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Species-specific environmental requirements to break seed dormancy: implications for selection of regeneration niches in three *Lonicera* (Caprifoliaceae) species

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Abstract: Environmental requirements for seed germination can operate as an important filter in determining the regeneration niche and ultimately the habitat preference of many plant species. We hypothesize that morphological and morphophysiological seed dormancy may play a major role in habitat selection, because underdeveloped embryos responsible for those dormancy types usually require strict species-specific environmental conditions to grow and to overcome dormancy, imposing marked constraints to recruitment and thus to species distribution. We analyzed the influence of temperature and light on embryo growth and seed germination, as well as germination phenology in three *Lonicera* (Caprifoliaceae) species. *Lonicera xylosteum* L. seeds had morphological dormancy. Those of *Lonicera etrusca* Santi had unusual within-species dormancy variability, with a fraction being able to show both morphological and morphophysiological dormancy. Seeds of *Lonicera arborea* had deep complex morphophysiological dormancy. The close correspondence between the environmental conditions that each *Lonicera* species requires to break seed dormancy and their altitudinal range suggests that morphological and morphophysiological dormancies act as important filters in determining the regeneration niches of species, probably because such dormancy mechanisms impose markedly specific environmental requirements during the earlier stages of recruitment.

Key words: altitudinal gradient, dormancy break, morphological seed dormancy, morphophysiological seed dormancy, regeneration niche, seed-germination phenology.

Résumé : Les besoins environnementaux pour la germination des graines peut constituer un filtre important dans la détermination des niches de régénération et ultimement de l'habitat préféré de plusieurs espèces de plantes. Les auteurs ont formulé l'hypothèse que la dormance morphologique et morphophysiologique des graines peut jouer un rôle majeur dans la sélection de l'habitat, puisque les embryons incomplètement développés responsables pour ces types de dormance, imposent des contraintes prononcées au recrutement et conséquemment à la distribution des espèces. Les auteurs ont analysé l'influence de la température et de la lumière sur la croissance de l'embryon et la germination des graines, ainsi que la phénologie de la germination chez trois espèces *Lonicera* (Caprifoliaceae). Les graines du *Lonicera xylosteum* L. ont une dormance morphologique. Celles du *Lonicera etrusca* Santi possèdent une variabilité intraspécifique inhabituelle de la dormance, une fraction étant capable de montrer à la fois une dormance morphologique et morphophysiologique. Les graines du *Lonicera arborea* montrent une dormance morphophysiologique complexe. L'étroite correspondance entre les conditions environnementales dont chaque espèce a besoin pour briser la dormance de ses graines et leurs aires altitudinales suggèrent que les dormances morphologiques et morphophysiologiques agissent comme filtre important pour déterminer les niches de régénération des espèces, probablement dû à ce que les mécanismes de dormance imposent des besoins environnementaux nettement spécifiques au cours des premiers stades du recrutement. [Traduit par la Rédaction]

Mots-clés : gradient altitudinal, bris de dormance, dormance morphologique des graines, dormance morphophysiologique des graines, niches de régénération, phénologie de la germination des graines.

Introduction

Dormancy provides seeds with a mechanism that enables them to skip periods favorable to germination but unfavorable to seedling establishment (Baskin and Baskin 1998; Fenner and Thompson 2005; Vandelook et al. 2008). Environmental conditions act as signals breaking seed dormancy and promoting germination at both a site and time likely for seedling survival. As a result, seed germination in nature is often restricted to particular locations, referred to as "safe sites" (Harper 1977) or "regeneration niches" (Grubb 1977), which provide appropriate environmental requirements. Seed dormancy breaking and germination requirements are specific for each species, to the point that environmental signals for seed germination can reflect habitat adaptations (Vandelook et al. 2008). Large-scale screening studies have indicated that germination strategies are, to a certain extent, related to the habitat of species (Grime et al. 1981; Baskin and Baskin 1988; Schütz and Rave 1999). Thus, species-specific environmental requirements for seed germination can strongly contribute to determine the habitats where plants can grow. This happens in species with morphological (MD) and morphophysiological (MPD) seed dormancy. MD results from the underdevelopment of embryo at dispersal time. In seeds with MPD, germination is prevented by a combination of both MD and physiological mechanisms. Embryos must attain a critical length before germination can take place (Baskin and Baskin 1998). Development of the rudimentary, underdeveloped

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embryos associated with these seed dormancy types often requires specific durations at defined temperature ranges (e.g., Kondo et al. 2006; Baskin et al. 2009; Herranz et al. 2010*a*; Copete et al. 2011). The strict requirements allowing embryo growth in seeds with MD or MPD may impose defined constraints to seedling recruitment, and consequently, influence habitat selection for the species.

Seed-germination ecology of species with MD and MPD (e.g., Ranunculaceae, Papaveraceae, Paeoniaceae, Umbelliferae, Liliaceae, Caprifoliaceae) has been studied in western North America (Baskin and Baskin 2008; Baskin et al. 2009), eastern Asia (Kondo et al. 2005; Kondo et al. 2006), and central Europe (Vandelook et al. 2007*a*, 2008). Nine levels of MPD have been described up to the present (see Baskin and Baskin 1998 and Baskin et al. 2008). However, few studies on this topic have focused on the Mediterranean Basin in spite of the region's rich plant biodiversity (about 25 000 species, half of them endemic; Quézel 1985) and strong representation of the families mentioned above.

Species within the family Caprifoliaceae produce seeds that vary considerably in their germination ecology and dormancy breaking requirements (Martin 1946; Hidayati et al. 2000*a*, 2000*b*, 2001, 2005). Different species within *Lonicera* exhibit both MD (Phartyal et al. 2009*a*) and MPD (Hidayati et al. 2000*a*), and a variation in MPD levels within-species has been observed (Hidayati et al. 2000*a*).

Comparisons of seed dormancy and germination phenology among closely related species may provide insight on the underlying mechanisms that lead to differentiation of regeneration niches (Vandelook et al. 2008). Here, we analyzed the germination requirements for three *Lonicera* shrub species differing in their habitat spectrum and geographical range but overlapping on the Iberian Peninsula, where they live along an altitudinal gradient.

Lonicera xylosteum L. is widely distributed in Europe and, in Spain, occupies the meso- and supra-Mediterranean bioclimatic levels at altitudes of 700–1800 m above sea level (a.s.l.). *Lonicera etrusca* Santi is also broadly distributed, occurring from the thermo- to the supra-Mediterranean bioclimate at altitudes of 0 to 1600 m a.s.l. *Lonicera arborea* is endemic to the Iberian Peninsula and Northern Africa and grows at 1350 to 2100 m a.s.l. in the high supra-Mediterranean. Wild populations of *Lonicera* are found in a variety of habitats, preferentially in gaps, with *L. xylosteum* associated with a wide spectrum of broad-leaved deciduous forests (e.g., *Fagus sylvatica* L., *Corylus avellana* L., *Castanea sativa* Mill.); *L. etrusca* is found with *Quercus pyrenaica* Willd. or *Quercus faginea* Lam. (cold-winter supra-Mediterranean) and *Quercus ilex* subsp. *rotundifolia* Lam. (warm dry-summer meso- or thermo-Mediterranean); and *L. arborea* is found in cold *Q. faginea* and *Pinus nigra* Arnold forests (Ruiz-Téllez and Devesa 2007).

Temperature is the major environmental factor regulating dormancy of seeds (Vleeshouwers et al. 1995). The temperature conditions in which recently dispersed, fresh seeds are exposed to are determined by both abiotic (e.g., seasonal changes in temperatures along with altitudinal gradient) and biotic (e.g., temporal seed-dispersal patterns by frugivory) factors. In Mediterranean habitats, ripe *Lonicera* berries may be particularly attractive to the most significant avian dispersers (small birds; Bartholomew and Cade 1963), because of their high water content in dry late summer – early autumn (Herrera 1982). Exploring jointly seed-germination requirements and phenology may also shed light on adaptive recruitment responses to temperature conditions modulated by both abiotic and biotic factors.

In this study, we (*i*) determined the temperature requirements for breaking dormancy and for growth of embryos, (*ii*) tested the effect of the duration of warm or cold stratification and light on germination, (*iii*) tested the effect of gibberellic acidon dormancy break and embryo growth, and (*iv*) analyzed the phenology of seed germination. The aim was two fold, as follows: (1) to determine the type of seed dormancy and the environmental requirements needed to break dormancy and (2) to define the phenological patterns of seed germination in three *Lonicera* species, to assess the extent to which differences in the earlier mechanisms of recruitment are responsible for differences in regeneration niches of the species and habitat differentiation.

Materials and methods

Seed source

Mature berries of the three *Lonicera* species were collected from wild populations in August and September 2008. Berries of *L. arborea* were collected on 23 September 2008 from a stand 1700 m a.s.l. in the Almenara mountain (30SWH4766; Albacete Province, central Spain); those of *L. xylosteum* were collected on 22 August from 1497 m a.s.l. in Orea (30TXK1085; Guadalajara Province, central Spain); and mature berries of *L. etrusca* were collected on 25 August from 1569 m a.s.l., in Bronchales (30TXK2183; Teruel Province, central Spain). Berries were considered mature by a change of colour to bright red (*L. xylostemum*), orange (*L. etrusca*), or whitish (*L. arborea*). Approximately 100 berries were harvested from each of 50–100 healthy-looking individuals selected randomly.

In the laboratory, seeds were separated from the pulp on the day of collection by manually crushing the fruit and washing the material with cold water (Bacchetta et al. 2008). The seeds were left to dry at room temperature (ca. 20–23 °C) for 6–7 days (Hidayati et al. 2000*a*), and testing began after the seeds had been cleaned and dried (0-month-old seeds). Preliminary tests with fresh seeds collected in 2007 showed that germination in *L. xylosteum* was complete within 4 weeks, whereas *L. etrusca* and *L. arborea* required prolonged exposure to warm or cold stratification to achieve a high germination percentage.

General conditions for embryo-growth and germination experiments

Germination tests were intended to simulate temperature conditions in central Spain from where the seeds were collected (Herranz et al. 2010*a*). Temperatures were programmed (Model F4, Ibercex, Madrid, Spain) at the mean maximum and minimum temperatures for each month to represent conditions in November and March (maximum 15 °C, minimum 4 °C; the notation 15/4 °C is used hereafter to express this range), October and April (20/7 °C), September and May (25/10 °C), August and June (28/14 °C), and July (32/18 °C). A constant temperature of 5 °C simulated the winter months of December through February. Light, in a 12 h light – 12 h dark cycle (light hereafter), was controlled using cold white fluorescent tubes (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$; 1350 lx). Seeds incubated in dark conditions (darkness hereinafter) were wrapped in a double layer of aluminum foil. Seeds were considered germinated when the radicle emerged.

For each species, incubation treatments were carried out. In addition, seeds of *L. etrusca* and *L. arborea* also required stratification treatments to germinate. Incubations consisted of short (i.e., 4 weeks) thermal treatments at either autumn or spring temperatures to promote germination of seeds. For each incubation test, four replications of 25 seeds each were used. Seeds were incubated in 9 cm diameter Petri dishes on two layers of filter paper moistened constantly with distilled water. The dishes were sealed with parafilm to minimize the loss of water. In tests that involved photoperiod as a treatment (hereinafter referred to as the light treatment), germination was checked every 2–3 days. Germination percentages were based on the number of viable seeds, following Baskin and Baskin's (1998) recommendations. Viability of ungerminated seeds at the end of the experiment was assessed on the basis of the appearance of embryos, paying special attention to their color and firmness; white and turgid embryos were taken as viable and light-brown, soft embryos were taken as dead. These indicators of embryo viability were confirmed as accurate in *Lonicera* by means of tetrazolium tests (Hidayati et al. 2000*a*).

After incubation treatments at the full range of experimental temperatures, mature seeds of *L. arborea* and *L. etrusca* showed low germination rates and were therefore considered dormant (Baskin and Baskin 1998). To break dormancy, seeds were subjected to stratifications, which consisted of lengthy (i.e., from 4 to 16 weeks) thermal treatments at either winter or summer temperatures. For each species, two seed lots were placed in two 14 cm diameter Petri dishes lined with a double layer of filter paper saturated with distilled water. One dish was cold stratified (5 °C) and the other warm was stratified (28/14 °C) in light. Additional species-specific stratification treatments were also carried out when appropriate to simulate conditions close to those in the natural habitat (see above). During stratification treatments, germination tests were carried out at 4-week intervals in both light and darkness at 15/4 °C, 20/7 °C, and 25/10 °C.

Effect of temperature and light conditions on germination

Incubation experiment

To detect MD in freshly matured seeds, on 1 September 2008 (*L. xylosteum* and *L. etrusca*) and 1 October 2008 (*L. arborea*), four replication of 25 seeds of each *Lonicera* species were incubated at 5 °C, 15/4 °C, 20/7 °C, 25/10 °C, 28/14 °C, and 32/18 °C, in both light and darkness. Germination in light was checked every 2–3 days for 4 weeks. In darkness tests, seed germination was recorded once a week.

Stratification experiment

For species that did not manifest uniquely MD, stratification treatments were carried out to assess MPD levels.

Lonicera etrusca

On 1 October, two dishes with 2400 seeds each were stratified from 4 to 12 weeks in light at 5 °C and 28/14 °C, respectively. Another 2400 seed lot was subjected to sequential warm stratification (4 weeks at 28/14 °C; 4 weeks at 25/10 °C; 4 weeks at 20/7 °C) in light during the same period.

Lonicera arborea

On 1 November 2008, three dishes with 2400 seeds each were stratified from 4 to 12 weeks in light at 5 °C, 20/7 °C, and 28/14 °C, respectively.

Control tests were also carried out with freshly matured seeds of those species. Four replicates with 25 seeds were incubated in both light and darkness at 15/4 °C, 20/7 °C, and 25/10 °C during the entire experiment (i.e., 16 weeks) and without any previous stratification pretreatment. Germination was checked once every 4 weeks; in darkness treatments, germination was checked under a dim green light (Vandelook et al. 2007*a*).

After stratification treatments, seeds were incubated at 15/4 °C, 20/7 °C, and 25/10 °C to test germination response.

Effect of temperature on embryo growth

To determine the initial embryo length, 25 freshly matured seeds of each of the three *Lonicera* species were placed for 24 h in a 9 cm Petri dish lined with a double layer of filter paper moistened with distilled water and kept at room temperature (20–23 °C) for imbibition. Subsequently, embryos were excised with a razor blade and their length measured using a microscope equipped with a micrometer.

For each species, the critical embryo length was assessed by measuring embryos from 40 seeds in which the seed coat was split but the radicle had not yet emerged (Vandelook et al. 2009; Phartyal et al. 2009*a*). The lowest species-specific value recorded in the range of those measurement sets represented the threshold embryo size, i.e., the minimum embryo length at which germination is possible. This parameter is a reliable indicator that the MD dormancy component is being surpassed (Herranz et al. 2010*a*, 2010*b*).

To determine embryo growth, on 1 September 2008 (*L. xylosteum* and *L. estrusca*) and on 1 October 2008 (*L. arborea*), four dishes with 25 seeds in each were placed in light at 5 °C, 15/4 °C, 20/7 °C, 25/10 °C, 28/14 °C, and 32/18 °C. Another set of four dishes was placed in darkness at the same temperatures. Embryos were extracted from 25 seeds for each temperature regime and light conditions after 1, 2, 3, and 4 weeks and their lengths were measured.

For species whose embryo did not complete growth within 4 weeks, stratification treatments were carried out as follows below.

Lonicera etrusca

On 1 October 2008, three dishes with 300 seeds each were submitted to cold (5 °C), warm (28/14 °C), or sequential warm (4 weeks at 28/14 °C; 4 weeks at 25/10 °C; 4 weeks at 20/7 °C, under light) stratification. Embryos were excised from 25 seeds in each dish after 4, 8, and 12 weeks and their lengths were measured.

Lonicera arborea

On 1 November 2008, three dishes with 300 seeds each were submitted to cold (5 °C) or warm (20/7 °C or 28/14 °C) stratification, and embryos of 25 seeds from each dish were extracted after 4, 6, 8, 10, 12, and 16 weeks and their lengths were measured.

Mean length and standard error were calculated for each batch of 25 embryos. For seeds that germinated during treatment, embryo length was recorded as the critical length (Hidayati et al. 2000; Herranz et al. 2010*a*).

Effect of gibberellic acid on germination

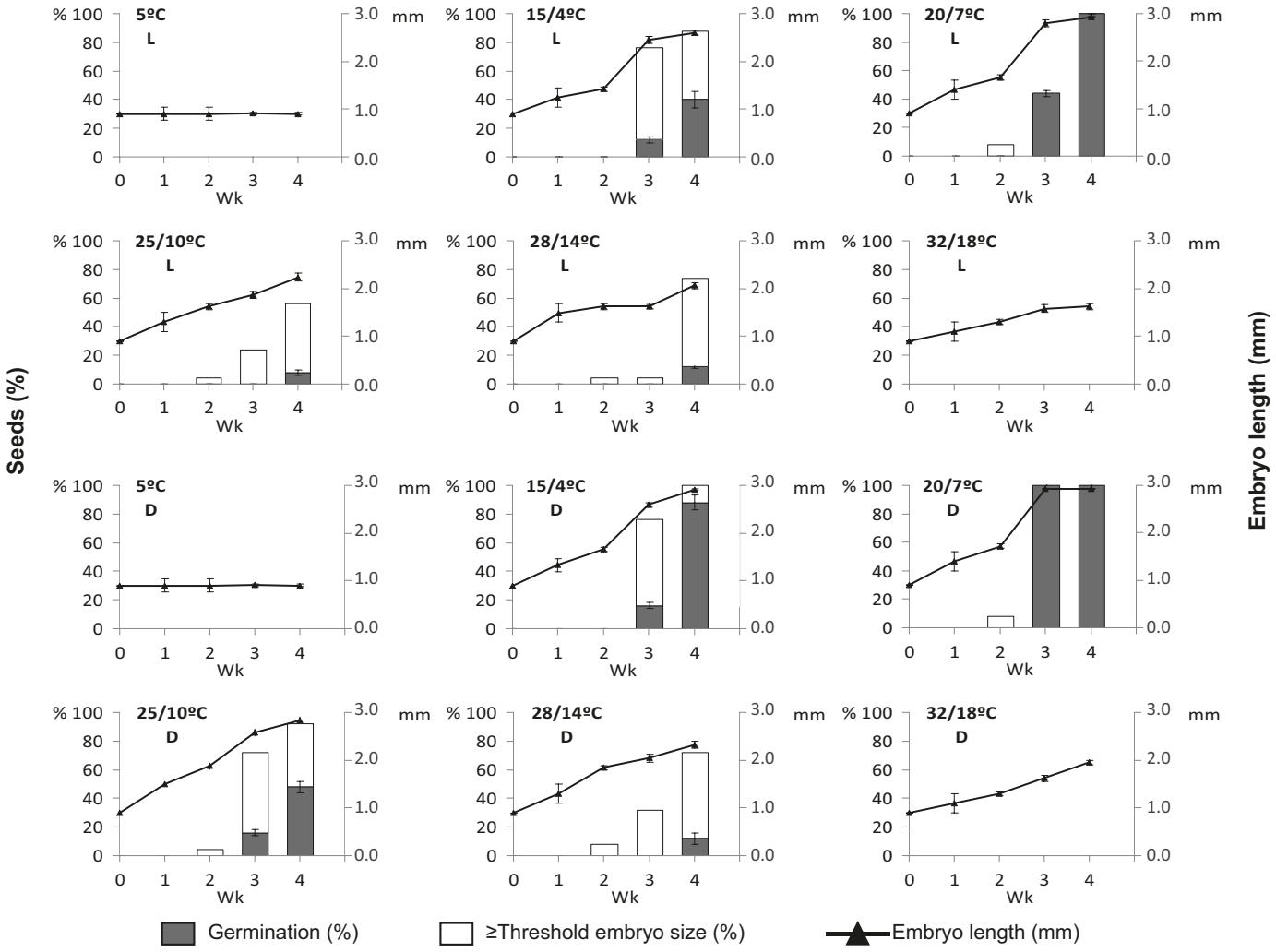
This test was performed only for *L. etrusca* and *L. arborea*. To ascertain whether gibberellic acid (GA_3) can substitute the effect of warm or cold stratification on seeds in overcoming dormancy, eight replicates of 25 seeds each for each species were placed in 9 cm diameter Petri dishes on two sheets of filter paper moistened with a solution of 2000 ppm GA_3 and sealed to avoid water evaporation. Of the eight replicates, four were placed at 5 °C and four at 20/7 °C in light for 16 weeks and checked for germination weekly until first germination and at intervals of 2–3 days thereafter. Seeds placed at the same temperatures and moistened with distilled water were used as a control.

Phenology of embryo growth and radicle emergence

To link laboratory studies with germination phenology under field conditions, a buried seed experiment was conducted in the experimental campus in Albacete, which is 100–150 km from the collection sites at an altitude of 686 m a.s.l. On 1 September 2008 (*L. xylosteum* and *L. etrusca*) and 1 October 2008 (*L. arborea*), 12 lots of freshly matured seeds for each species, 50 seeds to a lot, were mixed with sterilized sand. Each lot was placed in a bag of polyester mesh and buried 6 cm deep in pots with drainage holes. The pots were placed in a metal frame shadehouse with no temperature control system. To approximately simulate field conditions at the seed collection sites, the pots were watered to field capacity with an automatic micro-sprinkler system once a week, except in rainy weeks and when the substratum was frozen. Air temperatures in the shadehouse were recorded continuously with a meteorological data logger and their mean weekly maximum and minimum values were calculated.

One bag was exhumed every month for 12 months. Embryos of 25 healthy ungerminated seeds were dissected and measured using a microscope equipped with a micrometer. Mean embryo lengths and standard errors were calculated. In germinated seeds, the length of the embryo was assumed to be equal to the critical embryo-length value (see the section on the effect of temperature on embryo growth). Seeds that had germinated while being buried were counted, and germination percentages were calculated based on the number of viable seeds.

Fig. 1. Embryo growth (mm) in freshly matured *Lonicera xylosteum* seeds (mean ± SE; n = 4) incubated from 1 to 4 weeks with the complete range of temperatures in light (L) and in darkness (D). In each column, the shaded portion shows the percentage of seeds germinated (mean ± SE) and the open portion is the percentage of seeds with an embryo length ≥ the threshold embryo size at which radicle emergence is possible.



Phenology of seedling emergence

On 1 September 2008 (*L. xylosteum* and *L. etrusca*) and 1 October 2008 (*L. xylosteum* and *L. arborea*), three replicates of 200 seeds each for each species were sown 5 mm deep in a mixture of sterile peat and sand (2:1, *v*/*v*) in 20 cm × 30 cm × 8 cm plastic flats with drainage holes. The flats were placed in the nonheated shadehouse and watered using an automatic microsprinkler system, following the same irrigation schedule as described above. The flats were examined weekly, and the seedlings (i.e., plants at the cotyledon-emergence stage) that had emerged were counted and removed. The observation of seedling emergence was discontinued on 30 May 2009, when no more emergences took place. The data were expressed in terms of percent of cumulative emergence.

Statistical analysis

Germination was expressed as the final cumulative percentage. Means and standard errors were calculated both for germination (n = 4) and for embryo growth (n = 25). Effects of (*i*) light and (*ii*) temperature during stratification, as well as (*iii*) incubation temperatures, on embryo growth were analyzed with a multifactorial ANOVA. In all cases, factors associated with significant main effects were detected with a multiple-comparison Tukey's test, and significant interactions were explored by contrasting confidence intervals. A similar analysis was performed to determine the effects on final cumulative germination percentage. Prior to the analyses, normality (the Cochran test) and homoscedasticity (the David test) of the data were checked. Percentages were previously transformed into square-root arcsine values, but figures show nontransformed data. In all tests, the significance level (α) was 5%. Although all conditions in tests were contrasted statistically, significance is only shown when relevant. Detail of statistical contrasts associated with comparisons of embryo length and germination are available in Supplementary information[1].

Results

Lonicera xylosteum

Effect of temperature and light conditions on germination

Germination of seeds incubated at 20/7 °C in darkness was 100% by the third week and took 1 week longer to reach the same value when incubated in light. Furthermore, high germination values (e.g., 88%) were also achieved at 4 weeks of incubation at 15/4 °C in

[1]Supplementary data are available with the article through the journal Web site (http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2012-0169).

darkness. In contrast, germination during the first 4 weeks was poor at 5 °C, 28/14 °C, and 32/18 °C (Fig. 1).

Effect of temperature on embryo growth

Mean length of the spatulate embryos in freshly matured seeds was 0.92 mm. The critical length for germination was estimated to be 2.93 mm (SE = 0.26). The threshold embryo size was 2.27 mm.

Embryos of freshly matured seeds grew in the first 4 weeks at all combinations of temperature and light except 5 °C. At 15/4 °C, 20/7 °C, 25/10 °C, and 28/14 °C, the threshold embryo size was reached from the second to the third week of incubation irrespective of light conditions. At 20/7 °C, all embryos reached the critical length in 3 weeks in darkness and 4 weeks in light (Fig. 1).

Phenology of embryo growth and of radicle and seedling emergence

Nearly all embryos of seeds buried on 1 September 2008 reached the mean critical length and germinated in October 2008. Indeed, in mid-November 2008, 88% of seedlings had emerged (Fig. 2*a*). In contrast, seedling emergence from seeds buried on 1 October 2008 was 55% by late November 2008. After that, no seedling emerged until March 2009, reaching 86% by early April 2009 (Fig. 2*a*).

Lonicera etrusca

Effect of temperature and light conditions on germination

Light conditions did not have significant effects on germination ($P > 0.05$), regardless of stratification and incubation treatments. In the incubation experiment, germination was evident within the first 4 weeks of incubation under all temperatures except at 5 °C. At the fourth week, germination was the highest at 15/4 °C (35%, $P < 0.05$, data not shown).

In the stratification experiment, the highest germination percentage (99%, $P < 0.05$) was recorded in seeds warm stratified and then incubated at 15/4 °C. Seeds warm stratified and incubated at 20/7 °C also recorded high values (Table 1). Seeds also germinated after cold stratification, although the response was in general much lower. Germination decreased with the duration of cold stratification (Table 1).

Effect of temperature on embryo growth

Mean length of embryos in freshly matured seeds was 1.49 mm. The critical length was estimated to be 3.22 mm (SE = 0.40). The threshold embryo size 2.75 mm was achieved faster under the warm-stratification sequence (4 weeks at 28/14 °C; 4 weeks at 25/10 °C; 4 weeks at 20/7 °C) than in other treatments ($P < 0.05$) (Fig. 3). At the sequential treatment, 90% seeds achieved the critical embryo length after 12 weeks (Fig. 3).

The growth of embryos was significantly slower ($P < 0.05$) under cold stratification. Hence, after 12 weeks of cold stratification, the mean length was 2.23 mm, which was lower than the critical length. Nevertheless, 32% of the embryos cold stratified surpassed the threshold embryo size, which may explain the significant ($P < 0.05$) germination (14%) recorded in that treatment (Fig. 3).

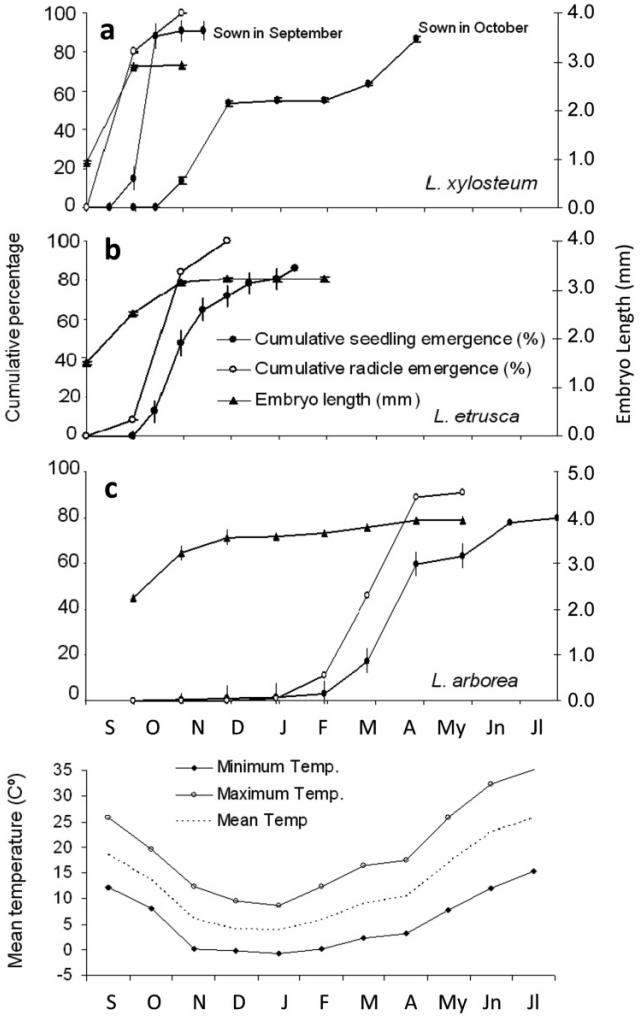
Effect of gibberellic acid on germination

Germination at 20/7 °C after 4 weeks in light was significantly higher with (88%) than without GA_3 (25%; $P < 0.05$, data not shown). In contrast, at 5 °C seeds did not respond to gibberellic acid.

Phenology of embryo growth and of radicle and seedling emergence

Nearly all embryos in seeds buried on 1 September 2008 reached the critical length during the first 2 months, coinciding with a high radicle emergence (Fig. 2*b*). Seedling emergence started in mid-October and became very high in late November 2008 (Fig. 2*b*).

Fig. 2. Phenology of embryo growth (mean ± SE, if SE value > 0.1; $n = 25$), radicle emergence, and seedling emergence (mean ± SE; $n = 3$) in seeds of (*a*) *Lonicera xylosteum*, (*b*) *Lonicera etrusca*, and (*c*) *Lonicera arborea*. Experiments were started on 1 September 2008 for *L. xylosteum* and *L. etrusca* and on 1 October 2008 for *L. arborea*. An additional seedling-emergence experiment with *L. xylosteum* seeds was repeated on 1 October 2008 (see Materials and methods). The lowest panel shows changes in temperature (mean maximum, mean minimum, and mean monthly temperatures) recorded in the shadehouse throughout the experiment.



Lonicera arborea

Effect of temperature and light conditions on germination

Effects of light conditions on germination were nonsignificant. In the incubation experiment, germination was not evident within the first 4 weeks of incubation.

In the stratification experiment, the highest germination percentage of 75%–84% ($P < 0.05$) was recorded in seeds cold stratified for 12 weeks and incubated at 20/7 and 15/4 °C, respectively (Table 1). Warm stratification did not promote germination (1%–3%) significantly (Table 1).

Effect of temperature on embryo growth

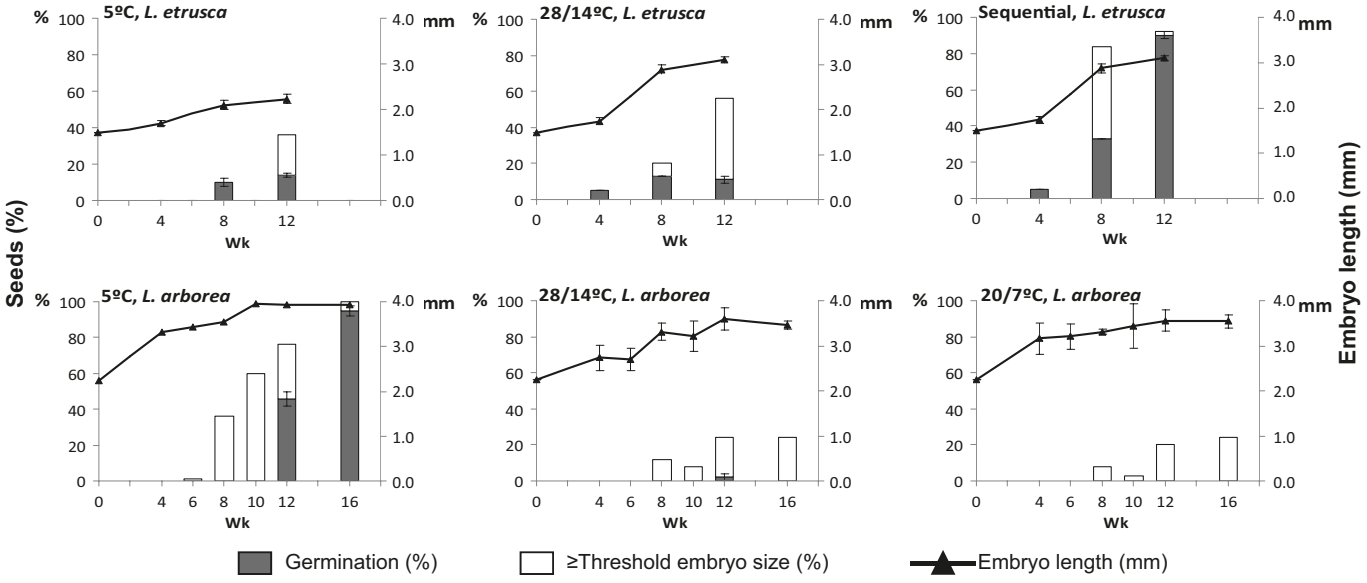
In freshly matured seeds, embryo length was 2.24 mm. The critical length was 3.93 mm (SE = 0.47). The threshold embryo size was 3.02 mm. Embryos of seeds cold stratified at 5 °C for 10 weeks grew over the critical length. However, germination did not begin until the 12th week (46%) and reached the highest value (95%) in the 16th week (Fig. 3). Embryos of seeds stratified at high temper-

Table 1. Effect of stratification treatments on *Lonicera etrusca* and *Lonicera arborea* germination.

Stratification		*L. etrusca*			*L. arborea*		
Temperature	Length (weeks)	15/4 °C	20/7 °C	25/10 °C	15/4 °C	20/7 °C	25/10 °C
Cold (5 °C)	4	41±4	33±1	12±1	9±2	7±1	11±1
	8	17±1	8±1	3±1	18±1	10±1	11±1
	12	6±1	2±1	2±1	84±6	75±2	67±3
Warm (28/14 °C)	4	95±1	81±2	23±3	3±2	4±1	2±0
	8	87±2	57±1	12±1	4±1	3±1	3±1
	12	99±1	63±1	11±1	3±2	1±1	2±1
Control	16	91±2	88±2	51±2	5±3	2±3	6±1

Note: Table shows final mean germinations, in % (±SE), of freshly matured seeds that were cold (5 °C) or warm (28/14 °C) stratified for 4, 8, or 12 weeks and then incubated at 15/4 °C, 20/7 °C, or 25/10 °C for 4 weeks. In control, seeds were not stratified but kept moist in dishes at each temperature regime during the entire experiment (i.e., 16 weeks). Because light conditions (photoperiod vs. darkness) during incubation phase had no significant effect on final germination, results in the table are pooled (n = 8).

Fig. 3. Embryo growth (mm) in freshly matured seeds of *Lonicera etrusca* and *Lonicera arborea* (mean ± SE; n = 4) submitted to cold (5 °C) or warm (28/14 °C) stratification during 12 (*L. etrusca*) or 16 (*L. arborea*) weeks. Embryo growth in two additional stratification treatments is also shown: sequential warm stratification (28/14 °C; 25/10 °C; 20/7 °C) at regular intervals of 4 weeks in light (*L. etrusca*), and 20/7 °C stratification (*L. arborea*). In each column, the shaded portion shows the percentage of seeds germinated (mean ± SE) and the open portion is the percentage of seeds with an embryo length ≥ the threshold embryo size at which radicle emergence is possible.



atures (28/14 °C and 20/7 °C) grew slowly and failed to attain the critical length. Indeed, the longest embryo at high temperatures was 3.60 mm (Fig. 3).

Effect of gibberellic acid on germination

Germination did not improve in seeds incubated with gibberellic acid. The highest germination with GA_3 treatment was 2% (data not shown).

Phenology of embryo growth and of radicle and seedling emergence

Embryos of freshly matured seeds buried on 1 October 2008 grew to 3.58 mm in December 2008. However, they did not reach the critical length until the end of March 2009 (Fig. 2*c*). Radicle emergence was not significant until February 2009, reaching 89% in April 2009 (Fig. 2*c*).

Temporal patterns of seedling emergence closely matched those of radicle emergence. By June 2009, most seedlings had emerged; no more emergence was recorded thereafter (Fig. 2*c*).

Discussion

Our results suggest MD and MPD are having a relevant filtering effect in determining habitat selection. Freshly matured seeds of the three *Lonicera* species have underdeveloped embryos (see Supplementary information). However, the environmental requirements to overcome seed dormancy, particularly those regarding temperature, substantially differed among the three species despite being closely related.

Lonicera xylosteum seeds were highly sensitive to warm temperatures (20/7 °C), attaining critical embryo length and completing germination (100%) within 1 month (Fig. 1). This germination pattern is unequivocally correspondent to species having MD (sensu Baskin and Baskin 1998). This class of seed dormancy was also reported in *L. caerulea* var. *emphyllocalyx* by Phartyal et al. (2009*a*).

Lonicera etrusca seeds had unusual within-species dormancy variability. In this species, some seeds (35%) germinated by the fourth week of 15/4 °C without any stratification pretreatment, so they were only morphologically dormant. The remaining seed fraction had nondeep simple MPD, because (*i*) germination was completed

after 12 weeks of warm (28/14 °C) stratification followed by incubation at 15/4 °C for 4 weeks (Table 1), and (*ii*) GA_3 also promoted germination of a high amount of seeds (see results) (Baskin and Baskin 1998). However, some of those seeds could also behave as deep complex morphophysiologically dormant, because a significant seed percentage (14%) also germinated after 12 weeks of cold (5 °C) stratification (see Fig. 3) and were not responsive to gibberellic acid. Thus, *L. etrusca* seeds can have (*i*) MD or (*ii*) nondeep simple MPD, the second group having a fraction able to show, and (*iii*) deep complex MPD. Adams et al. (2005) found that all seeds within a population of *Aristolochia californica* had either intermediate or deep complex MPD. However, only one precedent of a species with seeds showing simultaneously two levels of MPD is available in the literature; results obtained with *Lonicera mackii* (Hidayati et al. 2000*a*) suggest the existence of seeds with both MPD levels, as was detected here in *L. etrusca*. Although Hidayati et al. (2000*a*) recognized only nondeep simple MPD in *L. mackii* seeds, 84% of the seeds germinated after 12 weeks of either warm stratification or cold stratification. Having different classes and (or) levels of seed dormancy provides plant species with an obvious ecological advantage, because it should increase plasticity in the regeneration niche, allowing species to establish over a wider range of environments. Indeed, the *Lonicera* species with the widest variability in seed dormancy in this study corresponds to the one occupying the most contrasting elevations (from sea level to mid-mountain) and habitats (from warm dry-summer evergreen *Q. ilex* to cold-winter marcescent *Q. faginea* broadleaved forests).

None of the *L. arborea* seeds germinated in the first 4 weeks of incubation, so genuine MD should be dismissed. Although the seeds completed germination after cold stratification (5 °C) for 16 weeks (Fig. 3), and GA_3 did not stimulate germination, it can be concluded that *L. arborea* has deep complex MPD (Baskin and Baskin 1998). In this species, however, the critical embryo length was reached after 10 weeks of cold stratification, but germination at that time was nil. Indeed, germination needed two additional weeks of cold stratification to become apparent (Fig. 3). The breaking of deep physiological dormancy in the underdeveloped embryo and its elongation to full length (Nikolaeva's first and second stages of dormancy-break in seeds with MPD, in Baskin and Baskin 2008) probably occur simultaneously during the first 10 weeks of cold stratification. Once the two earliest phases are completed, additional period of cold stratification may be required to break remaining phase of the deep physiological dormancy in the seeds with fully developed embryos. Thus, for some species, the embryo size may be a prerequisite but not the only requirement for germination, as recorded for *Aegopodium podagraria* (Phartyal et al. 2009*b*) and for *L. arborea* in the present work.

Our results concerning the phenology of seed germination are fairly consistent with the classes and (or) levels of seed dormancy described above. In the shadehouse without a temperature control system, *L. arborea* seeds germinated in spring (Fig. 2*c*), after the winter cold stratification required by the seeds with deep complex MPD. However, embryo growth in *L. arborea* seeds occurred during October and November 2008. This is because temperatures during half of that period were low enough (0–10 °C) to simulate cold stratification. High embryo growth rates in autumn have also been recorded in other species with deep complex MPD, such as *Thaspium pinnatifidum* (Buckley) A. Gray (Baskin et al. 1992) and *Aegopodium podagraria* L.(Phartyal et al. 2009*b*), although in species with this MPD level, embryo growth is more likely to be completed during the winter months (e.g., *Delphinium tricorne* Michx., Baskin and Baskin 1994; *Chaerophyllum temulum* L., Vandelook et al. 2007*b*; *Lomatium dissectum* (Nutt.) Mathias & Constance, Scholten et al. 2009).

Lonicera xylosteum seeds completed their embryo development and germinated during autumn (Fig. 2*a*), coinciding with exposure to September and October temperatures, as expected for seeds with MD. Because the time required to complete embryo development in MD is relatively short (ca. 1 month), seedlings from seeds sown in early September emerged promptly in that season. In contrast, seeds sown in early October 2008 were able to delay seedling emergence until the following spring, although laboratory examination suggested that embryo growth and radicle emergence would be completed at October temperatures (Fig. 1). *Lonicera etrusca* seeds germinated in autumn (Fig. 2*b*), a result compatible with the requirement for warm stratification in seeds with nondeep simple MPD and that of cool temperatures in seeds with MD.

The overall ecological significance of these seed-germination traits helps to explain the altitudinal gradient of habitat occupation shown by these *Lonicera* species in the Iberian Peninsula. The deep complex MPD in *L. arborea* seeds constrains its geographical range to high elevations under Mediterranean climate conditions. Seed germination requiring cold stratification will therefore occur in spring, avoiding the exposure of sensitive seedlings to extremely cold winter temperatures recorded in high mountains. In addition, summers at that altitude are not dry, so young-seedling survival is not threatened by the drought that characterizes summers in genuine Mediterranean climates at lower elevations. In the opposite, we find *L. etrusca*, which inhabits from dry-summer lowland to cold-winter highland Mediterranean *Quercus* forests. Both MD and nondeep simple MPD detected in *L. etrusca* should induce seeds to germinate in autumn. In dry-summer environments at low Mediterranean elevations (0–1000 m a.s.l.; thermo- and meso-Mediterranean bioclimatic levels), winter temperatures are not extremely low. In consequence, seedlings that emerge in autumn will rely on a longer growth period to successfully face summer drought. In addition, deep complex MPD may be expressed in *L. etrusca* seeds at the highest elevations within its altitudinal range (1000–1600 m a.s.l.; supra-Mediterranean bioclimatic level), where the low winter temperatures may limit seedling recruitment. *Lonicera xylosteum* lives in temperate deciduous forests at low to medium elevations (300–1500 m a.s.l.), so a rapid autumn seed germination mediated by a MD mechanism should provide seedlings with higher probabilities of survival for the following summer, a period that can become water-stressful in Mediterranean environments at that altitude.

Of particular interest was the ability of *L. xylosteum* seedlings to delay emergence when seeds were sown later in autumn (i.e., October vs. September), as shown in the phenology study. Herrera (1995) demonstrated that fruit-removal efficiency by avian dispersers decreases with altitude in the western Mediterranean region. Water-rich (Herrera 1982) *L. xylosteum* berries may be quite attractive for birds in late summer – early autumn (A. Santiago, personal observation; Berger and Hart 1974; Fogden 1972; Langslow 1976). In lowlands, where fruit consumption is highly intense and competed, *L. xylosteum* seeds are likely to be dispersed early, so they could surpass MD, germinate, and emerge in autumn, before the onset of moderately cold winter temperatures. In highlands, effectiveness of dispersers is probably lower, so seeds are more likely to be released from the pulp and incorporate into the soil later, when cold temperature at this altitude begins to limit the survival of emergent seedlings. The facultative ability of *L. xylosteum* to delay seedling emergence until next spring may thus represent an ecological adaptive strategy to conciliate MD with low efficiency of avian seed dispersal and cold winter temperature, both of which are features of high elevations. Thus, temporal patterns of seedling emergence in species with seed dormancy may be modulated by the combined effects of abiotic (e.g., altitude) and biotic (e.g., disperser efficiency) factors, ultimately determining the temperature to which seedlings will be exposed.

As opposed to temperature, light conditions played an irrelevant role in breaking dormancy and promoting seed germination in the three *Lonicera* species studied. This record contrasts with the preference of canopy gaps shown by these species at maturity (Ruiz-Téllez and Devesa 2007). Probably, light demand may be

determinant in later stages of plant development other than seed germination and seedling emergence. In some species, gap occupation has been attributed to processes activated in subsequent phases of the life cycle (Houle 1992). Population resilience of many species characterizing mature plant community stages is based on maintaining arrested-growth shadow-tolerant saplings in the understory ready to detect and occupy new canopy gaps (Harper 1977; Fenner 1993). In such a case, light sensitivity of the three species may manifest itself only in those stages of plant growth associated with occupation of gaps.

Our study shows that the species-specific environmental requirements (i.e., temperature) to break seed dormancy are strongly associated with altitudinal ranges at which the three *Lonicera* species live. This observation suggests that MD and MPD associated with underdeveloped embryos may represent an important filter in determining regenerative niches of the species by imposing specific environmental requirements on the earlier stages of recruitment (e.g., Hidayati et al. 2000*a*; Phartyal et al. 2009*a*). The results show that *L. arborea* may be more sensitive to the effects of climate change, because their seeds strictly demand cold temperature to surpass dormancy. This study provides additional evidence of the unusual diversity in seed-dormancy categories in *Lonicera*. Such high variation may explain, at least partly, why some species in the genus have recently become invasive in both North America (Trisel 1997; Hidayati et al. 2000*a*; McCusker et al. 2010) and Europe (Sanz-Elorza et al. 2004). Finally, the study emphasizes the interest of *Lonicera* genus within the context of germination ecology, because it provides some evidence that between-species germinative mechanisms are not clear-cut, thus leading us to consider evolutionary gradients rather than well differentiated species-specific germination models.

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