	12
	RT	180711-03	26/04/2012	1.521	12	1	10
	VB	060810-02	23/02/2011	8.601	85	3	25
<i>Carex nigra</i>	ROJ	020811-04	26/04/2012	0.400	-	-	-
<i>Carex pulicaris</i>	RX	090910-01	23/02/2011	1.391	91	0	1
	RX	230911-13	26/04/2012	0.781	99	0	1
<i>Centaurium somedanum</i>	AG	061009-02	30/11/2011	0.005	98	1	0
	AR	061009-01	30/11/2011	0.082	64	0	0
	BO	101009-02	23/11/2009	0.004	96	2	0
	BR	151009-01	23/11/2009	0.036	1	2	1
	CA	101009-01	27/11/2009	0.008	86	0	3
	CM	020910-03	23/02/2011	0.025	20	1	2
	CM	110912-02	04/03/2013	0.020	72	1	3
	CM	131011-01	03/05/2012	0.045	83	2	2
	CM	170908-01	02/03/2009	0.068	98	3	3
	CM	240909-01	27/11/2009	0.132	37	3	6
	CU	081009-01	28/11/2009	0.006	86	0	1
	FM	101009-03	11/11/2009	0.020	56	1	1
	FU	290909-01	25/11/2009	0.025	70	1	1
	MA	021009-02	27/11/2009	0.059	46	3	1
	ML	230909-01	27/11/2009	0.044	24	1	1
	PU	011009-01	19/11/2009	0.102	15	9	1
	PU	170908-03	02/03/2009	0.190	-	-	-
	TE	011009-03	10/11/2009	0.066	43	1	0
	TR	021009-01	18/11/2009	0.024	50	0	2
	VA	020910-04	23/02/2011	0.135	33	3	3
	VA	091009-01	18/11/2009	0.137	51	4	1
	VA	110912-01	04/03/2013	0.067	98	4	11
	VA	131011-02	03/05/2012	0.064	95	2	2
	VA	170908-02	02/03/2009	0.777	95	3	3
	VV	011009-02	19/11/2009	0.001	57	3	1

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Species	Site	Accession code	Banking date	Weight (g) banked	G	C	E
<i>Eleocharis quinqueflora</i>	BR	310811-01	03/05/2012	0.193	0	1	4
<i>Eriophorum angustifolium</i>	CI	100712-03	04/03/2013	0.496	100	9	10
	LC	120712-17	04/03/2013	0.104	100	1	1
	ROJ	020811-05	03/05/2012	0.061	-	-	-
<i>Eriophorum latifolium</i>	AA	050810-03	23/02/2011	0.814	94	8	15
	BR	030810-04	23/02/2011	2.441	99	11	16
	BR	130711-07	03/05/2012	1.155	72	8	7
	KUB	030811-01	03/05/2012	0.640	-	-	-
	KUB	250811-03	03/05/2012	0.297	57	16	6
	KUB	290810-06	23/02/2011	1.672	87	4	6
	LIP	020811-11	03/05/2012	0.547	89	7	5
	LIP	290810-07	23/02/2011	0.375	-	-	-
	NI	030810-05	23/02/2011	0.370	85	3	3
	NI	130711-04	03/05/2012	2.138	99	7	17
	ROJ	020811-06	03/05/2012	0.127	98	18	10
	ROJ	290810-08	23/02/2011	0.338	-	-	-
	ROJ	290810-09	23/02/2011	0.254	96	18	25
	VB	060810-03	23/02/2011	0.759	88	8	4
VO	050810-04	23/02/2011	1.839	-	-	-	
<i>Eriophorum vaginatum</i>	CZ	230712-02	04/03/2013	4.541	99	11	4
	VN	230712-06	04/03/2013	2.746	100	6	8
<i>Juncus alpino-articulatus</i>	BR	130911-02	07/05/2012	0.158	92	0	0
	RT	140911-01	07/05/2012	0.050	72	0	0
<i>Parnassia palustris</i>	RX	230911-12	07/05/2012	1.729	85	4	3
	VB	230911-07	09/05/2012	0.886	87	6	4
<i>Pedicularis mixta</i>	BR	310811-02	09/05/2012	2.439	35	11	0
	MA	100811-03	09/05/2012	5.462	97	2	0
<i>Pinguicula grandiflora</i>	FM	130711-03	09/05/2012	0.134	95	3	7
	TI	220611-01	09/05/2012	0.180	86	0	0
<i>Primula farinosa</i>	TH	040811-02	09/05/2012	2.096	99	0	0
	TI	040811-01	09/05/2012	0.535	-	-	-
	TI	140711-01	09/05/2012	0.163	100	0	1
<i>Swertia perennis</i>	RS	200911-10	09/05/2012	0.526	54	0	0
	RS	290910-01	23/02/2011	0.251	88	9	3
	RT	140911-02	09/05/2012	0.791	40	0	0
	RT	280910-01	23/02/2011	2.548	98	10	8
	RX	250910-01	23/02/2011	0.492	99	2	1
<i>Triglochin palustris</i>	PU	130911-03	09/05/2012	0.612	100	1	39
	PU	160910-02	23/02/2011	1.620	100	8	40
	RS	290910-02	23/02/2011	0.134	-	-	-
	RT	020910-05	23/02/2011	0.083	-	-	-

**Supplementary table 3** Optimal germination conditions and effect of the conservation protocol on the study species. The accession code of the collection that was used in the experiment is indicated. The experiments were performed in Petri dishes (1 % agar). Stratification lasted 12 weeks in darkness. The germination incubation thermoperiods were coupled with 12 h-light/12-h darkness photoperiods and lasted four weeks. The germination results before and after the conservation protocol (drying to 5 % moisture content, freezing to -13 °C) are indicated as average percentage  $\pm$  SE. The results of a Generalized Linear Model (binomial distribution, logit link) to test differences in germination before and after are given.

Species	Accession code	Stratification (°C)	Incubation (°C)	Germination before (%)	Germination after (%)	$\chi^2$	p
<i>Blysmus compressus</i>	130711-02	3	30/20	100	97 $\pm$ 2	0.394	0.530
<i>Carex davalliana</i>	030810-01	3	30/20	74 $\pm$ 2	71 $\pm$ 3	0.138	0.710
<i>Carex diandra</i>	260809-01	3	30/20	94 $\pm$ 2	97 $\pm$ 1	1.059	0.303
<i>Carex echinata</i>	180711-02	3	22/12	100	97 $\pm$ 3	0.400	0.527
<i>Carex lepidocarpa</i>	130711-01	3	30/20	57 $\pm$ 9	84 $\pm$ 3	14.951	<0.001
<i>Carex pulicaris</i>	230911-13	25	22/12	92 $\pm$ 2	97 $\pm$ 1	2.094	0.148
<i>Centaurium somedanum</i>	131011-02	3	22/12	95 $\pm$ 1	99 $\pm$ 1	2.263	0.133
<i>Eriophorum latifolium</i>	130711-04	3	30/20	99 $\pm$ 1	71 $\pm$ 4	10.836	0.001
<i>Juncus alpino-articulatus</i>	130911-02	3	22/12	92 $\pm$ 2	49 $\pm$ 7	35.035	<0.001
<i>Parnassia palustris</i>	230911-07	3	30/20	87 $\pm$ 4	21 $\pm$ 5	56.242	<0.001
<i>Pedicularis mixta</i>	100811-03	3	14/4	97 $\pm$ 2	72 $\pm$ 3	14.729	<0.001
<i>Pinguicula grandiflora</i>	130711-03	3	22/12	95 $\pm$ 2	95 $\pm$ 5	0.000	1.000
<i>Primula farinosa</i>	040811-02	3	30/20	99 $\pm$ 1	99 $\pm$ 1	0.001	0.971
<i>Swertia perennis</i>	200911-10	3	30/20	54 $\pm$ 8	90 $\pm$ 6	6.963	0.008
<i>Triglochin palustris</i>	130911-03	3	22/12	98 $\pm$ 2	100	0.001	0.981

**Supplementary table 4** DNA samples of *Centaurium somedanum* individuals kept at the DNA bank of the Jardín Botánico Canario “Viera y Clavijo” (Las Palmas de Gran Canaria, Spain). The two letters at the beginning of the sample code indicate the sampling site, according to the site codes of Supplementary table 1. The sampling date is given in the format dd-mm (sampling was in year 2009). The DNA concentrations were quantified using a Biophotometer (Eppendorf, Hamburg, Germany).

Sample	UTM 29T ED50	Sampling date	[DNA] µg/ml	Sample	UTM 29T ED50	Sampling date	[DNA] µg/ml
AG01	722718 4776984	6-10	68.5	BO04	727406 4774331	10-10	1074.8
AG02	722718 4776984	6-10	139.9	BO05	727407 4774331	10-10	1070.6
AG03	722717 4776982	6-10	128.7	BO06	727408 4774341	10-10	349.6
AG04	722717 4776982	6-10	170.3	BO07	727409 4774344	10-10	308.1
AG05	722717 4776981	6-10	184.3	BO08	727408 4774345	10-10	547.3
AG06	722551 4776660	6-10	307.3	BO09	727409 4774343	10-10	503.5
AG07	722551 4776660	6-10	111.0	BO10	727408 4774346	10-10	283.6
AG08	722550 4776659	6-10	304.5	BO11	727410 4774353	10-10	210.4
AG09	722549 4776658	6-10	325.8	BO12	727411 4774356	10-10	334.6
AG10	722548 4776658	6-10	128.7	BR01	727303 4767227	15-10	237.0
AG11	722362 4776552	6-10	389.4	BR02	727304 4767230	15-10	308.5
AG12	722362 4776552	6-10	203.3	BR03	727302 4767230	15-10	422.6
AG13	722363 4776553	6-10	280.5	BR04	727303 4767231	15-10	122.7
AG14	722362 4776552	6-10	353.8	BR05	727304 4767231	15-10	198.0
AR01	734699 4774476	6-10	451.9	BR06	727304 4767233	15-10	194.8
AR02	734699 4774477	6-10	205.5	BR07	727303 4767235	15-10	468.2
AR03	734698 4774478	6-10	208.9	BR08	727303 4767237	15-10	102.5
AR04	734698 4774484	6-10	200.5	BR09	727303 4767237	15-10	198.4
AR05	734697 4774486	6-10	84.7	BR10	727302 4767240	15-10	815.1
AR06	734693 4774478	6-10	610.7	BR11	727307 4767264	15-10	273.8
AR07	734692 4774478	6-10	325.7	BR12	727306 4767270	15-10	245.6
AR08	734691 4774482	6-10	100.2	BR13	727316 4767309	15-10	332.1
AR09	734691 4774484	6-10	242.5	BR14	727333 4767345	15-10	437.3
AR10	734691 4774485	6-10	359.5	BR15	727290 4767384	15-10	476.2
AR11	734687 4774485	6-10	138.9	BR16	727292 4767329	15-10	392.3
AR12	734691 4774488	6-10	78.3	CA01	723850 4772106	10-10	259.6
AR13	734688 4774491	6-10	313.7	CA02	723850 4772106	10-10	308.3
AR14	734689 4774492	6-10	399.2	CA03	723851 4772106	10-10	291.6
AR15	734686 4774489	6-10	1124.8	CA04	723852 4772107	10-10	214.3
AR16	734685 4774495	6-10	169.4	CM01	723517 4777297	24-9	244.9
AR17	734684 4774496	6-10	364.8	CM02	723518 4777298	24-9	537.7
AR18	734687 4774500	6-10	254.0	CM03	723527 4777348	24-9	97.8
AR19	734683 4774499	6-10	107.7	CM04	723528 4777352	24-9	88.7
AR20	734687 4774500	6-10	161.1	CM05	723536 4777372	24-9	140.4
AR21	734687 4774500	6-10	868.5	CM06	723539 4777376	24-9	194.5
AR22	734687 4774501	6-10	407.7	CM07	723540 4777378	24-9	121.8
AR23	734685 4774505	6-10	270.3	CM08	723569 4777505	24-9	153.0
AR24	734680 4774506	6-10	293.3	CM09	723570 4777508	24-9	230.4
AR25	734681 4774509	6-10	188.1	CM10	723573 4777511	24-9	137.8
AR26	734680 4774511	6-10	255.0	CM11	723595 4777558	24-9	224.2
AR27	734680 4774513	6-10	276.2	CM12	723594 4777561	24-9	145.6
AR28	734683 4774511	6-10	216.7	CM13	723593 4777571	24-9	229.4
AR29	734683 4774511	6-10	43.8	CM14	723716 4777832	24-9	253.5
AR30	734682 4774512	6-10	202.1	CM15	723713 4777841	24-9	618.8

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Sample	UTM 29T ED50	Sampling date	[DNA] $\mu\text{g/ml}$	Sample	UTM 29T ED50	Sampling date	[DNA] $\mu\text{g/ml}$
CM14	723716 4777832	24-9	253.5	FM12	723504 4777749	10-10	466.7
CM15	723713 4777841	24-9	618.8	FM13	723503 4777748	10-10	285.6
CM16	723709 4777849	24-9	138.7	FM14	723501 4777745	10-10	364.6
CM17	723708 4777850	24-9	161.8	FM15	723498 4777741	10-10	417.1
CM18	723645 4777907	24-9	82.2	FM16	723497 4777740	10-10	332.5
CM19	723642 4777914	24-9	254.7	FM17	723497 4777740	10-10	109.0
CM20	723644 4777915	24-9	95.3	FM18	723496 4777739	10-10	483.5
CM21	723642 4777919	24-9	242.4	FU01	721851 4776019	29-9	177.7
CM22	723641 4777924	24-9	118.4	FU02	721851 4776018	29-9	133.0
CM23	723620 4777959	24-9	175.4	FU03	721851 4776015	29-9	102.9
CM24	723620 4777963	24-9	339.3	FU04	721852 4776013	29-9	64.2
CM25	723615 4777977	24-9	166.7	FU05	721851 4776013	29-9	252.4
CM26	723614 4777981	24-9	336.6	FU06	721851 4776010	29-9	171.5
CM27	723602 4778021	24-9	269.4	FU07	721852 4776009	29-9	220.7
CM28	723605 4778037	24-9	182.6	FU08	721851 4776007	29-9	121.5
CM29	723618 4778071	24-9	228.1	FU09	721851 4776005	29-9	231.6
CM30	723624 4778079	24-9	330.1	FU10	721851 4776002	29-9	256.1
CM31	723617 4778117	24-9	1210.8	FU11	721850 4775999	29-9	105.2
CM32	723615 4778116	24-9	408.7	FU12	721850 4775997	29-9	144.1
CM33	723625 4778142	24-9	115.9	FU13	721850 4775993	29-9	238.7
CM34	723630 4778150	24-9	763.0	FU14	721849 4775990	29-9	203.1
CM35	723632 4778153	24-9	132.8	FU15	721848 4775988	29-9	154.0
CM36	723639 4778169	24-9	273.6	FU16	721847 4775984	29-9	238.6
CM37	723643 4778174	24-9	240.4	MA01	735532 4766046	2-10	596.2
CM38	723655 4778190	24-9	200.4	MA02	735535 4766055	2-10	97.6
CM39	723663 4778195	24-9	228.7	MA03	735534 4766061	2-10	181.5
CM40	723673 4778201	24-9	320.5	MA04	735533 4766064	2-10	131.2
CM41	723570 4778526	24-9	71.0	MA05	735533 4766065	2-10	161.6
CM42	723570 4778528	24-9	158.8	MA06	735533 4766065	2-10	456.0
CU01	727578 4784774	8-10	501.5	MA07	735533 4766067	2-10	105.5
CU02	727579 4784776	8-10	314.9	MA08	735531 4766068	2-10	149.5
CU03	727579 4784778	8-10	295.1	MA09	735529 4766072	2-10	291.0
CU04	727580 4784777	8-10	175.6	MA10	735526 4766074	2-10	328.0
CU05	727580 4784777	8-10	186.6	MA11	735525 4766076	2-10	156.6
CU06	727580 4784777	8-10	77.9	MA12	735514 4766078	2-10	548.4
CU07	727582 4784779	8-10	248.8	MA13	735499 4766090	2-10	153.0
CU08	727582 4784778	8-10	258.8	MA14	735501 4766091	2-10	236.8
CU09	727583 4784776	8-10	174.9	MA15	735495 4766100	2-10	261.1
CU10	727583 4784776	8-10	173.9	ML01	731844 4770263	23-9	45.4
FM01	723507 4777732	10-10	384.3	ML02	731835 4770269	23-9	82.7
FM02	723508 4777733	10-10	657.6	ML03	731834 4770269	23-9	113.8
FM03	723509 4777733	10-10	751.6	ML04	731829 4770269	23-9	275.2
FM04	723509 4777734	10-10	376.8	ML05	731802 4770258	23-9	183.6
FM05	723509 4777734	10-10	259.0	ML06	731797 4770262	23-9	416.3
FM06	723513 4777738	10-10	82.9	ML07	731772 4770262	23-9	97.9
FM07	723513 4777739	10-10	226.1	ML08	731773 4770264	23-9	94.9
FM08	723513 4777738	10-10	123.3	ML09	731772 4770263	23-9	81.2
FM09	723514 4777739	10-10	438.6	ML10	731767 4770264	23-9	387.5
FM10	723514 4777739	10-10	308.1	ML11	731765 4770264	23-9	166.8
FM11	723489 4777756	10-10	146.7	ML12	731764 4770266	23-9	377.1

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*Supplementary materials*

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Sample	UTM 29T ED50	Sampling date	[DNA] µg/ml	Sample	UTM 29T ED50	Sampling date	[DNA] µg/ml
ML13	731760 4770267	23-9	274.4	TE07	735567 4764365	1-10	473.9
ML14	731759 4770267	23-9	412.7	TE08	735582 4764357	1-10	327.6
ML15	731757 4770269	23-9	206.1	TE09	735579 4764354	1-10	188.9
ML16	731756 4770270	23-9	190.1	TE10	735583 4764351	1-10	319.9
ML17	731756 4770270	23-9	228.3	TE11	735584 4764349	1-10	509.5
ML18	731755 4770270	23-9	310.8	TE12	735582 4764346	1-10	457.0
ML19	731754 4770270	23-9	341.0	TE13	735583 4764339	1-10	197.6
ML21	731750 4770270	23-9	147.0	TE14	735583 4764334	1-10	411.8
ML22	731752 4770273	23-9	328.2	TE15	735583 4764332	1-10	375.3
ML23	731750 4770273	23-9	239.2	TE16	735583 4764332	1-10	234.3
ML24	731751 4770273	23-9	296.5	TE17	735584 4764329	1-10	318.7
ML25	731746 4770272	23-9	109.5	TE18	735589 4764326	1-10	235.8
ML26	731745 4770267	23-9	106.1	TE19	735588 4764318	1-10	367.5
ML27	731748 4770263	23-9	179.8	TE20	735593 4764314	1-10	403.7
ML28	731750 4770263	23-9	323.2	TE21	735597 4764310	1-10	183.0
ML29	731749 4770261	23-9	487.1	TE22	735597 4764307	1-10	133.8
ML30	731746 4770256	23-9	246.7	TE23	735599 4764309	1-10	191.5
ML31	731751 4770254	23-9	285.4	TE24	735597 4764306	1-10	232.4
ML32	731752 4770253	23-9	285.3	TE25	735597 4764306	1-10	141.2
ML33	731755 4770252	23-9	322.6	TE26	735602 4764306	1-10	384.5
ML34	731791 4770252	23-9	166.4	TE27	735599 4764303	1-10	275.8
PU01	726226 4765804	1-10	426.3	TE28	735608 4764300	1-10	186.8
PU02	726229 4765800	1-10	124.6	TE29	735611 4764296	1-10	138.3
PU03	726232 4765798	1-10	80.3	TE30	735612 4764295	1-10	311.6
PU04	726231 4765794	1-10	217.1	TE31	735643 4764257	1-10	661.7
PU05	726232 4765775	1-10	456.0	TE32	735662 4764223	1-10	284.5
PU06	726235 4765770	1-10	55.6	TE33	735778 4764172	1-10	182.6
PU07	726234 4765769	1-10	132.4	TR01	734716 4766020	2-10	219.6
PU08	726238 4765797	1-10	638.9	TR02	734715 4766021	2-10	321.9
PU09	726238 4765802	1-10	99.6	TR03	734714 4766030	2-10	208.4
PU10	726241 4765800	1-10	1402.4	TR04	734715 4766031	2-10	61.6
PU11	726241 4765797	1-10	124.7	TR05	734715 4766030	2-10	177.2
PU12	726244 4765794	1-10	428.0	TR06	734716 4766031	2-10	120.1
PU13	726241 4765803	1-10	302.2	TR07	734715 4766031	2-10	87.3
PU14	726245 4765799	1-10	84.5	TR08	734716 4766034	2-10	244.2
PU15	726249 4765810	1-10	350.3	TR09	734718 4766028	2-10	186.1
PU16	726205 4765816	1-10	577.9	TR10	734715 4766026	2-10	184.6
PU17	726205 4765811	1-10	559.7	VA01	728350 4772835	9-10	392.2
PU18	726211 4765792	1-10	781.0	VA02	728348 4772836	9-10	272.0
PU19	726212 4765789	1-10	286.7	VA03	728349 4772839	9-10	383.1
PU20	726201 4765793	1-10	964.9	VA04	728345 4772838	9-10	226.1
PU21	726200 4765793	1-10	366.0	VA05	728343 4772839	9-10	463.9
PU22	726181 4765782	1-10	293.5	VA06	728354 4772845	9-10	82.3
TE01	735556 4764385	1-10	185.4	VA07	728356 4772847	9-10	121.0
TE02	735566 4764383	1-10	482.3	VA08	728342 4772843	9-10	403.7
TE03	735567 4764378	1-10	728.3	VA09	728335 4772844	9-10	909.2
TE04	735580 4764367	1-10	422.0	VA10	728333 4772845	9-10	269.4
TE05	735571 4764367	1-10	237.3	VA11	728336 4772845	9-10	180.3
TE06	735568 4764365	1-10	452.7	VA12	728334 4772846	9-10	227.4

(Continues)

*(Continues)*

Sample	UTM 29T ED50	Sampling date	[DNA] $\mu\text{g/ml}$
VA13	728337 4772846	9-10	248.9
VA14	728336 4772851	9-10	350.1
VA15	728336 4772852	9-10	246.4
VA16	728338 4772853	9-10	482.0
VA17	728340 4772853	9-10	390.4
VA18	728342 4772851	9-10	254.6
VA19	728340 4772850	9-10	577.7
VA20	728342 4772853	9-10	332.3
VA21	728343 4772855	9-10	442.2
VA22	728345 4772853	9-10	215.0
VA23	728343 4772862	9-10	558.5
VA24	728346 4772861	9-10	380.9
VA25	728348 4772863	9-10	288.5
VA26	728350 4772864	9-10	364.5
VA27	728349 4772860	9-10	933.3
VA28	728361 4772855	9-10	52.1
VA29	728363 4772853	9-10	1161.8
VA30	728365 4772855	9-10	323.8
VA31	728366 4772856	9-10	690.1
VA32	728371 4772853	9-10	271.7
VA33	728363 4772850	9-10	92.3
VA34	728375 4772845	9-10	213.8
VA35	728373 4772847	9-10	176.8
VA36	728375 4772850	9-10	205.6
VA37	728375 4772854	9-10	1279.8
VA38	728381 4772855	9-10	479.6
VA39	728381 4772854	9-10	119.8
VA40	728382 4772854	9-10	284.6
VA41	728383 4772857	9-10	536.7
VA42	728445 4772821	9-10	1016.1
VA43	728445 4772821	9-10	568.7
VA44	728443 4772824	9-10	285.4
VA45	728449 4772815	9-10	143.6
VV01	725119 4768885	1-10	265.7
VV02	725118 4768884	1-10	425.6
VV03	725118 4768884	1-10	335.4

**Supplementary table 5** Impact factor (IF) of the publications. This report is included in compliance with the University regulations. Source: Journal Citation Reports 2012 Copyright © 2013 Thomson Reuters.

Journal title	Indexed in (ranking)	ISSN	IF 2012	IF 5-year
Plant Ecology	Plant Sciences (86/195, Q2), Ecology (85/136, Q3), Forestry (19/60, Q2)	1385-0237	1.534	1.866
Seed Science Research	Plant Sciences (66/195, Q2)	0960-2585	1.931	1.767
Annals of Botany	Plant Sciences (27/195, Q1)	0305-7364	3.449	4.051











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**Fernández-Pascual E (2013) The Role of Temperature in the Seed Germination Ecology of Mountain Springs and Fens. Doctoral thesis, University of Oviedo. 129 pp.**

Mountain calcareous springs and fens are floristically diverse wetlands fed by carbonate-rich groundwater. This thesis studies their seed ecology, focusing on the interaction between environmental temperature and germination. Through laboratory germination experiments, it characterizes the temperature dimension of the germination niche and identifies specific germination strategies. It explores the special thermal characteristics of fen soils and their consequences on germination. Using the Cantabrian endemic *Centaureum somedanum* as a study species, it investigates the germination ecology of spring specialists and performs a common garden experiment supported by ISSR fingerprinting to determine the genetic and environmental influences on seed dormancy variation. Based on the results, it proposes two germination strategies. Fen species generally follow a warm strategy, characterized by a heat response capable of overriding dormancy during episodes of unusual heat. Spring species lack the heat response and germinate only at cool temperatures. Fen soils have a buffer effect that makes their temperature less extreme than predicted, especially during the night and in winter. However, the germination niche breadth of fen species shows plasticity and is narrower in regions with harsher winters. The germination ecology of the spring specialist *C. somedanum* departs from the general germination patterns of its genus, showing morphophysiological dormancy and cool germination. This species has a local dormancy cline that is related to the genetic composition of the populations and to site climate, but that also responds in the short term to the seed maturation environment. These results highlight the potential of germination traits to react to climate changes at different spatial and temporal scales.



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