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Minimal temperature of pollen germination controls species distribution along a temperature gradient

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• **Background and Aims** Although plant distribution patterns are well documented, our understanding of the ecophysiological mechanisms that control the geographical ranges of plant species remains poor. We used a largely ignored method, the performance of the male gametophyte *in vitro*, to assess whether the thermal range of pollen germination and tube growth controls species distribution ranges, in this case along an elevational gradient.
• **Methods** Using *in vitro* pollen germination experiments, we obtained cardinal temperatures (minimal, optimal and maximal) of pollen germination and pollen tube growth for 25 herbaceous species along a mean annual temperature gradient of about 5 °C. These temperatures were correlated with temperatures of the sites where the species were collected. The presence of a phylogenetic signal in the data set as well as an effect of species flowering phenology were also estimated.
• **Key Results and Conclusions** We found a strong positive relationship between temperature conditions at our collection sites and the minimum temperature for both pollen germination and pollen tube growth. In addition, a significant correlation between maximum temperature of pollen tube growth and temperature of flowering month was apparent. We conclude that the restriction of pollen germination and growth by low temperatures is an important contributor to the climatic restriction of plant species distributions. Improved knowledge of this thermal precursor to seed production could, from a functional perspective, enhance our understanding of species distributions along climatic gradients and our ability to predict how anthropogenic climate change might affect plant community composition.

Key words: pollen germination, pollen tube growth, plant distribution, elevational gradient, biogeography, temperature, climate change.

INTRODUCTION

Temperature has been long recognized as a major factor controlling plant distribution along latitudinal and elevational gradients (Salisbury, 1926; Woodward and Jones, 1984; Archibold, 1995). The identification of plant–environment relationships has catalysed the generation of many vegetation–climate models to describe plant distribution patterns at different geographical scales (Meusel *et al*., 1965; Woodward, 1987; Prentice *et al*., 1992). Although these models are highly informative, the exact ecophysiological mechanisms that shape the distribution of species with respect to temperature range remain unclear.

One suggested mechanism regulating climatic restriction of plant species relates to the sensitivity of the shoot. From studies on plant persistence at low temperatures it has been proposed that the level of frost resistance of vegetative organs, such as buds, leaves and stems, can predict the extent to which species are distributed in regions with harsh climates (Sakai and Larcher, 1987; Körner, 1999; Larcher, 2000; Taschler and Neuner, 2004). However, differences in distributional ranges are not always clear-cut; plant species from different biomes often overlap in their frost tolerance (Sakai and Larcher, 1987; Körner, 1999; Larcher, 2003). Thus, this ecophysiological mechanism provides only a partial explanation of the large-scale discontinuities in plant distributions. This 'frost-sensitivity' explanation also fails to consider the fact that the geographical limits of vegetative survival may be determined at stages of the plant life cycle other than that of the mature plant, and by very different environmental conditions (see Woodward, 1987, 1997; García *et al*., 2000). Despite its fundamental importance in shaping the distribution of species and vegetation types globally, and the uncertainties surrounding future impacts of climate warming (Parmesan and Hanley, 2015), an accurate mechanistic appreciation of how temperature shapes the distribution of a plant species remains elusive. The identification of the rule base that explains how climate restricts plant distribution remains one of the major challenges of modern ecology (Bykova *et al*., 2012).

In contrast to the ambiguous relationships between climate and vegetative growth, reproduction processes are intimately related to ambient temperatures (Zinn *et al*., 2010; Bykova *et al*., 2012). They may therefore be a primary determinant of the geographical boundaries of species (e.g. Pigott and Huntley, 1981; Pigott, 1992; García *et al*., 2000; Jump and Woodward, 2003). For a given species, 'environmental favourability' declines from the core to the periphery of its distributional area, a fact that may negatively affect the reproduction performance of the plant. Indeed, seed set progressively decreases from a maximal capacity at the centre of this area to conditions where the quality and quantity of the produced seeds are below that

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necessary for successful and long-term regeneration at the distributional edge (García *et al*., 2000; Jump and Woodward, 2003). Consequently, the inability to reproduce, or poor reproduction, beyond its ecological optimum limits the further geographical expansion of a species (Grubb, 1977; Woodward and Jones, 1984; Pigott, 1992; McKee and Richards, 1996). For example, seed production of lowland species decreases drastically with increasing elevation (Hofgaard, 1993; Kullman, 1993), while alpine species are able to complete reproduction even under the extreme climatic conditions of high elevations (Ladinig and Wagner, 2005; Wagner *et al*., 2010). However, although the climatic control of species ranges may be mediated through seed production (Grubb, 1977; Woodward, 1987), we remain uncertain as to the key stage of the reproduction process: the stage that is critical in ecophysiological terms and has therefore strong predictive value in defining the distributional range of a species.

Variation in seed crop production in relation to climate may stem from a specific physiological limitation of seed development (Pigott, 1992; Zinn *et al*., 2010). Although various stages of seed development are temperature-dependent (e.g. Henttonen *et al*., 1986; Peet *et al*., 1997), numerous experimental studies have demonstrated that for the completion of successful fertilization, both pollen germination (PG) and pollen tube growth (PTG) are also highly temperature-dependent (Weinbaum *et al*., 1984; Elgersma *et al*., 1989; Kakani *et al*., 2005; Boavida and McCormick, 2007; Steinacher and Wagner, 2012). Despite dependence on habitat temperature for the success of both the PG and the PTG components of the progamic phase of fertilization, however, few studies have related temperature requirements of PG and PTG to the geographical range of the investigated species. Nonetheless, Pigott and Huntley (1981) demonstrated that the temperature sensitivity of PTG and the short period of stigmoid and stylar receptivity in *Tilia cordata* may account for its northern distributional limit in the British Isles (see also Pigott and Warr, 1989; Pigott, 1992).

Here, we attempt to take the ground-breaking but still unrecognized work of ecologists such as Pigott and Huntley (1981) to the next level. We explore the extent to which temperature requirements of PG and pollen tube elongation of contrasted distributional ranges may be predicted from the temperatures associated with their natural habitat. More specifically, we test the hypothesis that the specific temperature requirements of PG and PTG *in vitro* predict positively species occurrence along a gradient of mean annual temperature (MAT). The distribution of a species with a high temperature requirement for these two processes is expected to be limited to the higher part of the temperature gradient, due to increasing negative temperature stress.

To test our hypothesis, we selected an elevational gradient as a study system, because it is one of the most powerful 'natural experiments' for testing ecological and evolutionary responses of biota to geophysical influences, such as temperature (Körner, 2007*a*, *b*). The strong negative correlation between MAT and elevation (0·6 K per 100 m; Sakai and Larcher, 1987; Körner, 1999, 2007*a*, *b*) offers an ideal opportunity to explore macroecological mechanisms of plant–climate interactions over short spatial distances and offers additional insight into the likely impact of anthropogenic climate change on plant ecophysiology and distribution (Dunne *et al*., 2003).

MATERIALS AND METHODS

Study system: study area and species selection

Fieldwork was carried out in the Berchtesgaden National Park located in the Bavarian Alps (south-east Germany). The National Park is approx. 200 km^2 in area and characterized as typically alpine topography, with steep mountain peaks composed of Triassic limestone and dolomite (Marke *et al*., 2013). The climate is typically montane with large altitudinal decrease in mean annual air temperatures from +7 to – 2 °C [from 603 to 2713 m above sea level (a.s.l.), respectively]. Mean annual precipitation in the region varies, ranging from approx. 1500 to 2600 mm (Marke *et al*., 2013).

For the purpose of this study, 25 herbaceous species with different elevational distributions (and therefore with different distributional ranges along the MAT gradient) within southern Germany were selected (see Table 1; Supplementary Data, Table S1). All chosen species occur in a single vegetation type (calcareous grasslands) and share similar habitat preferences with regard to light, water, soil characteristics and chemistry (Ellenberg *et al*., 1991; Oberdorfer, 2001); thus, temperature is likely to be the main explanatory variable for their distributional ranges. Nomenclature follows Oberdorfer (2001).

We surveyed vegetation at 40 sites along an altitudinal gradient from 650 to 2570 m a.s.l. to determine distributional (and therefore climatic) ranges of our target species. Ten random 1-m^2 plots were set up at each site and in each plot we recorded the cover of each vascular plant species. The relative abundance of a species at a site was calculated as the mean value (% cover) of its abundance in all plots. The species' relative abundances among sites were compared, and the site with the highest value for that species (i.e. with putative optimal ecological conditions) was identified. Pollen grains of the selected species were subsequently collected from this 'optimal' site. Pollen samples were mainly collected in the Berchtesgaden National Park from between 800 and 2050 m a.s.l., although pollen grains from *Anemone pulsatilla* L., *Globularia cordifolia* L. and *Primula veris* L. were collected from calcareous grassland located at 450 m a.s.l. (Garchinger Heide, southern Bavaria, Germany). For every collection site, data on MAT as well as mean monthly temperature from March to September (see below) were obtained from the closest weather station (difference in elevation less than 50 m and none more than 2 km from the site). Data are presented as mean values for the last 10 years of records.

MAT as a proxy for habitat temperature environment

Using temperature of the flowering period as a main factor influencing cardinal temperatures of PT and PTG would be the optimal approach in the current study, as this better reflects the conditions the pollen actually experiences. However, we were compelled to use MAT as a proxy for species habitat temperature conditions, for the following reasons. Flowering time, especially in mountain plants, is a highly flexible trait, strongly dependent on both habitat characteristics (e.g. altitude, slope aspect and inclination, shadow from surrounding trees or rocks) and particular weather conditions (thickness and distribution of snow, inter-annual temperature variation, etc.). Due to these

TABLE 1. *Cardinal temperatures of pollen germination (PG) and pollen tube growth (PTG) for the investigated species*

Species	Species distribution characteristics		Flowering phenology		Pollen germination			Pollen tube growth		
	Range of MAT (°C)	MAT of collection site (°C)	Flowering month	TFM (°C)	T_{min}	T_{opt}	T_{max}	T_{min}	T_{opt}	T_{max}
Aconitum napellus L.	2·4–4·4	3·8	VIII	11·3	–	–	–	2·4	21·2	37·5
Anemone nemorosa L.	3·9–9·0	6·1	IV	5·0	7·5	26·3	36·0	5·0	31·1	34·0
Anemone pulsatilla L.	6·2–9·0	8·0	IV	8·1	9·0	17·3	37·2	9·0	32·2	34·0
Caltha palustris L.	4·9–9·0	6·8	IV	6·0	9·0	23·2	34·0	8·9	28·4	34·0
Campanula alpina Jacq.	1·9–3·6	2·7	VI	8·6	0·0	16·4	34·0	0·0	17·8	34·0
Campanula scheuchzeri Vill.	2·2–6·2	3·5	VII	10·6	0·0	17·3	35·9	0·0	16·8	39·0
Carex caryophyllea Latourr.	4·9–9·0	6·3	IV	15·7	0·0	15·9	40·0	0·0	34·3	40·0
Carex firma Host	1·8–6·7	6·3	V	10·5	0·2	15·8	35·0	0·0	23·9	35·7
Carex flacca Schreb.	4·9–9·0	6·3	V	14·1	–	–	–	4·0	30·0	34·0
Erica carnea L.	3·1–7·0	6·3	IV	15·7	0·0	26·0	34·8	0·0	28·8	40·0
Gentiana asclepiadea L.	3·4–7·5	5·4	VIII	9·3	–	–	–	8·0	19·2	35·6
Gentiana pannonica Scop.	2·6–5·6	5·1	VII	12·8	0·9	28·5	35·0	2·0	19·6	36·0
Gentianella aspera (Hegetschw. & Heer) Dostál ex Skalický, Chrtek & Gill	1·9–3·6	3·7	VIII	11·3	2·5	26·1	34·8	1·0	16·4	40·0
Globularia cordifolia L.	4·9–9·0	8·0	IV	8·1	9·0	30·4	40·0	9·0	29·6	40·0
Helleborus niger L.	4·9–7·5	6·3	III	5·5	0·0	14·5	33·4	5·0	23·5	34·4
Parnassia palustris L.	1·8–7·4	2·8	VIII	10·2	6·5	31·8	34·0	0·0	31·0	34·0
Phyteuma orbiculare L.	2·3–4·9	3·5	VI	10·6	0·0	21·6	33·9	0·0	16·3	30·5
Primula minima L.	1·9–3·6	2·7	IV	5·8	0·0	16·1	34·0	0·0	26·9	34·0
Plantago lanceolata L.	4·4–7·4	6·1	VII	12·9	5·0	20·6	36·8	4·7	25·8	38·2
Primula veris L.	6·2–9·0	8·0	IV	8·1	9·0	22·7	33·9	9·0	24·8	34·7
Ranunculus acris L.	5·9–9·0	6·3	VI	13·4	12·2	31·0	35·0	12·0	26·2	35·9
Ranunculus serpens subsp.polyanthemophyllus (W.Koch & H.E.Hess) Kerguélen	5·9–9·0	6·7	V	11·0	5·0	21·7	35·0	5·0	22·8	35·0
Silene flos-cuculi (L.) Greuter & Burdet	3·9–9·0	6·1	VI	12·9	9·0	17·8	34·0	9·0	25·3	34·0
Soldanella alpina L.	1·9–6·2	4·4	IV	8·0	2·0	18·6	33·6	2·5	23·3	33·9
Trollius europaeus L.	2·6–5·6	5·4	VI	12·1	–	–	–	6·3	30·7	34·0

Species distribution characteristics are based on a vegetation survey carried out at 40 sites along a gradient from 650 to 2570 m a.s.l. in the Berchtesgaden National Park. The cardinal temperatures were estimated by fitting the generalized plant growth model to PG rate and PTG length, which were obtained in *in vitro* germination experiments along a temperature gradient from 5 to 35 °C (for details see Material and Methods section). T_{min}, lowest temperature; T_{opt}, optimum temperature; T_{max}, maximum temperature of PG or PTG; MAT, mean annual temperature.

two factors, individual plants may flower over a prolonged period: for example, in 2010, *Sesleria albicans*, one of the most common species in the study region, flowering began on 1 May at 800 m a.s.l. and ended on 10 July at 2430 m a.s.l. Furthermore, ground-level temperatures, where flowers are actually located, differ from meteorological records measured to international standards at 2 m above the ground (Geiger *et al*., 2009). Thus, to obtain reliable temperature data for the flowering period for any given species, it was necessary to observe flowering phenology within entire distribution ranges over a long period, as well as conduct direct measurements of ambient temperatures at flower height. Due to logistical limitations (phenological observations and fresh pollen collections were simultaneously carried out by one person only in a remote area at sites located from between 800 and 2050 m a.s.l.), phenological observations for a given species were confined to one year (either 2010 or 2011) and only in a site with putative optimal ecological conditions. For similar reasons, we were unable to obtain near-ground temperatures during species flowering (a pollen collection site was visited for several hours only). Under these circumstances and despite the possible confounding effect of coldest month, MAT was therefore the only feasible proxy for temperature conditions during flowering and seed set for sites located within a relatively small geographically compact region.

Flowering phenology

As temperature conditions during flowering may also be correlated with the temperature requirements of PG and PTG (e.g. early-flowering species show lower temperature thresholds for these two processes; Weinbaum *et al*., 1984; Luza *et al*., 1987), the flowering phenology of the studied species was also considered. To that end, phenological observations of flower development were conducted two to three times a month from March to September in either 2010 or 2011 (Table S1) in every 'optimal' site (see above) following the Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (BBCH) code. This code provides a detailed growth stage key that includes intermediate stages as well as stages marking the end of phenophases and thus allows observation of the entire development cycle of all mono- and dicotyledonous plants using a decimal coding system (Meier, 2001). Using this key also obviates the necessity of being present at the exact start of each phenological stage and the frequency distribution of phenophases within the population can instead be assessed at each sampling date (Cornelius *et al*., 2013). We focused here on three key phenological events: the onset of flowering (non-graminoids: first flowers open; graminoids: first anthers visible), full flowering (non-graminoids: 50 % of flowers open; graminoids: 50 % of anthers mature) and the cessation of flowering (non-graminoids: petals dehydrated or fallen; graminoids: all spikelets/panicles have completed flowering but some dehydrated anthers may remain). On each sampling date, the phenological stage of the majority of individuals of a species was recorded. The timing of the onset of phenophases was defined as the sampling date when the stage was first observed. When the majority of individuals of the target species were in flower, this was adjudged to be in phenological terms the flowering date and flower buds were collected for the PG experiments.

Pollen collection

To minimize the impact of intraspecific variation on PG measurements (Kakani *et al*., 2005), fresh flower buds (1–3 d before opening) were randomly collected in the field from at least 30 individuals separated by a distance of 2–5 m from each other. After collection, buds were taken immediately to the laboratory and disinfected by spraying with 96 % ethanol. The anthers were then removed manually and left to dry for 2–3 d at room temperature in a desiccator filled with silica gel (relative humidity approx. 30 %). To extract the pollen grains, the dried anthers were subsequently crushed into small pieces and passed through a 200-μm sieve. Prior to the experiments, the pollen grains were stored at 5 °C for not more than 7 d.

Pollen germination experiments

The media used for PG contained nutrient salts (0·01 % H_3BO_4, 1 mM $Ca(NO_3)_2$, 1 mM $CaCl_2$ and 1 mM $MgSO_4$) and different concentrations of sucrose, from 10 to 30 % (Brewbaker and Kwack, 1963). To reduce the risk of microbial and fungal contamination, the glassware and stock solutions of the salts were autoclaved before use; the PG media were sterilized by filtration. Fresh germination media were prepared and stored at 5 °C for no longer than 3 d prior to use.

Preliminary tests were required to determine the species-specific pollen sensitivity to the sugar concentration of the germination media (Bajaj, 1987). To this end, hydrated pollen (see below) was mixed with germination media that varied in sucrose content (from 10 to 30 % with a 2 % step) and allowed to germinate at room temperature (22 °C) for 18 h. The medium with the highest germination rates and longest pollen tubes was considered as optimal and subsequently used in the germination experiments.

To avoid the pollen grains bursting, a hydration procedure was performed over a saturated KCl solution for 6 h at 5 °C (Connor and Towill, 1993). After hydration, the pollen was mixed with the appropriate PG media, and 150 μL of the mixture was pipetted into a germination chamber produced by cutting a 96-well PCR plate into 24 pieces (with four wells each). The pre-processed pollen samples were maintained at nine different temperatures (5, 9, 12, 16, 20, 23, 27, 31 and 34 °C) on a thermogradient table (RUMED 5990, Rubarth Apparate GmbH, Laatzen, Germany). There were six replicates at each temperature except for *Globularia cordifolia* for which, due to a shortage of pollen, the number of replicates was reduced to four. After 18 h, PG was terminated by pipetting approx. 100 μL of formalin acetic alcohol (Pigott and Huntley, 1981) into each germination chamber, then stored at 5 °C before measurement.

PG was estimated by examining 300 pollen grains in wells (inverted microscope; approx. 10–15 microscopic fields of view) for each replicate. Germination was defined as having occurred when the length of the pollen tube was at least double grain diameter (Kakani *et al*., 2005). PG was determined by dividing the number of germinated pollen grains per field of

view by the total number of pollen grains per field of view, expressed as a percentage.

To estimate the effect of temperature on PTG, images of 25 randomly selected pollen tubes from each replicate were captured with Axiovision version 4.3. software using an AxioCamMR camera (Carl Zeiss, Oberkochen, Germany). The pollen tube lengths were measured manually using ImageJ software (Abramoff *et al*., 2004).

Statistical analysis

All statistical calculations were performed in R software version 3.2.0 (R Core Development Team, 2012).

Cardinal temperatures

To quantify minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}) temperatures of PG and PTG, the generalized plant growth model (eqn 1; Yin and Kropff, 1996) was fitted to PG rate and PTG length versus test temperatures. This flexible model, which is directly specified from the cardinal temperatures, is found to be the best predictive model for the cardinal temperatures of plant growth processes [e.g. seed germination (Derakhshan *et al*., 2014) or flowering time (Yin *et al*., 1995)]. An iterative optimization method was applied to estimate the model parameters based on the *port* algorithm in library *nls* implemented in R software version 3.2.0 (R Development Core Team, 2012). For this, residual sums of squares were used to detect the best estimates of parameters.

$$R = R_{max}\left[\left(\frac{T - T_{min}}{T_{opt} - T_{min}}\right)\left(\frac{T_{max} - T}{T_{max} - Topt}\right)^{\left(\frac{Tmax - Topt}{Topt - Tmin}\right)}\right]^{a} \quad (1)$$

where T_{min}, T_{opt} and T_{max} are the minimum, optimum and maximum temperatures for PG rate or pollen tube length (R), T is the temperature at which germination and tube growth were studied, R_{max} is a maximum value of R at T_{opt} and a is coefficient defining the curvature of the relationship.

Phylogenetic signal in the cardinal temperatures of PG and PTG

As related species are likely to share similar attributes (Harvey and Pagel, 1991), prior to estimating the relationship between the cardinal temperatures of PG and PTG and MAT, we calculated an estimated value of K, a Brownian motion-based metric of the strength of phylogenetic signal (Blomberg *et al*., 2003), using the *phylosignal* function in the 'picante' library (Kembel *et al*., 2010). $K = 1$ indicates that closely related species have trait values that are similar to those expected given Brownian motion; $K < 1$ indicates that closely related species have trait values that are less similar than expected given a Brownian model of evolution.

Relationship between PG/PTG and MAT

To assess the relationship between the temperature experienced by the plant in its natural habitat and the temperature requirements of the progamic phase of fertilization, cardinal temperatures (response variables) were correlated with collection site MATs (predictor) using phylogenetic generalized least squares (PGLS) regression models implemented in the *cape* library (Orme *et al*., 2013). We preferred to use PGLS for our regression models, because closely related species are likely to have similar trait values (Harvey and Pagel, 1991) and therefore violate assumptions of independence for traditional statistical analysis (Kraft *et al*., 2015). Whereas components of the error term in an ordinary least squares regression are assumed to follow a normal distribution around a mean of zero and variance σ^2, PGLS corrects for the effect of non-independence in observations by incorporating an expected model of evolution and phylogeny into the variance–covariance matrix specified for the error term of a linear model (Kraft *et al*., 2015). Our analysis assumed a simple continuous evolutionary model of Brownian motion subject to the scaling factor, λ (Pagel, 1999). λ, estimated in each model using a maximum-likelihood (ML) approach, is a constant that allows one to assess the strength of a phylogenetic signal in the residuals of a regression model (Kraft *et al*., 2015), which ranges between 0 and 1. More specifically, $\lambda = 0$ indicates phylogenetic independence among observations (i.e. the variation of a trait is modelled as a function of an independent evolution along the branches leading to the tips) and thus the resulting PGLS is equivalent to ordinary least squares (Kraft *et al*., 2015). When $\lambda = 1$, the trait shows variation expected under the Brownian motion model of evolution (Freckleton *et al*., 2003). Intermediate values of λ indicate varying degrees of phylogenetic dependence in the data. The phylogenetic tree used in the analysis was based a documented phylogeny of a large European flora (Durka and Michalski, 2012). In all the models, non-linear terms were also considered ($T_{cardinal} \sim MAT + MAT^2$).

Effect of flowering phenology on PG and PTG variations

To account for any possible effect of flowering phenology on PG and PTG variations among the studied species, we also included in all PGLS models temperature of the flowering month (TFM) both as an independent variable and as an interaction with MAT (TFM:MAT) as predictors of a cardinal temperature. The PGLS models with all variables included were reduced via backward selection of the least significant variables until we achieved the minimal adequate model (Crawley, 2007). Following model-fitting, the model requirements (a normal distribution and homogeneous variances in the residuals) were checked. All explanatory variables were considered to be not collinear, because they were not correlated to each other.

RESULTS

Distributional ranges and flowering phenology

Distributional ranges along the MAT gradient differed considerably among the study species from typical lowland species, such as *Anemone pulsatilla* or *Primula veris* (MAT range 6·2–9·0 °C for both), to species with main distribution in the alpine belt, e.g. *Campanula alpina* and *Primula minima* (MAT range 1·9–3·6 °C for both species; Table 1).

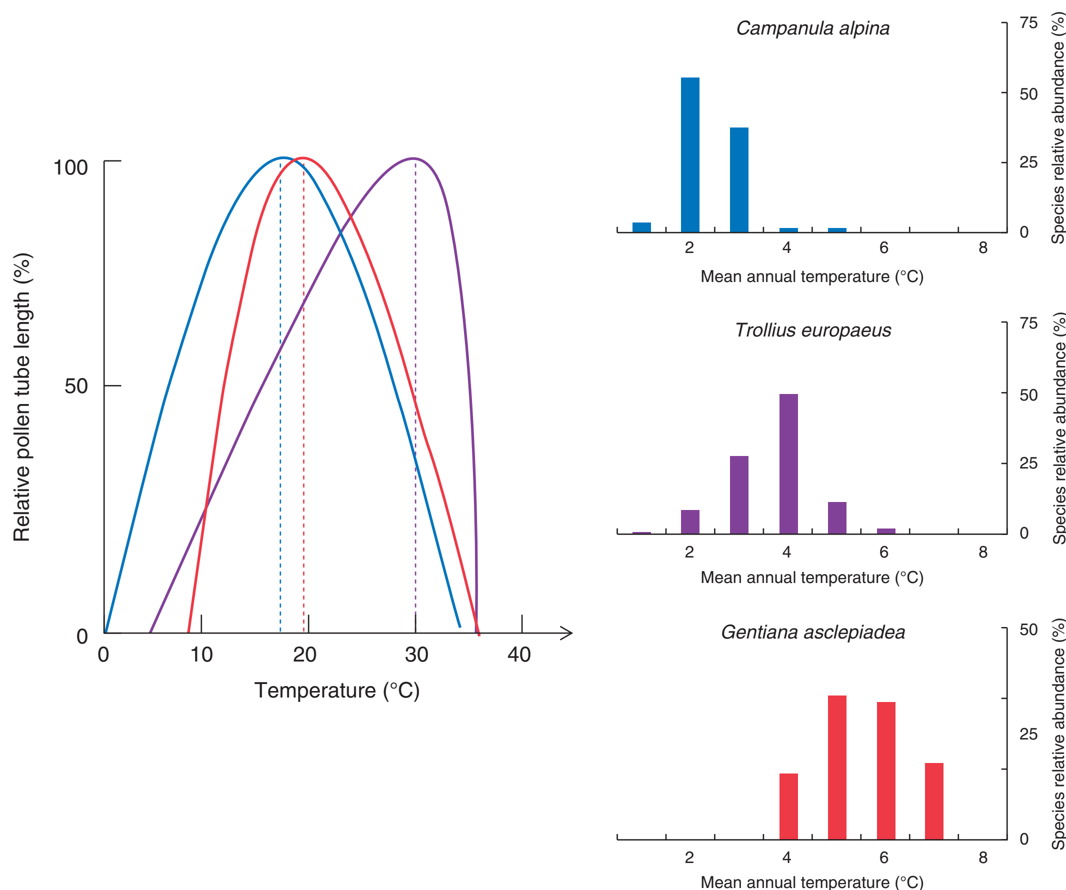

Fig. 1. Differences in the temperature requirements for PTG of three species (*Campanula alpina*, *Trollius europaeus* and *Gentiana asclepiadea*) occurring in climatically contrasting habitats. The cardinal temperatures were estimated by fitting the generalized plant growth model to the experimental data for pollen tube length (see Materials and Methods section). The species relative abundances along the gradient of mean annual temperatures relate to the vegetation survey in the study area (see Materials and Methods section).

Flowering phenology also varied, from early-flowering species, such as *Helleborus niger* or *Soldanella alpina* (March and April, respectively) to late-flowering species, e.g. *Aconitum napellus* to *Parnassia palustris* (both flower in August). Correspondingly, TFM varied from 5 °C (*Anemone nemorosa*) to 15·7 °C (*Erica carnea* and *Carex caryophyllea*) with an average of 10·3 °C.

Cardinal temperatures of PG

Cardinal temperatures for PG differed greatly; T_{min} ranged from 0 °C (e.g. *Campanula alpina*) to 12·2 °C (*Ranunculus acris*), with a mean of 4·1 °C. The optimum temperature (T_{opt}) ranged from 14·5 °C (*Helleborus niger*) to 31·8 °C (*Parnassia palustris*), with a mean of 21·9 °C. The T_{max} values varied from 33·4 °C for *Helleborus niger* to 40·0 °C for *Carex caryophyllea* and *Globularia cordifolia*, with a mean of 35·3 °C (Table 1).

Due to the presence of anther debris obscuring pollen grains, PG rates could not be measured exactly for *Aconitum napellus*, *Carex flacca*, *Gentiana asclepiadea* or *Trollius europaeus*.

Cardinal temperatures of PTG

We found considerable variation in pollen tube length at the minimum, optimum and maximum cardinal temperatures (Table 1). The values of T_{min} ranged from 0 °C (e.g. *Erica carnea* and *Phyteuma orbiculare*) to 12 °C (*Ranunculus acris*). The magnitude of T_{opt} ranged from 16·3 °C (*Phyteuma orbiculare*) to 34·3 °C (*Carex caryophyllea*). The T_{max} values ranged from 30·5 °C for *Phyteuma orbiculare* to 40 °C for, for example, *Erica carnea* and *Gentianella aspera*. The mean values for T_{min}, T_{opt} and T_{max} were 4·1, 25·0 and 35·7 °C, respectively.

PTG responses to the temperature treatments of three species (*Campanula alpina*, *Trollius europaeus* and *Gentiana asclepiadea*) from climatically contrasted habitats showing reduction in T_{min} as MAT decreases are illustrated in Fig. 1. Estimated cardinal temperatures for PG and PTG are presented in Table 1.

Phylogenetic signal

A phylogenetic signal was not detected in PG, irrespective of the cardinal temperature [T_{min} ($K = 0{\cdot}10$, $P = 0{\cdot}63$); T_{opt} ($K = 0{\cdot}10$, $P = 0{\cdot}67$); T_{max} ($K = 0{\cdot}36$, $P = 0{\cdot}15$)]. Among cardinal temperatures for PTG, a moderate phylogenetic signal was only detected in T_{opt} ($K = 0{\cdot}43$, $P = 0{\cdot}03$). The low and non-significant K values ($K = 0{\cdot}11$, $P = 0{\cdot}72$ and $K = 0{\cdot}23$, $P = 0{\cdot}37$, respectively) indicate that T_{min} and T_{max} of PTG are not phylogenetically constrained. Measures of the phylogenetic signal (measured as Bloomberg's K) are presented in Table 2.

TABLE 2. *Phylogenetic conservatism in cardinal temperatures of PG and PTG rate according to Bloomberg's* K*-statistics (for details see Materials and Methods section)*

Trait	Pollen germination		Pollen tube growth	
	K	*P*	*K*	*P*
T_{min}	0·10	0·63	0·11	0·72
T_{opt}	0·10	0·67	0·43	**0·03**
T_{max}	0·36	0·15	0·23	0·37

$K = 1$ indicates that closely related species have trait values that are similar to those expected given Brownian motion. $K < 1$ indicates that closely related species have trait values that are less similar than expected given a Brownian model of evolution.

TABLE 3. *The relationship between PG and PTG, habitat mean annual temperature (MAT) and mean temperature of flowering month (TFM) estimated by phylogenetic least squares analysis (see Material and Methods section for details)*

	Adj. R^2	*P*	λ
Pollen germination			
$T_{min} \sim$ MAT	0·25	**0·01**	0·118
$T_{opt} \sim$ MAT	–0·02	0·45	0
$T_{max} \sim$ MAT	0·14	0·06	0·113
Pollen tube growth			
$T_{min} \sim$ MAT	0·46	**<0·001**	0·281
$T_{opt} \sim$ MAT	0·07	0·11	0·69
$T_{max} \sim$ TFM	0·19	**0·03**	0

The minimum adequate models with corresponding coefficient of determination (Adj. R^2), significance of model terms (*P*) and measure of phylogenetical signal included as parameter in the models (λ; shown as here as a maximum-likelihood estimate) are shown.

Correlations between cardinal temperatures and habitat temperatures

Among studied cardinal temperatures of PG, only T_{min} was positively linearly correlated with MAT (Table 3; Fig. 2A, $r^2 = 0{\cdot}25$, $P = 0{\cdot}01$; test accounted for phylogeny via PGLS).

An analysis of the PTG data showed that minimum temperature was also a significant predictor of the species occurrence along the MAT gradient (Table 3; Fig. 3A, $r^2 = 0{\cdot}46$, $P < 0{\cdot}001$; test accounted for phylogeny via PGLS). In contrast, the optimum and maximum temperatures of PTG were not correlated with MAT.

Effect of flowering phenology on PG and PTG variations

A significant correlation between TFM and temperature requirements of the progamic phase of fertilization was found only for T_{max} of PTG (Table 3; Fig. 3C; $r^2 = 0{\cdot}19$, $P = 0{\cdot}03$; tests accounted for phylogeny via PGLS), suggesting that PTG of species which flower under relatively high ambient temperatures is suppressed by higher temperatures.

DISCUSSION

It has been suggested that because of their association with more favourable climatic conditions, pollen grains of species from habitats with a higher MAT are adapted to germinate and grow under relatively high temperatures (Weinbaum *et al.*, 1984; Jakobsen and Martens, 1994; Pasonen *et al.*, 2000; Kremer and Jemrić, 2006). Our results are consistent with this hypothesis: the pollen of the majority of 'low-altitude' species began to germinate and grow at relatively high temperatures. For example, species collected in the warmest habitat in our study system (MAT 8 °C; Table 1), namely *Anemone pulsatilla*, *Globularia cordifolia* and *Primula veris*, had the highest temperature requirements (except *Ranunculus acris*) for both PG and PTG (in all cases 9 °C).

A decreasing MAT along a climatic gradient is coupled with an increasing probability of negative temperature stress: this may take the form of freeze–thawing cycles in spring or autumn or as freezing episodes during the growing season (Sakai and Larcher, 1987; Körner, 1999). In species of cold habitats, fertilization can be adapted to this stress by plants reducing their temperature requirements for PG and pollen tube elongation (Zamir *et al.*, 1981; Steinacher and Wagner, 2012). Our results are again consistent with previous findings: the minimal temperature of PG and PTG was on average 4·3 °C less for species from relatively cold habitats (MAT < 5·4 °C; Table 1). Thus, the minimal temperatures for both PG and PTG are strongly negatively correlated with the mean annual habitat temperature (Figs 2A and 3A). Moreover, these specific temperature requirements were good predictors of species occurrence along a MAT gradient (for example PTG; Fig. 1).

In addition to habitat temperature conditions, flowering phenology was also correlated with the temperature requirements of PG and PTG *in vitro*. It is known from crop plants that pollen of species and cultivars that flower under relatively high temperatures germinates and grows tubes at relatively high temperatures (e.g. Luza *et al.*, 1987; Kakani *et al.*, 2005). Our results partially confirm this pattern; the positive correlation between TFM and cardinal temperatures of PG and PTG was only found in the linear model for T_{max} of PTG (Table 3; Fig. 3C). This finding suggests that regardless of MAT of a habitat, species flowering at different times of year differ in their upper temperature thresholds for PTG. As an example from our data set, *Carex caryophyllea*, which flowers at below 15·7 °C, shows a difference of PTG T_{min} (0 °C) of 6 °C to *Anemone nemorosa* (TFM 5 °C). The increased tolerance of PTG of species flowering under relatively warmer environmental conditions to high temperatures could be explained by higher temperature regimes within their flowers. However, in our dataset the overall effect of flowering phenology on the expression of maximal temperature of PTG is minor: TFM explained only 12 % of the trait variation (Table 3).

The strong correlation between habitat temperature and the two studied components of the progamic phase was consistent with the temperature requirements of PG and PTG impacting upon species distribution. The PG and PTG of species characteristic of warm habitats both require relatively high temperatures to induce the progamic phase of fertilization successfully after pollen adhesion on stigmas. When moving along the temperature gradient, a decreased MAT may result in increasingly suboptimal temperatures for PG and PTG and negatively affect seed production. Potentially, this reduction in reproductive output will in turn affect a species distribution, limiting its capacity to expand its geographical range or even to maintain existing

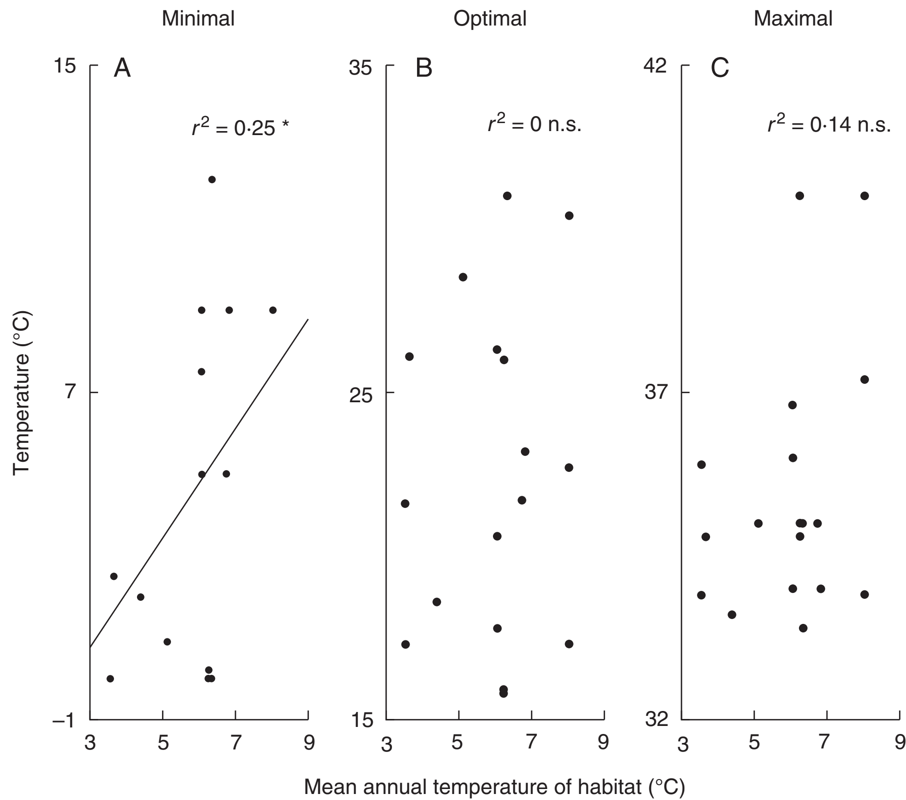

Fig. 2. Relationships between the temperature requirements of pollen germination (PG) and mean annual temperature of habitats where species occur: (A) minimum temperature of PG; (B) optimum temperature of PG; (C) maximum temperature of PG. Statistical significances: $*0·01 < P < 0·05$, n.s. (not significant) $P > 0·05$.

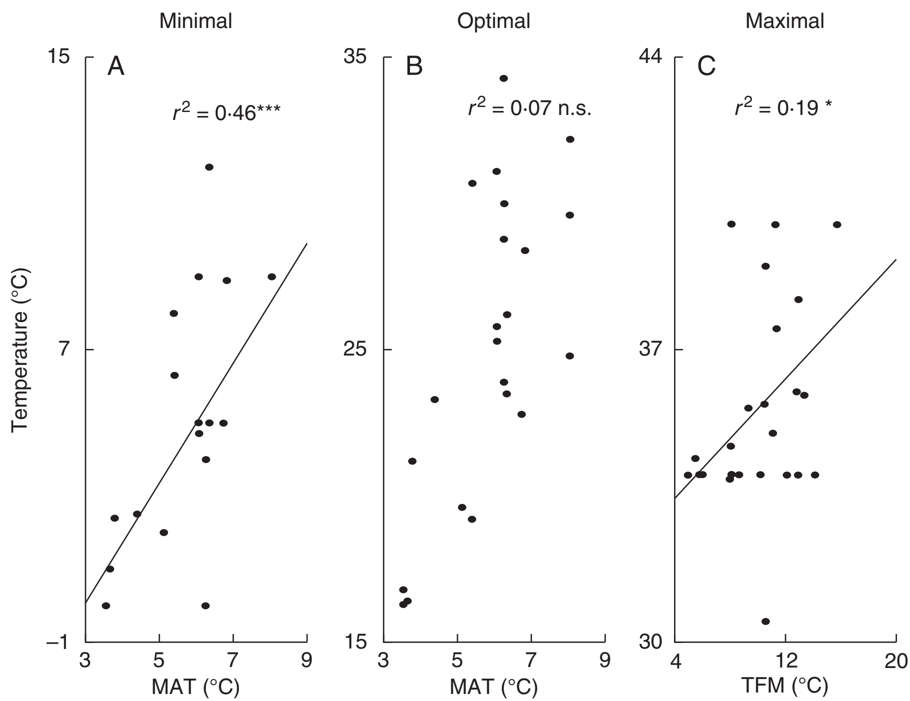

Fig. 3. Relationships between the temperature requirements of pollen tube growth (PTG) and mean annual temperature (MAT) of habitats where species occur (A and B are minimum and optimum temperature of PTG, respectively). (C) Relationship between the maximum temperature of PTG and temperature of flowering month (TFM) when pollen was collected. Statistical significances: $***P < 0·001$, $*0·01 < P < 0·05$, n.s. (not significant) $P > 0·05$.

populations (Grubb, 1977; Pigott and Huntley, 1981; Turnbull *et al*., 2000). In contrast, species whose pollen can germinate and grow at lower temperatures can successfully complete the progamic phase of fertilization, even in regions with a low MAT. This allows range expansions poleward or into higher elevations. Notwithstanding this, the influence of traits such as slow growth rate and short stature, which lead to low competitive ability (Woodward, 1975; Körner, 1999), may limit expansion into higher MAT regions.

Our results suggest that the inclusion of a 'pollen dimension' enables improved understanding of existing patterns of plant biogeography and probably plant distributional responses to anthropogenic climate change. For example, the strong correlation between PG and PTG minimal temperature and habitat temperature found here suggests that upward range shifts and changes in plant abundances in alpine vegetation (Gottfried *et al*., 2012; Pauli *et al*., 2012; Rosbakh *et al*., 2014; Mondoni *et al*., 2015) could be partly due to changes in seed set. We speculate that the temperature rise and associated phenological shifts observed in the Alps in recent decades have alleviated restrictions of harsh high-altitude environments (both extreme weather events, such as frosts, and generally low average temperature) on sexual reproduction for lowland species. These species, which require relatively high temperatures to initiate PG and PTG, could benefit from the relaxation of the low-temperature filter by increasing seed production and contribute to range extension into alpine vegetation. Furthermore, in the last decade, a strong emphasis has been placed on modelling of vegetation–climate interactions (Parmesan and Hanley, 2015). However, these models still suffer from an essential lack of temperature-specific ecological data and a mechanistic understanding of how environmental factors shape plant ecophysiology and current species distributions (Mondoni *et al*., 2015; Parmesan and Hanley, 2015). We suggest that the temperature requirements of PG and PTG *in vitro* could easily be integrated into environmental niche models as they can quantitatively estimate the success of reproduction under specific climatic conditions and are technically more easily measured than those relating to many other stages of the plant life cycle.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: Collection sites of the studies species.

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