STUDIES

Seed germination in Narcissus yepesii (Amaryllidaceae): clinal variation in the morphophysiological dormancy levels

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Abstract

Seed dormancy classes determine both population and species-level processes which can be crucial in the life cycle of many plants. However, there are no studies of a dormancy cline between levels of morphophysiological dormancy (MPD). We aimed to determine the class of seed dormancy of Narcissus yepesii exhibits in order to explore links between different dormancy levels, previously characterized in two closely related phylogenetic congeners, N. alcaracensis and N. longispathus. Experiments were carried out under both near-natural temperature and controlled laboratory conditions. The parameters calculated were mean embryo length, radicle and shoot emergence percentages. The effects of different periods of storage; and different periods with or without GA3 of warm, cold or warm plus cold were analysed. The Narcissus populations from the Baetic System of mountain ranges in south-eastern Spain show clinal variation in a northeast–southwest gradient from intermediate to non-deep complex MPD, through the coexistence of intermediate and non-deep complex MPD in N. yepesii (21 % and 74 %, respectively). In addition, 54 % of stored seeds were able to show both levels of MPD. Narcissus yepesii occupies an intermediate position between N. alcaracensis and N. longispathus in the geographical distribution and in the clinal germination ranges. It strongly suggests an evolutionary gradient, which connects the intermediate complex MPD with the non-deep complex MPD in southern Iberian daffodils. This is the first study showing a gradient in the evolution between levels of MPD. Our results demonstrate a cline in these levels in response to both an environmental gradient and genetic differences.

Keywords: Gibberellic acid; Narcissus alcaracensis; Narcissus longispathus; radicle emergence; secondary dormancy; seed storage; seed stratification.

Introduction

Seed germination and the recruitment of new individuals from seeds are currently recognized as crucial phases of the plant's life that are strongly dependent on environmental filters which act as signals breaking seed dormancy (Donohue et al. 2010). As a result, seed germination in nature is often restricted to particular locations, which provide appropriate environmental requirements, to the point that different environmental signals for seed germination can reflect habitat adaptations (Grubb 1977; Vandelook and Van Assche 2008). The dormancy class can influence both population and species-level processes such as colonization,
adaptation, speciation and extinction (Willis et al. 2014). Between the different dormancy classes, morphophysiological dormancy (MPD) is the most difficult to overcome because embryos must attain a critical length before germination can take place and growth of underdeveloped embryos requires specific durations of warm and/or cold stratification depending on the level of MPD (Baskin and Baskin 2014).

In the short term, the successful of germination requires specific dormancy-breaking cues that have to be present in the habitat. However, trait evolution from one to other level of MPD can occur as adaptation to environment in the long term. The evolution of levels of MPD can be consequence of divergence of the taxa in disjunct congeners, such as North American–Asian species of Osmorhiza with non-deep and deep complex, respectively (Walck et al. 2002) and Eurasian–North American species of Sambucus with intermediate complex and deep simple, respectively (Hidayati et al. 2000b). Also, this evolution has been studied in close-related species, whose populations are separated by just a few kilometres, such as Narcissus alcaracensis with intermediate complex MPD (Herranz et al. 2013b) and N. longispathus with non-deep complex MPD (Herranz et al. 2013a).

Narcissus (Amaryllidaceae) is a genus of perennial geophytes whose main centre of diversity is the Iberian Peninsula and Morocco, but it also occurs elsewhere along the coasts of the Mediterranean and the European Atlantic. Taxonomic difficulties arise from weak reproductive barriers, resulting in the widespread occurrence of hybridization and a high morphological variability. Consequently, the number of wild daffodil species varies considerably between different taxonomic treatments from 36 differentiated by Zonneveld (2008) to 64 recognized by Blanchard (1990).

A new taxon belonging to this group has been described in the last decades: Narcissus yepesi from the Pseudonarcissi section (Ríos-Ruiz et al. 1999). This daffodil grows along the banks of small permanent streams in small populations, and the number of individuals ranges between several dozens to a few hundred. Its main distribution area occurs in Segura Mountains, which are part of the Baetic System of mountain ranges (in the south-east of the Iberian Peninsula). The other two species above mentioned from the same section are found in nearby areas from the same Baetic mountain system: (i) N. alcaracensis in the Alcaraz Mountains (Juan-Vicedo et al. 2018), and (ii) N. longispathus in mountains of Cazorla, Castril and Magina (Hernández-Bermejo et al. 2006). The Euclidean distance between the location of the populations of N. alcaracensis and N. longispathus is ~97 km, while N. yepesi grows in the middle (~37 km from N. alcaracensis and 60 km from N. longispathus). A clinal geographic variation in a northeast–southwest gradient exists between these three species (Fig. 1) and this geographical variation involves a smooth increase of mean annual temperature (from 13.1 to 15.7 °C), which could influence on germination requirements of the three species. It is known that a cline in different morphological traits within a genus can be a key factor in the speciation process, e.g. Coincya (Gómez-Campo et al. 2001). Also, Montesinos-Navarro et al. (2012) have observed a clinal variation in seed germination

![Figure 1. Seed populations in Baetic System: N. alcaracensis in Alcaraz Mountains (Albacete province); N. yepesi in Segura Mountains (Jaén province); and N. longispathus in mountains of Cazorla, Castril and Magina (Jaén province).](image-url)
of Arabidopsis thaliana related with an altitudinal and climatic gradient from warmer summers and wetter winters to cooler summers and drier winters.

There are differences between N. longispathus and N. alcaracensis at three levels: physiological (Herranz et al. 2013a, b), morphological (Ríos-Ruiz et al. 1999) and genetic (Jiménez et al. 2009). Therefore, these differences support the taxonomical separation in two different species. On the other hand, many morphological features of N. yepesi and the other two Narcissus taxa show a large degree of overlap (Table 1), which casts doubt on the taxonomic treatment by Ríos-Ruiz et al. (1999). Therefore, with regards to population distribution and morphological appearance, because N. yepesi is intermediate to N. longispathus and N. alcaracensis in many morphological traits, it is of interest to determine which germinative behaviour best describes N. yepesi and to relate it with the requirements of the other two close species.

In the Pseudonarcissi section, all the previous studies report species with some level of MPD (Vandelaar and Van Assche 2008; Copete et al. 2011a, 2014; Herranz et al. 2013a; Newton et al. 2015, 2020), meaning freshly matured seeds have underdeveloped dormant embryos (i.e. differentiated but that occupies <50 % of the endosperm length) at the moment of dispersal. A previous laboratory study (unpublished data) showed that embryo length in mature seeds of N. yepesi was 1.28 ± 0.05 mm while endosperm length was 3.14 ± 0.05 mm (mean ± standard error [SE], n = 25).

In addition, these seeds incubated under a wide range of light-temperature conditions (5, 15/4, 20/7, 25/10, 28/14 and 32/18 °C in light/darkness) had not germinated after 30 days. Both results suggest that seeds of N. yepesi have an underdeveloped embryo that is physiologically dormant at dispersal time, i.e. this species has some level of MPD. This is also true for the closely related taxa. In N. alcaracensis (Herranz et al. 2013b), dormancy break and embryo growth just require several months at 5 °C since these seeds have intermediate complex MPD. In N. longispathus (Herranz et al. 2013a), dormancy break, embryo growth and radicle emergence require warm temperatures followed by cold stratification, corresponding with non-deep complex MPD (Baskin and Baskin 2014).

The present work focuses on verification of the following hypotheses: (i) N. yepesi may have different dormancy-breaking requirements than the phylogenetically closely related Narcissus species; and (ii) the requirements to break seed dormancy in Narcissus populations from the Baetic System may show clinal variation along a northeast–southwest gradient. With a focus on the seed germination of N. yepesi, the specific aims of the current study were to investigate: (i) the phenotype of embryo growth and radicle and shoot emergence; (ii) dormancy break in seeds buried under natural conditions; (iii) the influence of temperature and light on embryo growth and radicle emergence; (iv) the influence of seed storage duration on embryo growth; (v) the effect of cool temperatures to overcome seed dormancy; (vi) the effect of gibberellic acid (GA, hereafter) on embryo growth; (vii) the influence of cold stratification on shoot emergence; and (viii) induction of dormancy by warm temperatures in non-dormant seeds.

Materials and Methods

Seed material

Mature fruits were randomly collected when they changed from green to yellow and began to open for seed dispersal on 7 June 2016 and on 13 June 2017 from a sample around 400 vigorous plants growing in Siles (Jaén province, Spain), 1340 m above sea level (m a.s.l.), 38°23′N, 2°38′W. It is a single homogeneous population compounded by 2000 or 3000 individuals. Ripe capsules were spread out in the laboratory to allow the capsules to open and the seeds to fall out (Newton et al. 2013). In recently released seeds, moisture content (fresh weight basis) was 9.4 %.

Seeds were dried in the laboratory (22–23 °C) until the initial germination tests began on 1 July 2016 and 2017; at this time seeds were considered to be 0 months old and the seed moisture content was 3.9 %. To analyse seed age effect, seeds were stored dry in paper bags under laboratory conditions: 22–23 °C and 50–60 % air humidity, moisture content remained constant (3.9 %). Unless otherwise stated, experiments were conducted using the 2016 seed lot. Samples of seeds were randomly selected for the several different studies.

Shadehouse experiments

The purpose of these experiments was to analyse the phenology of dormancy break, i.e. embryo growth, radicle emergence and shoot emergence in N. yepesi seeds under near-natural temperature conditions. Therefore, these experiments were conducted in a non-heated metal frame shadehouse (12 threads per cm and 50 % sunlight interception) located at the experimental campus of the Technological School of Agronomy in Albacete (690 m a.s.l., 90 km from Siles). Temperatures were recorded continuously with a TinytagPlus TGP-0020 data-logger (Gemini Data Loggers, Chichester, West Sussex, UK), and mean daily maximum and minimum temperatures were calculated. Pots and trays containing the seeds were filled with a mixture of peat and sand (2:1 v/v) and watered to field capacity once a week throughout the year, with two exceptions. The first exception was to simulate summer water deficit that is common in the Mediterranean area; in July and August, the pots and trays were only watered twice a month. The second exception is that water was withheld when the substratum was frozen in winter. Thus, temperature and soil moisture conditions were similar to those in the natural habitat of N. yepesi. Each of the shadehouse experiments was initiated in July and in December to test the effect of warm summer temperatures.

Phenology of embryo growth. On 1 July 2016, eight samples of 30 seeds were mixed with fine-grained sterilized sand, and each sample was placed in a fine-mesh nylon cloth bag. Bags were buried 5-cm deep in an opaque white plastic pot (24 cm of diameter) and placed in the previously described metal-framed shadehouse. Each bag was labelled, but the label was not buried, which made it easy to recover each bag individually. One bag was exhumed each month from August 2016 to March 2017. The bag content was sieved (1-mm mesh size) to separate seeds

| Table 1. Morphological traits of each species (Ríos-Ruiz et al. 1999; personal observations). |
|---------------------------------------------------------------|
| Morphological traits | Narcissus alcaracensis | Narcissus yepesi | Narcissus longispathus |
| Leaves length (cm) | 11–43 | 19–31 | 40–60 |
| Floral scape (cm) | 10–26 | 16–35 | 30–70 |
| Pedicel (mm) | 15–46 | 18–55 | 40–90 |
| Perianth segments (mm) | 14–22 | 19–25 | 25–32 |
| Spathe length (mm) | 43–60 | 40–90 | 60–100 |
| Corona length (mm) | 15–23 | 15–30 | 14–22 |
| Number of flowers/scape | 1–2 (3 rarely) | 1 | 1 |
from sand. Embryos were excised from 25 healthy-looking seeds, i.e. seeds whose embryos were white and firm. More than 95% of the seeds were viable. Then, embryo length was measured. These values were used in two ways: (i) monthly mean embryo length was calculated to analyse embryo growth throughout the experiment; and (ii) each month these 25 values were grouped into size classes to monitor temporal changes in the distribution of embryo-size structure from August 2016 to March 2017. The study described above was initiated again on 1 December 2016, using four samples of 30 seeds. Embryos from seeds whose radicles had emerged during burial were recorded as having a critical embryo length (Hidayati et al. 2000a; Herranz et al. 2010). The critical embryo length for germination is the length of the embryo at the time the seed coat splits but before the radicle emerges (Vandeloek and Van Assche 2008). As revealed in preliminary tests in the laboratory, the critical embryo length for germination in N. yepesii was 2.75 mm (SE = 0.05 mm, n = 40, range = 2.10–3.20 mm) calculated as the mean embryo length in 40 seeds with split seed coat. The E:S ratio is the mean quotient between embryo (E) and endosperm (S) lengths (0.89 ± 0.01). The threshold E:S ratio is a good indicator that the morphological dormancy (MD) component is being broken and seeds are ready to germinate (Copete et al. 2011a). It was calculated as the minimum E:S ratio in those 40 seeds (range = 0.76–0.97, where the threshold E:S ratio is 0.76).

Phenology of radicle emergence and breaking dormancy phase during burial experiment. Each sample of 100 seeds was mixed with sand and placed in a nylon permeable bag. In 2016, eight seed samples were buried in a pot (above-mentioned characteristics) on 1 July and four seed samples on 1 December. One bag was exhumed on the first day of each month for 8 months, respectively. Non-germinated healthy seeds were buried in a pot (above-mentioned characteristics) on 1 July and four seed samples on 1 December. Eight seed samples were buried in a pot (above-mentioned characteristics) on 1 July and four seed samples on 1 December. These experiments were carried out in germination chambers (Ibercex model F-4, Madrid, Spain) with a digital temperature and light control system (±0.1 °C, cool white fluorescent light, 25 μmol m⁻² s⁻¹). One hundred seeds (four replicates × 25 seeds) were placed in Petri dishes on two layers of filter paper (Whatman grade 1) moistened with distilled water. Germination tests were conducted under a 12-h daily photoperiod (hereafter ‘light’) and under continuous darkness (hereafter ‘darkness’); darkness was achieved by wrapping Petri dishes in a double layer of aluminium foil (Baskin and Baskin 2014). Dark-incubated seeds were handled under a dim green light (Vandeloek et al. 2007) to reduce the interruption of the continuous darkness.

Germination tests were conducted using alternating temperature regimes (12/12 h) that simulate mean maximum and mean minimum monthly temperatures during the annual growth cycle at the seed-source region: 15/4 °C, November and March; 20/7 °C, October and April; 25/10 °C, September and May; 28/14 °C, August and June; and 32/18 °C, July. In these 12/12 h alternating temperature regimes, the high temperature coincided with the light phase and the low temperature with darkness to simulate day/night conditions. A constant temperature of 5 °C simulated the mean temperature recorded during winter months: December, January and February. Other low temperatures (9/5 and 10 °C) were chosen because they are within the effective temperature range for cold stratification (0–10 °C) (Nikolaeva 1969). Percentages of germination were based on the number of apparently viable seeds. Embryo growth was calculated throughout each experiment. Embryos excised from 25 seeds at each condition were measured at 30-day intervals.

How do temperature and light conditions influence embryo growth? The purpose of this experiment was to determine which temperatures promote embryo growth; this information is needed to determine if the MPD level is simple or complex. Twenty-five seed samples were stratified for 4 months at each of three conditions: (i) cold at 5 °C, (ii) warm at 28/14 °C and (iii) warm followed by cold at 20/7 + 15/4 + 5 + 5 °C (1 month at each temperature). At each condition, ten 25-seed samples were in light and 10 in darkness. Monthly, a 25-seed sample in light and in darkness at each of the three conditions was used to measure mean embryo length (four samples × three treatments). After 4 months of stratification, a 25-seed sample was incubated in light and in darkness at 5, 15/4, 20/7, 25/10, 28/14 and 32/18 °C for 1 month, after which embryos were measured.

Does dry storage improve embryo growth? The aim of this experiment was to verify whether dry storage can promote embryo growth as detected in some species with intermediate complex MPD (Herranz et al. 2013b). The effect of seed age on embryo growth was determined in seeds that were dry-stored for 0 (freshly matured seeds) or 20 months. After both dry-storage periods, seeds were incubated in darkness at 5, 15/4 and 20/7 °C and at a 20/7 + 15/4 + 5 + 5 °C sequence of temperatures (1 month for each part of the sequence) for 4 months. At monthly intervals, the embryo length was measured of 25 seeds from each temperature regime.

Do low temperatures overcome seed dormancy? Seeds dry-stored for 0 or 8 months were cold-stratified at 9/5 and 10 °C for 3–4 months, and after 3 months seeds were incubated in light and darkness at 5, 15/4, 20/7 °C for 1 month. Embryo length was determined monthly and germination percentage was calculated at the end of the experiment.

How do temperature–light conditions and seed storage influence radicle emergence of seeds with an almost fully developed embryo? This experiment was carried out using 0-month-old seeds collected in 2017, and it was repeated when seeds in the same seed sample were 8 and 20 months old. Firstly, two samples of >1200 seeds were stratified for 4 months at 20/7 + 15/4 + 5 + 5 °C (1 month for each part of the temperature regime); one sample was kept in light and the other in darkness. Secondly, from each one of these stratified samples, 12 groups of 100 randomly selected
seeds were incubated for 30 days at 5, 15/4, 20/7, 25/10, 28/14 and 32/18 °C in either light or darkness (six temperatures × two light conditions).

**Does GA₃ promote embryo growth?** The aim of this experiment was to evaluate the effect of GA₃ on embryo growth, which is an important trait differentiating levels of MPD since GA₃ only promotes embryo growth in non-deep or intermediate levels, but not in deep ones (Baskin and Baskin 2014). Samples of 25 seeds were placed in Petri dishes on two sheets of filter paper moistened with a GA₃ solution (1000 ppm). Then, three dishes each were sealed with Parafilm and placed at each temperature/light condition (5, 25/10 or 28/14 °C in light and in darkness) for 1, 3 or 5 months. The embryo length was measured for one Petri dish from each temperature after 1, 3 and 5 months and to calculate the percentage of seeds whose E:S ratio was higher than the threshold E:S ratio. In a control test, filter paper was moistened with distilled water (GA₃ 0 ppm), and a dish placed at each of the same six temperature/light conditions; the embryo length was measured only after 5 months.

**Are seedlings dormant after radicle emergence?** If seedlings are dormant after radicle emergence, the epicotyl would require cold stratification to overcome dormancy (Baskin and Baskin 2014). Two samples of 50 seeds with an emerged radicle (2–3 mm) were placed on two sheets of filter paper moistened with distilled water and incubated in light for 70 days. One of the samples was stratified to overcome dormancy (Baskin and Baskin 2014). If seeds were dormant until October (mean minimum temperature 7 °C). Embryo length increased from 1.59 ± 0.07 mm on 1 October to 2.64 ± 0.07 mm on 1 November, reaching the largest size class. Similarly, in seeds buried on 1 December 2016 (Fig. 2C), the embryos grew continuously from December to March; during this period, the mean minimum daily temperature was −0.7 °C and the mean maximum daily temperature was 13 °C. Most of the embryos were not in the >2.20-mm size class until March [see Supporting Information—Fig. S1B].

**Can warm temperatures induce physiological dormancy after the embryo has grown?** The purpose of this experiment was to determine if warm temperature (28/14 °C) induced dormancy in seeds with an almost fully developed embryo after warm plus cold stratification, and if so, to quantify the period of warm plus cold stratification required for overcoming secondary dormancy. Complete stratification in light lasted 9 months divided into three phases: (i) 4-month dormancy break stratification by warm + cold temperatures (20/7 + 15/4 + 5 + 5 °C); (ii) 1-month dormancy induction by warm temperatures (28/14 °C); and (iii) 4-month secondary-dormancy break stratification by warm + cold temperatures (20/7 + 15/4 + 5 + 5 °C). Germination tests were carried out during stratification to check if seeds were dormant or not dormant after phase 1, after phase 1 + 2, and after phase 1 + 2 + 3 (monthly during phase 3). Each test was conducted with four replicates of 25 seeds at 15/4 °C in both light and darkness for 30 days (optimal conditions for germination in non-dormant seeds). The seed lot was from 2017.

**Statistical analysis**

Means and SEs were calculated for percentages of radicle and shoot emergence and for embryo lengths. All the factors analysed were stratification temperature, incubation temperature, light condition during stratification-incubation, time of stratification, concentration of GA₃ (0 and 1000 ppm) and seed age. An independent statistical analysis was performed in each part of this study. Thus, the effects of the factors involved in each experiment on both embryo length and germination were analysed by multifactor analysis of variance (ANOVA) using Statgraphics centurion XVI. Seed germinability was evaluated by the final cumulative germination percentage of the number of viable seeds. When the effect of a factor was significant, differences were compared by a multiple comparison Tukey test. Prior to analyses, normality (Cochran test) and homoscedasticity (David test) of the data were checked. Values of the final cumulative germination percentages were square-root arcsine transformed.

**Results**

**Shadehouse experiments**

**Phenology of embryo growth.** For the seeds buried on 1 July 2016 (Fig. 2B), embryos did not grow from 1 July to 1 October; during this period, the mean minimum daily temperature was 14 °C and the mean maximum daily temperature was 31 °C (Fig. 2A). Main part of the embryos (>60 %) was in the three smaller size classes (<1.60 mm) [see Supporting Information—Fig. S1B]. However, embryos grew rapidly completing their development in October (mean minimum temperature 7 °C). Embryo length increased from 1.59 ± 0.07 mm on 1 October to 2.64 ± 0.07 mm on 1 November, reaching the largest size class. Secondly, in seeds buried on 1 December 2016 (Fig. 2C), the embryos grew continuously from December to March; during this period, the mean minimum daily temperature was −0.7 °C and the mean maximum daily temperature was 13 °C. Most of the embryos were not in the >2.20-mm size class until March [see Supporting Information—Fig. S1C].

**Phenology of radicle emergence and breaking dormancy phase during burial experiment.** In seeds buried on 1 July 2016 (Fig. 2B), all viable seeds were dormant until October (Fig. 3A), after which radicle emergence began. Cumulative radicle emergence was 13 % in November, 33 % in December, 52 % in January, 84 % in February and 91 % in March. The mean minimum and maximum daily temperatures during this period were −1 and 13 °C, respectively. In seeds buried on 1 December 2016 (Fig. 2C), emergence was 13 % in March and 51 % in April, while 42 % of non-germinated seeds in April were dormant and viable (Fig. 3B).

**Phenology of shoot emergence.** In seeds buried on 1 July 2016 (Fig. 2B), the first shoot emergence was observed on 1 March 2017 (7 %); during the previous month the minimum daily temperature was 2 °C and the maximum daily temperature was 16 °C (Fig. 2A). Cumulative shoot emergence was 72 % on 1 April and 79 % on 1 May. A few seedlings (4 %) emerged in the second spring (April 2018). In seeds buried on 1 December 2016 (Fig. 2C), 11 % of shoots had emerged by 1 March 2017 and 79 % emerged in the following spring (April 2018).

**Laboratory experiments**

**How do temperature and light conditions influence embryo growth?** Embryos grew significantly more in darkness than in light at all temperature conditions (P-value < 0.05) (Table 2). After 4 months of stratification, the percentages of seeds whose E:S ratio was higher than threshold ratio were 32, 10 and 96 % at 5, 28/14 and the sequence 20/7 + 15/4 + 5 + 5 °C, respectively (Table 2). The highest embryo growth and radicle emergence percentage (74 %) occurred after warm + cold stratification followed by incubation at 15/4 °C in darkness; 21 % of radicles emerged at 20/7 °C in darkness following 4 months at 5 °C. No germination occurred in the warm-stratified seeds subsequently incubated at the six incubation temperatures.
Does dry storage improve embryo growth? Embryos from freshly matured seeds grew slower than those from 20-month-old seeds ($P$-value < 0.05) (Fig. 4). After 4-month stratification at 5°C, 32% of embryos exceeded the threshold E:S ratio in fresh seeds and 92% in stored seeds; there was no radicle emergence in fresh seeds, and 75% radicle emergence in the stored seeds.

Figure 2. Mean daily minimum and maximum air temperatures (A) and phenology of embryo growth (■) and of root (▲) and seedling shoot (○) emergence from seeds sown on soil in a non-heated shadehouse in July (B) and in December 2016 (C).

Figure 3. Changes in the percentage of dormant, non-dormant, non-viable and germinated seeds of N. yepesii buried on 1 July 2016 (A) and on 1 December 2016 (B) and exhumed monthly for 8 and 4 months, respectively.
After 4 months at 15/4 °C, 60 % of embryos of fresh seeds (not dry-stored) exceeded the threshold E:S and there was 10 % radicle emergence. After a 3-month stratification at 5 °C, 76 % of embryos of seeds that had been dry-stored for 20 months exceeded the threshold E:S and there was 8 % radicle emergence. At 20/7 °C and at the sequence of temperatures, seed storage only slightly increased the embryo growth.

Do cool temperatures overcome seed dormancy? At 9/5 and 10 °C, the main embryo growth and germination occurred in 8-month-old seeds incubated in darkness (P-value < 0.05) (Table 3). Mean embryo length in 3-month-stratified seeds at 10 °C ranged from 2.04 ± 0.08 mm in 0-month-old seeds in light to 2.51 ± 0.07 mm in 8-month-old seeds in darkness (72 % of radicle emergence) (Table 3). After 4 months of stratification, radicle emergence in
8-month-old seeds was 89 % at 9/5 °C in darkness and 86 % at 10 °C in darkness.

How do temperature–light conditions and seed storage influence radicle emergence of seeds with a fully developed embryo? Radicle emergence increased significantly in 20-month-old seed stratified and incubated in darkness. The optimal incubation temperature was 15/4 °C (P-value < 0.001) with germination ranging from 52 to 82 % (Fig. 5). The lowest germination percentages (<20 %) were observed at 28/14 and 32/18 °C.

Does GA3 improve embryo growth? There were no significant differences (P-value > 0.05) between seeds tested with GA3 (1000 ppm) and distilled water (control) at 25/10 and 28/14 °C (Table 4). After 5 months of stratification at 5 °C in darkness, embryo length was 2.35 ± 0.11 mm with GA3 and 2.23 ± 0.09 mm in the control (the difference was not significant); radicle emergence was 27 % with GA3 and 8 % in the control.

Are seedlings dormant after radicle emergence? An 80 % of shoot emergence was reached in radicle-emerged seeds after 67 or 24 days depending on whether seeds were previously cold-stratified or not (Fig. 6). Shoots began to emerge after 6 days at 20/7 °C or after 44 days at 5 °C; however, after this delay, emergence rate was similar in both experiments, around 90 % of shoots emerged within 30 days.

Can warm temperatures induce physiological dormancy after the embryo has grown? Seed receiving warm + cold stratification and then incubated in light at 15/4 °C had 55 % radicle emergence, and seeds receiving similar stratification treatment but incubated in darkness had 75 % radicle emergence (Table 5). There was no germination of seed receiving warm + cold + warm stratification and then incubated in light at 15/4 °C. Seeds receiving similar stratification and temperature treatment, but incubated in darkness had 20 % germination. Seeds receiving (warm + cold) + warm + (warm + cold) and transferred monthly during the last 4-month stratification to incubation conditions had low radicle emergence until the last of the 4 months when warm + cold sequence was completed; germination reached 73 % at 15/4 °C in light.

Discussion
Diverse evolutionary processes with different selection pressures have produced a wide array of ecophysiological traits, such as different classes of seed dormancy (Walck and Hidayati 2004; Baskin and Baskin 2014). Within the genus Narcissus, there are species with fully developed embryos at dispersal time (Jonquillae and Tazettæ sections) and species with underdeveloped embryos at dispersal time (Bulbocodium and Pseudonarcissi sections) (Santiago 2012). According to Forbis et al. (2002), early diverging species have an underdeveloped embryo, and therefore exhibit MD or MPD, while the other dormancy classes are predicted to be derived from them (Baskin and Baskin 2004; Willis et al. 2014). Therefore, the Pseudonarcissi section occupies an ancestral place in the trait evolution process of the genus.

Narcissus yepesii, belonging to this last section, has an underdeveloped embryo, whose length is 1.28 ± 0.05 mm in mature seeds, which is 40 % of seed length. The seed is not able to germinate unless the embryo reaches at least the threshold length (2.1 mm), equivalent to 75 % of seed length. The lapse of time required for embryo growth and radicle emergence in optimal conditions is much longer than 30 days since a stratification period is necessary. This fact indicates that the embryo has to overcome both components of MPD: (i) MD or MPD, while the other dormancy classes are predicted to be derived from them (Baskin and Baskin 2004; Willis et al. 2014). Therefore, the Pseudonarcissi section occupies an ancestral place in the trait evolution process of the genus.

The embryo growth in N. yepesii occurs mainly during cold winter temperatures (5 °C) following warm summer/autumn temperatures (Table 2) or at cool temperatures (9/5 and 10 °C) (Table 3). Therefore, the level of MPD is complex since...
Embryo growth occurs from 0 to 10 °C (Nikolaeva 1969; Baskin and Baskin 2014). The most favourable temperature sequence is a warm stratification (20/7 + 15/4 °C, 1 month each) followed by 2 months at 5 °C, whereupon 96% of seeds have an E:S ratio ≥ threshold E:S ratio, (Table 2), meaning that the morphological component of MPD has been overcome (Copete et al. 2011a). After the embryo grew, 74% of the seeds incubated in darkness to autumn temperatures (15/4 °C) germinated.

To gain a better understanding of physiological component of MPD in N. yepesii, the effects of GA3 and of seed storage on overcoming dormancy were analysed. Gibberelic acid produced some stimulation of germination at low temperatures (5 °C in darkness) (Table 4), and dry laboratory storage had a significant positive influence on radicle emergence in 20-month-old seeds following a warm + cold stratification (Fig. 5). These results strongly support non-deep complex MPD in most of the seeds, which also occurs in seeds of N. longispathus (Herranz et al. 2013a).

The embryo growth was reduced when there was not a previous warm period before cold stratification. In seeds stratified for 4 months at 9/5 or 10 °C, 84 and 88% of embryos reached the threshold E:S ratio, respectively (Table 3). The explanation for high percentages is that both 10 and 9 °C (12 h day−1) are within the range of temperatures effective to overcome dormancy in

Table 4. Effect of GA3 (1000 ppm) on embryo growth (mean ± SE, mm, n = 25). Values followed by different uppercase letters within a column or different lowercase letters within a row are significantly different at the P-value < 0.05 level (Tukey multiple comparisons test). The first number in parentheses is the percentage of radicle emergence, and the second is the percentage of seeds with an E:S ratio greater than the threshold E:S ratio.

| Incubation (months) | 5 °C Light | 5 °C Darkness | 25/10 °C Light | 25/10 °C Darkness | 28/14 °C Light | 28/14 °C Darkness |
|---------------------|------------|--------------|----------------|------------------|----------------|------------------|
| 1                   | 1.40 ± 0.05Aa | 1.51 ± 0.06Aab | 1.60 ± 0.06Aab | 1.65 ± 0.05Ab    | 1.66 ± 0.07Ab  | 1.62 ± 0.07Ab    |
|                     | (0, 0)     | (0, 0)       | (0, 0)         | (0, 0)           | (0, 0)         | (0, 0)           |
| 3                   | 1.65 ± 0.07Ab | 2.06 ± 0.12Ab | 1.76 ± 0.06Aab | 1.87 ± 0.07Ab    | 1.84 ± 0.06Ab  | 1.96 ± 0.06Ab    |
|                     | (0, 10)    | (0, 32)      | (0, 0)         | (0, 4)           | (0, 10)        | (0, 0)           |
| 5                   | 1.75 ± 0.07Ab | 2.35 ± 0.11Ab | 1.87 ± 0.05Ab  | 2.07 ± 0.06Ab    | 2.16 ± 0.07Ab  | 1.96 ± 0.06Ab    |
|                     | (4, 10)    | (27, 46)     | (0, 0)         | (0, 10)          | (0, 24)        | (0, 4)           |
| Control (0 ppm)     | 1.73 ± 0.09Ab | 2.23 ± 0.09Ab | 1.98 ± 0.07Ab  | 2.05 ± 0.07Ab    | 2.18 ± 0.08Ab  | 2.11 ± 0.08Ab    |
|                     | (0, 10)    | (8, 44)      | (0, 0)         | (0, 10)          | (0, 24)        | (0, 20)          |
Incubation at 15/4 °C which is like seeds of the closely related N. alcaracensis 21 % of the 0-month seeds had intermediate complex MPD, slow embryo growth and no germination (Fig. 4). Therefore, 0-month seeds incubated under the same conditions exhibited for 4 months without a previous warm stratification, whereas storage, this shortened the cold stratification period required (Herranz et al. 2013b). Percentages of radicle emergence (mean ± SE, n = 4) after incubation at 15/4 °C in light and darkness following different stratification sequences. Seed age = 20 months. (W + C) + W + (20/7 °C) 0 ± 0 ---------a

| Stratification | Incubation at 15/4 °C |
|----------------|-----------------------|
| Temperature sequences | Light | Darkness |
| (W + C) + W | 55 ± 3 | 75 ± 3 |
| (W + C) + W + (20/7 °C) | 0 ± 0 | 20 ± 3 |
| (W + C) + W + (20/7 + 15/4 °C) | 0 ± 0 | ---------a |
| (W + C) + W + (20/7 + 15/4 + 5 °C) | 4 ± 0 | ---------a |
| (W + C) + W + (20/7 + 15/4 + 5 + 5 °C) | 73 ± 2 | ---------a |

This is because of the positive effect of seed dry storage during summer/autumn drought on germination.

In relation to shadehouse experiments, values of germination close to 100 % have been reached under natural conditions in contrast to laboratory tests. The coexistence of both dormancy levels implies a high final germination but over a period of several months, from November to March in seeds sown in July (Fig. 28). Firstly, non-deep dormant seeds germinate from November to January after a warm summer followed by a cool autumn, and then cold winter temperatures. Secondly, those seeds with an intermediate dormancy germinate from February to March because a longer cold period is required to overcome dormancy. Consequently, requirements of both levels are satisfied under natural conditions, and so final germination is close to 100 %.

The epicotyl is non-dormant in germinated seeds of N. yepesii since 80 % of shoots emerge from emerged-radicle seeds throughout 25 days of incubation at spring temperatures (20/7 °C) without a previous cold stratification (Fig. 6). The time (2 months) between radicle and shoot emergence could be due to low winter temperatures in the natural habitat, which means that a rapid shoot emergence can be avoided; this is similar to other species with non-deep complex MPD such as Merendera montana (Copete et al. 2011b).

Radicle emergence in N. yepesii seeds is promoted by darkness rather than light conditions (Fig. 5). Although this prevents the formation of a soil seed bank, the non-germinated seeds during the first spring after seed dispersal can re-enter secondary dormancy through the effect of warm summer temperatures (Table 5); this occurs in N. longispathus and N. alcaracensis (Herranz et al. 2016), and the seeds of these species will only be able to germinate in the second spring (Figs 2 and 7).

In view of the obtained results, the proposed hypothesis that N. yepesii may have different dormancy-breaking requirements to the phylogenetically closely related Narcissus species is not supported. In fact, remarkably the level of MPD present in seeds of N. alcaracensis and that in N. longispathus are both present in seeds of N. yepesii. However, the second hypothesis is satisfied since we have verified that seed dormancy breaking requirements in Narcissus populations from the Baetic System mountains show clinal variation in a northeast-southwest gradient from intermediate to non-deep
complex, through the unusual coexistence of both levels in *N. yepesi* plants living in an intermediate location of this geographical range (Fig. 1). Such a co-occurrence of dormancy levels should reflect the adaptation to the particular environmental conditions (Donohue et al. 2010).

Based on chromosomic studies, Fernandes (1975) showed *N. nevadensis* as being the most early diverging species from the *Pseudonarcissi* section. Afterwards, Herranz et al. (2013a) assigned intermediate complex MPD to the seeds of this species and suggested this dormancy level (3–4 months of cold stratification) as a possible way leading to non-deep complex MPD (2 months of warm + 2 months of cold stratification) throughout the adaptation to wetter environments, i.e. with an increase of mean temperature of the coldest month from 3.5 to 5.5 °C in the south-west area (Rivas-Martínez 1987). The anthropogenic climate change, with a progressive reduction of the cold period in the natural environment (Parmesan and Hanley 2015), could accentuate this tendency. However, the confirmation of this evolutionary scheme between levels of MPD requires delving into the knowledge of the phylogeny in the *Narcissus* group. Although Medrano et al. (2014) have analysed the genetic structure of daffodil populations in the Baetic System, further studies are still needed to evaluate the taxonomic status of some of these populations.

A similar model occurs in species of *Osmorhiza* (Apiaceae) from North America. So, *O. occidentalis* and *O. depauperata* in the West have deep complex MPD (ancestral dormancy condition), whereas *O. claytonii* and *O. longistylis* in the East have non-deep complex MPD (derived condition) (Walck and Hidayati 2004). Nevertheless, a species with both levels of MPD, which could play the role of *N. yepesi* in this study, was not identified in that model.

A dormancy cline related to altitude (m a.s.l.), mean annual temperature (°C) and summer precipitation (mm) has been shown in *Centaurium somedanum* (Gentianaceae), an endemic perennial herb of north-western Spain whose seeds have non-deep simple MPD (Fernández-Pascual et al. 2013). Nevertheless, this is the first study to show a gradient between levels of intermediate complex and non-deep complex MPD. Thus, the seed dormancy evolution across the cline from one level of MPD to another level of MPD, as adaptation to environmental changes, can be a key factor determining the course of the plant trait evolution process in species with underdeveloped embryos.

**Supporting Information**

The following additional information is available in the online version of this article—

**Figure S1.** Shows changes in size class distribution of embryos in seeds of *Narcissus yepesi*: at seed dispersal time (A), sown in July (B) and in December (C) and recovered each month for 4 months.
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Contributions by the Authors
All authors have contributed throughout the different phases of this work in a coordinated way: designed and performed the experiments, analysed data, wrote the manuscript, discussed the results and contributed to the final manuscript.

Conflict of Interest
None declared.

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Data Availability
All data are available in the online version of the article as Supporting Information.

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