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RESEARCH PAPER

Temperature controls seed germination and dormancy in the European woodland herbaceous perennial *Erythronium dens-canis* (Liliaceae)

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Keywords

Ecological adaptation; embryo growth; epicotyl morphophysiological dormancy; radicle and cotyledon emergence; seed germination.

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ABSTRACT

We examined the germination ecology and the temperature requirements for germination of *Erythronium dens-canis*, under both outdoor and laboratory conditions. *E. dens-canis* is a spring flowering woodland geophyte widely distributed across Europe. Germination phenology, including embryo development and radicle and cotyledon emergence, were investigated in a natural population growing in Northern Italy. Immediately after harvest, seeds of *E. dens-canis* were either sown on agar in the laboratory under simulated seasonal temperatures or placed in nylon mesh sachets and buried in the wild. Embryos, undifferentiated at the time of seed dispersal, grew during summer and autumn conditions in the laboratory and in the wild, culminating in radicle emergence in winter when temperatures fell to ~5 °C. Emergence of cotyledons did not occur immediately after radicle emergence, but was delayed until the end of winter. Laboratory experiments showed that temperature is the main factor controlling dormancy and germination, with seeds becoming non-dormant only when given warmth, followed by cold stratification. Unlike seeds of *E. dens-canis* that germinate in winter, in other *Erythronium* species radicle emergence occurs in autumn, while in some it is delayed until seeds are transferred from winter to spring conditions. Our results suggest that there is genetic and environmental control of the expression of seed dormancy amongst *Erythronium* species, which is related to local climate.

INTRODUCTION

Erythronium dens-canis (Liliaceae) is a herbaceous woodland perennial herb, widely distributed in Central and South Europe (Tutin *et al.* 1964). It is the only species of the genus *Erythronium* growing in Europe, with another ~30 species found in northern temperate regions of Asia and North America (Brian 1992). In Italy, it is found mostly in deciduous woodlands (Pignatti 1982) ascribed to *Carpino-Fagetea sylvatica* and less frequently in mesic meadows (Aeschimann *et al.* 2004) in lowland and mountain (N Apennines and Alps) locations from 0 to 600 m-a.s.l. At present, lowland populations have become rare (Pignatti 1982) due to an extensive replacement of natural woodland areas with agriculture fields.

Erythronium dens-canis undoubtedly has great aesthetic appeal, which gives this species potential for use in gardens or in habitat restoration activities (Rossi *et al.* 2009). It is an early-flowering plant that develops leaves and flower shoots from buds on perennial bulbs. Reproductively mature individuals are 10–30 cm high, with two opposite leaves at the base of the scape, and three stigmas of variable length. Flowering begins in March. The fruit is a capsule containing numerous elaiosome-bearing, ant-dispersed seeds. The capsules dehisce in May.

Despite the considerable information available on non-European *Erythronium* species, the biology of *E. dens-canis* is poorly understood (Guitián *et al.* 1999). Some of the aspects investigated include its reproductive success and seed dispersal strategies. *E. dens-canis* is characterised by floral phenotypes, with small-flowered plants producing fewer seeds than large-flowered plants, and this seems to be due to the less efficient pollination of small flowers (Guitián *et al.* 1999). Primary seed dispersal of *E. dens-canis* is negligible, since most seeds fall within 10 cm of the mother plant (Guitián *et al.* 2003). Myrmecochory is the only secondary seed dispersal mechanism for this species, although the efficiency of ant dispersal is highly variable, depending on habitat characteristics.

As far as we are aware there have been no previous studies on seed germination behaviour on *E. dens-canis*. However, studies on germination ecophysiology have been extensively reported for other species, such as *E. japonicum* (Kondo *et al.* 2002), *E. albidum* (Baskin & Baskin 1985), *E. grandiflorum* (Baskin *et al.* 1995), *E. americanum* and *E. rostratum* (Baskin & Baskin 1998). In all these cases, seeds could not immediately germinate after dispersal in early summer because they have underdeveloped embryos, consisting of no more than a clump of cells. Embryo elongation occurs slowly throughout the summer and mainly during autumn, but radicles do not emerge until seeds have experienced winter conditions. Seeds

of all these species have been described as having morpho-physiological dormancy (*sensu* Baskin & Baskin 1998). However, radicle emergence was concluded by early winter in all species except *E. albidum*, for which first germination occurred at the end of winter. Embryo growth of *E. grandiflorum* was apparently not dependent on summer and autumn temperatures prior to germination in winter, while embryo growth of *E. japonicum* is distinguished by epicotyl seed dormancy. The extent to which the subtle differences in germination phenology observed might indicate variation in dormancy patterns in the genus *Erythronium*, rather then represent adaptations to the local habitat conditions, remains to be investigated.

In this paper, in an attempt to further map dormancy patterns in the genus *Erythronium*, we examined the germination ecology and temperature requirements for germination of *E. dens-canis* under both outdoor and laboratory conditions.

METHODS

Seed collection

Collections of seed were made at the time of natural dispersal (Hay & Smith 2003), on 21 May 2010, from a population of *E. dens-canis* growing in a shady, deciduous woodland (ascribed to *Carpino-Fagetea sylvatica*) in the Ticino Natural Park, Po Plain in Lombardy; ~79 m a.s.l., in northern Italy.

Phenology of embryo growth and germination in the laboratory

All laboratory experiments involved sowing three replicates of 30 seeds each on 1% distilled water agar held in 9-cm Petri dishes. Treatments were incubated in temperature- and light-controlled incubators using a 12-h daily photoperiod (photosynthetically active radiation 40–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

On the day of harvest, seeds were placed into a temperature regime simulating seasonal changes occurring in the west Po plain (Mariani *et al.* 2001). Spring–summer conditions (May to September) were simulated by 140 days at 25/15 °C; early autumn (October) by 28 days at 20/10 °C; late autumn (November) by 28 days at 15/5 °C; winter (December to February) by 84 days at 5/0 °C; early spring (March) by 28 days at 15/5 °C; and late spring (April) by 28 days at 20/10 °C. In each case, a 12 h/12 h thermoperiod was used and illumination was provided during the warm phase. This cycle of temperatures was also used in completely dark conditions.

On the same day, ten seeds were dissected and the embryo length measured under a binocular microscope equipped with a micrometer. The ratio embryo length to seed length was calculated as a percentage on an endosperm length basis, since at the time of seed dispersal the endosperm represents almost the entire seed. Observations of embryo growth and root and cotyledon emergence were made every 28 days during summer and then at 7-days intervals (14-days in the case of embryo growth) until the end of the simulated cycle. Germination was defined as radicle protrusion and elongation of more than 2 mm. Following radicle emergence, the development of cotyledons were monitored throughout, either by leaving seedlings to experience the rest of the simulated cycle of seasonal temperature or by immediately moving them to warm conditions (e.g. late spring, 20/10 °C). This was set up to investigate the hypothesis that, following radical emergence in winter, further exposure of seedlings to winter temperatures is necessary to overcome physiological dormancy in the epicotyl.

Alternating temperatures were used because in the natural habitat seeds experience diurnal temperature variation (Baskin & Baskin 1998). However, because the forest cover and leaf litter are effective insulators of daily temperature variations (Ellenberg 1988; Mondoni *et al.* 2008), additional germination tests were set up to investigate the effect of constant temperatures. The following regimes were used: 140 days at 20 °C (summer) followed by 28 days at 15 °C (early autumn), 28 days at 10 °C (late autumn), 84 days at 5 °C (winter), 28 days at 10 °C (early spring) and 28 days at 15 °C (late spring). This cycle of temperatures was also performed, but with winter conditions set at 0 °C, which can be considered as the minimum temperature occurring under leaf litter at the study site (Mondoni *et al.* 2008).

Controls were also set up to investigate the effects of prolonged incubation at each of the mean seasonal alternating temperatures (5/0, 15/5, 20/10 and 25/15 °C). Seeds were checked for root and cotyledon emergence at weekly intervals over 300 days.

Effects of summer, autumn and winter conditions on root emergence

To examine the importance of summer conditions, samples of seeds were held at 25/15 °C for 0, 30 and 140 days. After each period, seeds were moved to autumn and then to winter conditions. The importance of autumn and winter conditions was examined after seeds had experienced the simulated summer condition (140 days at 25/15 °C), moving seeds to the other seasonal conditions, either skipping the autumn (28 days at 20/10 °C, followed by 28 days at 15/5 °C) or the winter (84 days at 5/0 °C). Furthermore, to examine the level of physiological dormancy, GA_3 (722 μM) was applied during a simulated early spring period in the absence of preceding winter conditions. The numbers of seeds and replications for each test, method of sowing, light conditions and observations were as described above.

Phenology of embryo growth and germination in the wild

At the time of collection, 25 fine-mesh polyester bags with 50 seeds each were buried approximately 5 cm under leaf litter at the collection site. Sachets were retrieved at 30-days intervals from May to September and then at 15-days intervals until mid-February. Embryo growth, radicle emergence and cotyledon emergence were monitored throughout. Soil temperature at the level of the sachets was recorded at hourly intervals using Tiny Tag data loggers (Gemini Data Logger Ltd, Chichester, UK).

Data analysis

Embryo growth data were analysed using linear regression in Minitab 14 (Minitab Inc., State College, PA, USA) on subsets of the data. To describe the progress of germination in the laboratory, the Gompertz function was fitted to cumulative germination data using Origin 6.1, according to the following formula:

$$y = a * \exp(-\exp(-t - t_{50})/b)$$

where y = germination percentage at time t (days), a = final average percentage germination, t_{50} = time to 50% germination and b = germination rate.

In addition, the mean germination time (MTG) was calculated using the formula:

$$MTG = \Sigma_{i,n} n_i t_i / N$$

where n_i is the number of seeds that germinated within consecutive intervals of time, t_i is the time between the beginning of the test and the end of a particular interval of measurement, and N is the total number of seeds that germinated.

Finally, *t*-tests were carried out in Minitab 14 to compare the final proportions of germinated seeds and the mean germination time under different conditions.

RESULTS

Phenology of embryo growth and germination in the laboratory

Embryos of *E. dens-canis*, undifferentiated at the time of dispersal, showed a slow, but significant ($F_{2,3}$ = 11.92, P = 0.041), linear increase in size (0.0065 mm·day^{-1}) over a period of ~140 days under continuous, simulated summer conditions in the laboratory (25/15 °C), when they occupied approximately 23% of the whole endosperm length (3.64 mm; Fig. 1A). Following transfer to autumn conditions (28 days at 20/10 °C followed by 28 days at 15/5 °C), embryo growth rate accelerated fivefold to 0.033 mm·day^{-1}, eventually occupying ~87% of the whole endosperm length ($F_{3,4}$ = 183.51, P = 0.005). However, seeds did not germinate during the simulated autumn conditions. Indeed, radicles of *E. dens-canis* began to emerge 28 days after transfer from autumn to winter conditions (0/5 °C) and growth was completed 56 days later (Fig. 1B). Under dark conditions, the progress of germination was similar to that observed in the light, with about 100% of seeds germinating over the same period (t = 2.02, P = 0.181).

In the simulated regime of constant temperatures, seed germination was significantly faster compared to that of the simulated alternating temperature regime (t = 24.04, P < 0.002), but there was no difference in final germination percentage. At constant temperatures, germination began 14 days after seeds were transferred to 5 °C, with 100% of seeds having germinated by 21 days. However, when winter temperature was held at constant 0 °C, seed germination did not occur until seeds were moved to early spring conditions (10 °C; Fig. 2).

In all cases, the cotyledon did not emerge immediately after radicle emergence, but was delayed until the end of winter. Furthermore, when seeds with an emerged radicle were immediately moved to late spring temperature (20/10 °C), cotyledon emergence did not exceed 40% (Fig. 1B).

Effects of summer conditions on root emergence

Increasing the length of the simulated summer conditions from 0 to 30 days resulted in a reduction in the mean germination time (t = 16.97, P = 0.003) when seeds were moved from autumn to winter temperature. In contrast, the total percentage of seeds germinating was unchanged (t = 2.14, P = 0.166). There was 71% radicle emergence after 77 days of winter conditions for seeds given 0 days of summer conditions, compared with 75% after 56 days for seeds given 30 days of summer (Fig. 3). Longer periods of summer conditions (140 days) resulted in a further increase in final germination (100%), although mean germination time was unchanged (t = −1.99, P = 0.185).

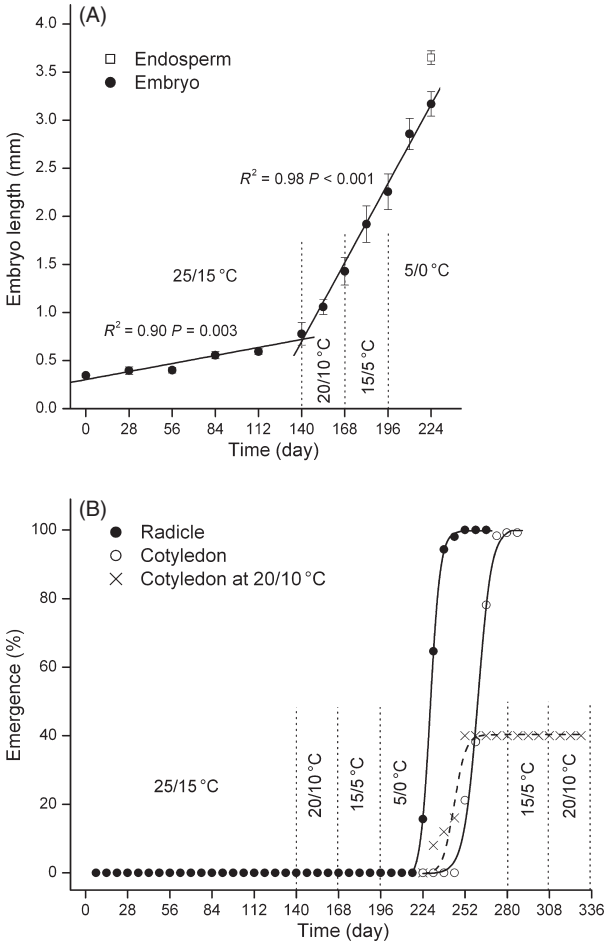

Fig. 1. A: Linear regressions of embryo growth of *E. dens-canis* in the laboratory at simulated summer (25/15 °C), autumn (20/10 and 15/5 °C) and winter (5/0 °C) temperatures. Bars are ±SE. Also shown is the length of the mucilaginous endosperm (open square), within which the embryo develops. B: Germination progress curves for seeds of *E. dens-canis* at simulated seasonal temperatures. Radicle (closed symbols) and cotyledon (open symbols). Also shown is cotyledon emergence when seeds with an emerged radicle were immediately moved to 20/10 °C (X symbol, broken line). Curves were fitted using the Gompertz function.

Effects of autumn and winter conditions on seed germination

In the absence of autumn conditions (30 days at 20/10 °C followed by 30 days at 15/5 °C), ~60% of summer-treated seeds (140 days at 25/15 °C) had an emerged radicle by the end of winter, whereupon no further germination occurred. In contrast, when summer- and autumn-treated seeds were

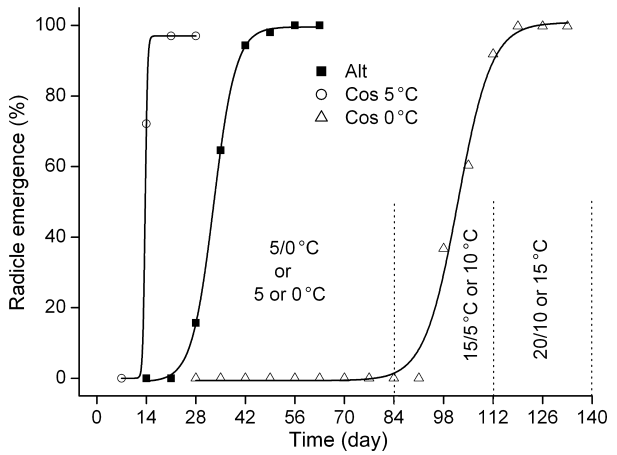

Fig. 2. Radicle emergence for seeds of *E. dens-canis* held at simulated seasonal constant (open symbols) and daily alternating temperatures (closed symbols), after summer (20 °C or 25/15 °C) and autumn (20/10 °C or 15 °C and 15/5 °C or 10 °C) temperatures (not shown in the graph). In the simulated seasonal cycle of constant temperatures, winter was either kept at 5 °C or 0 °C as indicated.

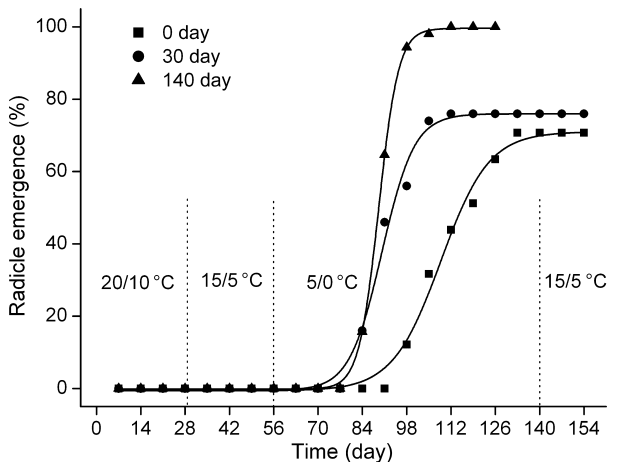

Fig. 3. Radicle emergence for seeds of *E. dens-canis* at simulated seasonal winter temperatures (5/0 °C), after increasing durations of summer conditions (0, 30 and 140 days at 25/15 °C, as indicated) and after autumn temperatures (20/10 and 15/5 °C). Curves were fitted using the Gompertz function.

placed directly into spring conditions (28 days at 15/5 °C followed by 28 days at 20/10 °C), there was no germination. However, under the same cycle of simulated temperatures with GA_3 (250 $ml \cdot l^{-1}$) applied during the early spring (15/5 °C), 83% of the seed population germinated (Fig. 4).

Controls

No seeds germinated in the absence of summer and autumn conditions or when they were kept under continuous 25/15, 20/10 and 5/0 °C conditions. After 175 days at 15/5 °C, however, seeds began to germinate slowly, with 50% radicle emergence recorded after a further 160 days (data not shown).

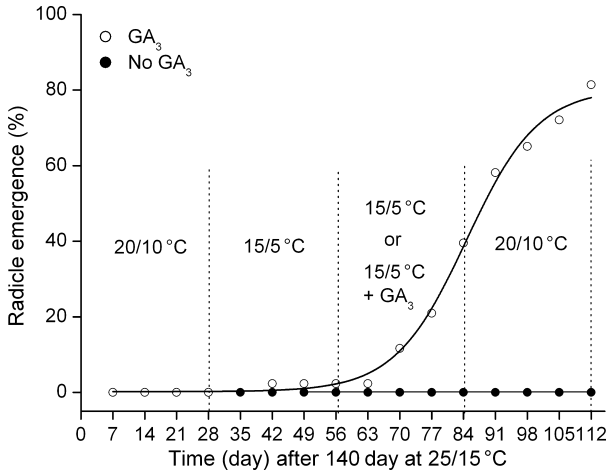

Fig. 4. Radicle emergence progress curves for seeds of *E. dens-canis* at simulated seasonal temperatures after 140 days at 25/15 °C in the absence of winter conditions (closed symbols) and under the same conditions but with applied GA_3 (open symbols) during a phase representing early spring (15/5 °C). Curves were fitted using the Gompertz function.

Phenology of germination under natural conditions

In agreement with the germination phenology observed in the laboratory, embryos in seeds of *E. dens-canis* buried in the wild grew slowly during the summer season (May–September), when soil surface temperature was ~20 °C. Embryos grew faster in autumn (October–November), when the temperature had dropped to ~10 °C (data not shown). Radicle emergence was first recorded at the end of November, when temperatures had dropped further to ~5 °C, and was almost completed by the end of December (Fig. 5). Cotyledon emergence occurred at the same temperatures, but first emergence was delayed under natural conditions until the beginning of February.

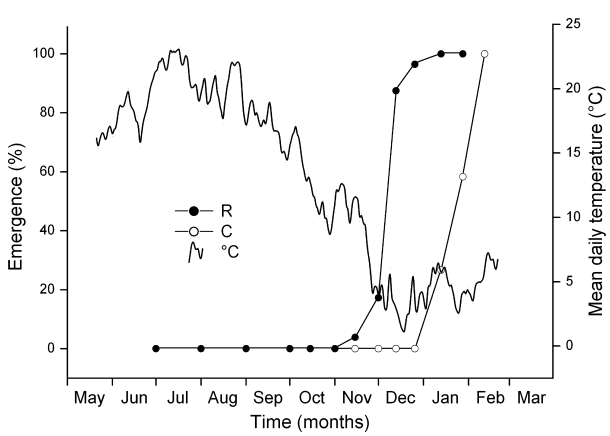

Fig. 5. Radicle (R) and cotyledon (C) emergence from seeds of *E. dens-canis* in the wild. Also shown is the mean daily soil surface temperature (°C) calculated from measurements made at hourly intervals at the study site.

DISCUSSION

Embryos of *E. dens-canis* were underdeveloped at time of seed dispersal in early May; differentiation and growth occurred slowly during the summer (May–September), accelerated when temperatures fell in the autumn (October–November), culminating in radicle and cotyledon emergence when temperatures dropped further in the winter (December).

Under simulated seasonal temperatures in the laboratory, germination phenology of *E. dens-canis* was remarkably similar to that observed in the wild, with 100% of seeds germinating in the winter, after having experienced summer and autumn simulated seasons, during which embryos had differentiated and grown. Despite the fact that 70% of autumn-treated seeds were capable of germination when moved to winter, summer followed by autumn conditions resulted in more complete germination (Fig. 3). It is therefore tempting to speculate that summer temperature may lead to morpho-physiological changes in seeds that speed up germination when they are moved to winter temperatures. Seed germination was <60% when summer-treated seeds were moved directly to winter conditions, indicating that embryo growth is also possible in winter. However, the possibility that a longer exposure to winter temperatures may lead to higher germination cannot be ruled out. Moreover, no seed germination occurred in the absence of both summer and autumn simulated seasons, indicating that high followed by medium or low temperature is needed for embryo development and germination. This evidence indicates that, according to the Baskin & Baskin (1998) system of dormancy classification, seeds of *E. dens-canis* should be described as having morpho-physiological dormancy (MPD). Furthermore, summer- and autumn-treated seeds required cold stratification or GA_3 treatment before they were able to germinate; therefore the type of MPD is best described as intermediate simple.

Kondo *et al.* (2002) reported that, while radicle emergence of *E. japonicum* was ended by November, cotyledon emergence was completed by the time of snowmelt, in early April. They suggested that such a delay could be due to physiological dormancy in the epicotyl of the seedlings. Here, to investigate the possibility that seeds of *E. dens-canis* could be described as having epicotyl dormancy, seeds with an emerged radicle were immediately moved to warmer conditions (simulating late spring, 20/10 °C) and seedling development was monitored throughout. About 40% of the seedlings moved to spring temperature developed cotyledons, against the 100% recorded for those left at winter temperatures after germination (Fig. 1B), indicating that *E. dens-canis* shows a degree of epicotyl dormancy. Epicotyl dormancy in species with autumn/winter germination, such as *E. dens-canis* and *E. japonicum* probably ensures that seedlings emerging from seeds during late winter remain below the leaf litter or snow cover, where they will be insulated from damaging frosts.

Under continuous 15/5 °C conditions, seeds started to germinate after 175 days. About 50% of seeds had an emerged radicle after 335 days at this temperature, indicating that slow and poorly synchronised embryo growth occurs in seeds placed at 15/5 °C. According to Baskin & Baskin (2003), temperatures suitable for cold stratification range from 0 to 10 °C, while those for warm stratification should be at least 15 °C; therefore 15/5 °C might have had a dual effect in seeds of *E. dens-canis*, acting as both a high temperature pre-treatment required to release the physiological block to embryo growth and a low temperature effect that promoted embryo growth and germination.

Under a simulated cycle of daily constant seasonal temperatures, germination of *E. dens-canis* occurred again in winter (5 °C), but was advanced and faster compared than that in the cycle of daily alternating temperatures (5/0 °C). However, germination was inhibited when summer- and autumn-treated seeds were moved to constant 0 °C, indicating that the slower germination rate at 5/0 °C was not due to the alternating temperature *per se*, but rather to the likelihood that the optimum temperature for germination is probably close 5 °C.

The sensitivity to amplitude of diurnal temperatures has been described as a mechanism that serves to detect canopy shade (Kos & Poschlod 2007), with germination of canopy-associated species being inhibited by high amplitude, diurnal alternating temperatures typical of matrix (open) sites (Ellenberg 1988). Interestingly, unlike some woodland species restricted to closed-canopy conditions, such as *Anemone ranunculoides*, which only germinates at constant temperatures (Mondoni *et al.* 2009), germination of *E. dens-canis* [found in both open- and closed-canopy habitats (Aeschimann *et al.* 2004)] was possible regardless of whether the temperatures were constant or alternating. However, since germination was faster under the constant temperature regime (5 °C) close to that observed in the natural habitat (Fig. 5), the seed population reported here is clearly well adapted to its shaded woodland habitat.

Although subtle differences could be highlighted in natural conditions or in simulated conditions in the laboratory, the pattern of germination phenology in seeds of *E. dens-canis* was similar to that reported for other *Erythronium* species from non-European locations. For example, similar to *E. dens-canis*, seeds of *E. albidum*, *E. paviflorum* and *E. americanum* became non-dormant when given warm followed by cold stratification (Baskin & Baskin 1985, 1998; Baskin *et al.* 1995). However, unlike seeds of *E. dens-canis* that germinated during the cold stratification treatment, in the other *Erythronium* species listed above radicle emergence occurred only after seeds were transferred from winter (5 °C) to spring conditions (*e.g.* 15/6 °C). In contrast, physiological dormancy in seeds of *E. japonicum* was broken by high summer temperature (25/15 or 20 °C), and transfer to winter (5 °C) temperature induced both embryo growth and germination (Kondo *et al.* 2002), as also found in *E. dens-canis*. However, unlike summer-treated seeds of *E. dens-canis*, which were capable of germination only when transferred to winter, about 60% of seeds of *E. japonicum* had germinated in the autumn (15/5 °C), by the time they were moved to the winter temperature (5 °C). Different expressions of dormancy resulting in autumn, winter or spring germination have therefore evolved in these related species.

The subtle differences in germination response reported here, compared to previous reports on other *Erythronium* species, indicate that the timing of germination can be tuned to the local climate. For example, while both *E. dens-canis* and *E. japonicum*, from locations where the coldest winter soil surface temperature is around 0 °C, are adapted to ger-

minate in autumn or winter, seeds of *E. albidum* from Lexington, Kentucky (USA), where winter temperatures can be as low as −20 °C, germinate in the spring when the threat of frost has passed. In support of this, we have shown that when the temperature was lower than 5 °C, there was no germination of *E. dens-canis* seeds. Unlike seeds of *E. dens-canis* and *E. japonicum*, where almost 100% of warm-stratified seeds germinated when transferred to 5 °C, seeds of *E. albidum* failed to geminate when the winter temperature was maintained at 5 °C. Therefore, there appears to be clear genetic and environmental control of the expression of seed dormancy in these species, which results in germination and emergence phenology that is perfectly adapted to the climate of origin.

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