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Published Version

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Newton, R. J., Hay, F. R. and Ellis, R. H. ORCID: <https://orcid.org/0000-0002-3695-6894> (2013) Seed development and maturation in early spring-flowering Galanthus nivalis and Narcissus pseudonarcissus continues post-shedding with little evidence of maturation in planta. Annals of Botany, 111 (5). pp. 945-955. ISSN 1095-8290 doi: <https://doi.org/10.1093/aob/mct051> Available at <https://centaur.reading.ac.uk/30948/>

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To link to this article DOI: <http://dx.doi.org/10.1093/aob/mct051>

Publisher: Oxford University Press

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## Seed development and maturation in early spring-flowering *Galanthus nivalis* and *Narcissus pseudonarcissus* continues post-shedding with little evidence of maturation *in planta*

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Received: 15 November 2012 Returned for revision: 7 January 2013 Accepted: 21 January 2013

- **Background and Aims** Seeds of the moist temperate woodland species *Galanthus nivalis* and *Narcissus pseudonarcissus*, dispersed during spring or early summer, germinated poorly in laboratory tests. Seed development and maturation were studied to better understand the progression from developmental to germinable mode in order to improve seed collection and germination practices in these and similar species.
- **Methods** Phenology, seed mass, moisture content and ability to germinate and tolerate desiccation were monitored during seed development until shedding. Embryo elongation within seeds was investigated during seed development and under several temperature regimes after shedding.
- **Key Results** Seeds were shed at high moisture content (>59 %) with little evidence that dry mass accumulation or embryo elongation were complete. Ability to germinate developed prior to the ability of some seeds to tolerate enforced desiccation. Germination was sporadic and slow. Embryo elongation occurred post-shedding in moist environments, most rapidly at 20 °C in *G. nivalis* and 15 °C in *N. pseudonarcissus*. The greatest germination also occurred in these regimes, 78 and 48 %, respectively, after 700 d.
- **Conclusions** Seeds of *G. nivalis* and *N. pseudonarcissus* were comparatively immature at shedding and substantial embryo elongation occurred post-shedding. Seeds showed limited desiccation tolerance at dispersal.

**Key words:** Amaryllidaceae, germination, *Galanthus nivalis*, *Narcissus pseudonarcissus*, seed development, desiccation tolerance, embryo growth, temperate woodland geophytes.

### INTRODUCTION

Orthodox seed (Roberts, 1973) development typically may be divided into three distinct phases following ovule fertilization: histodifferentiation, during which embryonic tissues are formed; seed filling, when reserve deposition takes place; and maturation drying, characterized by water loss and reduced metabolism (Kermode *et al.*, 1986; Bewley and Black, 1994). Seed filling is terminated at mass maturity (Ellis and Pieta Filho, 1992), with maturation drying thereafter. During orthodox seed development, the ability to germinate (Kermode and Bewley, 1985), seed desiccation tolerance (Ellis *et al.*, 1987) and potential longevity (Pieta-Filho and Ellis, 1991) develop and increase, usually in that order (Sanhewé and Ellis, 1996). In some species, drying promotes germinability; fresh seeds that have not undergone desiccation will not germinate or germinate only poorly (Adams and Rinne, 1981; Samarah *et al.*, 2004). In recalcitrant seeds (Roberts, 1973), maturation drying is absent or much reduced; at dispersal, seeds show little water loss, are metabolically active and may still be accumulating dry weight (Farrant *et al.*, 1993; Kermode and Finch-Savage, 2002).

Ideally, orthodox seeds should be collected when, for the majority of the seeds in the targeted population, germinability,

desiccation tolerance and longevity are at their maximum. At this point, it is important that seeds are dried to low moisture contents to ensure that this potential for considerable longevity is maintained (Harrington, 1972; Roberts, 1973). Recommended conditions for the conventional long-term storage of orthodox seeds, after drying to 3–7 % moisture content (fresh mass basis), are in air-tight containers at –18 °C or less (Cromarty *et al.*, 1982; FAO/IPGRI, 1994).

The Amaryllidaceae are extremely variable in fruit and seed characters, particularly among the American, African and Eurasian clades. Eurasian species usually have dry, wedge-shaped seeds that frequently have an elaiosome (oil-rich food body attractive to ants) at the chalazal end of the seed (Meerow *et al.*, 1999). Seeds comprise a small, linear embryo embedded in endosperm (Church, 1908; Martin, 1946). Seed storage behaviour ranges from predominantly orthodox in the American clade to recalcitrant in the African clade (Priestley, 1986; Sershen *et al.*, 2008). Seed storage behaviour in the Eurasian clade is unknown. The investigations reported here, of seed development in *Galanthus nivalis* L. (common snowdrop) and *Narcissus pseudonarcissus* L. (wild daffodil), arose from difficulties in germinating

seeds from collections of European species of the Amaryllidaceae in the Millennium Seed Bank and concerns regarding survival of stored seed (R. J. Probert, RBG Kew, UK, pers. comm.).

*Galanthus nivalis*, indigenous to western, southern and central Europe, and *N. pseudonarcissus*, native to southern Europe and the Western Mediterranean, are typically found in temperate deciduous woodland growing in damp soil which, in the case of *G. nivalis*, may be prone to short periods of flooding (Church, 1908; Caldwell and Wallace, 1955; Budnikov and Kricsfalussy, 1994; Bishop *et al.*, 2001). These plants are unique within these habitats as they are the first understorey plants to emerge and flower in late winter/early spring, with seed shed occurring near the end of spring. Rapid vegetative growth and flowering is possible in these species at these cold temperatures because shoots and flowers develop and differentiate internally in the bulbs during the previous summer (Church, 1908; Bishop *et al.*, 2001). Light conditions in the understorey of deciduous woodland are maximal during the period between leaf fall in autumn and leaf development in spring, compared with shaded conditions during summer when trees are in leaf (Salisbury, 1916), light being the limiting factor for vegetative growth and reproduction in woodland herbs (Whigham, 2004). Hence, their strategy of early development enables *G. nivalis* and *N. pseudonarcissus* to take advantage of the availability of light by completing vegetative and reproductive development, including the major proportion of seed development, before tree canopy closure. Little is known about the phenology of seed development physiology in early spring-flowering temperate species. Consequently, these two species were appropriate study subjects to improve our understanding of the acquisition of germinability and desiccation tolerance at colder temperatures.

The main objective of this study was to develop an understanding of seed development in these species and so improve the management of the seeds of these and similar species in *ex situ* conservation. We describe the phenology of seed development and maturation, characterize seed germinability and desiccation tolerance during seed development and on shedding, and investigate embryo elongation pre- and post-shedding in order to test the hypothesis that seed development and maturation are complete prior to dispersal.

## MATERIALS AND METHODS

### *Study sites and environmental conditions during seed development*

*Narcissus pseudonarcissus* seeds were collected from a wild population within a 200 × 200 m area in the Loder Valley Nature Reserve, West Sussex, UK (51°03'29"N, 00°05'33"W). *Galanthus nivalis* seeds were collected from an introduced population at Wakehurst Place, West Sussex, UK (51°04'07"N, 00°05'16"W). The latter population of approx. 20 000 plants was established from bulbs planted in September 2005 supplied by The Boston Bulb Company (Cradge Bank, Spalding, UK) from a UK grower who collected the naturalized plants under licence and with permission from landowners in Norfolk, Yorkshire and Scotland. A Tiny Tag

PT 100 logger [Gemini Loggers (UK) Ltd, Chichester, UK], positioned approx. 100 mm above the ground within the study sites, recorded relative humidity (RH) and temperature every 20 min during the period of capsule collection; the means of which were 75.1 % ( $\pm 0.47$  s.e.) and 15.5 °C ( $\pm 0.12$  s.e.), respectively. Daily maximum and minimum temperatures, obtained from the Horticulture Section at Wakehurst Place, were used to calculate the weighted mean daily temperature (taking photoperiod into account) during seed development.

### *Capsule collection*

Flowers of *N. pseudonarcissus* (in 2006) and *G. nivalis* (in 2008) were tagged every few days (1–3 times a week) from when they first opened in March and January, respectively. At least twice as many flowers were tagged as capsules collected due to flower and fruit loss during flowering and seed development. All flowers that had opened in the case of the first tagging, or had opened subsequent to the previous tagging, were tagged. This was continued within the designated sampling area until all flowers had opened. Capsules were sampled regularly to monitor seed development (from both species and all flower cohorts, where tagged), from the beginning of May (Fig. 1) until capsule opening and seed shed. Closed *G. nivalis* capsules, in which the flower stalk had started to disintegrate (typically around mid-May), thereby cutting the vascular connection to the mother plant, were collected and arranged in a monolayer on blotting paper in a plastic tray at 15 °C and 75 % RH in order to mimic the natural environment for continued ripening *ex planta*. The majority of seed development data for *G. nivalis* were obtained from capsules ripened *ex planta* in these conditions for 1–20 d. A few *G. nivalis* capsules dried without dehiscing; seeds from these capsules were excluded from analyses. As the flower stalk did not disintegrate in *N. pseudonarcissus*, capsules were left to ripen naturally on the plant throughout seed development.

### *Capsule and seed measurements*

In total, 47 *N. pseudonarcissus* capsules were collected in 2006 and 46 in 2009; 260 *G. nivalis* capsules were collected in 2008. Capsule colour was recorded and seeds from individual capsules were divided equally into five groups ( $n \leq 10$  seeds per group) to determine: (1) individual seed fresh weight, dry weight and hence moisture content; (2) the ability of fresh (i.e. not dried) seeds to germinate; (3) the ability of dried seeds to germinate (and so desiccation tolerance); (4) embryo length and viability of fresh seeds; and (5) embryo length and viability of dried seeds. To estimate desiccation tolerance, seeds from individual capsules were rapidly dried by placing in a monolayer in 10 mL clear, open glass vials (Scientific Laboratory Supplies Ltd, Nottingham, UK) at 15 °C and 15 % RH for 28 d prior to germination testing. Data from individual capsules were then combined for analysis.

The effect of drying at 15 °C and 75 % RH (reflecting the mean ambient temperature and RH at the study site at the time of capsule opening) on seed moisture content was also determined for separate samples of freshly dispersed *N. pseudonarcissus* and *G. nivalis* seeds.

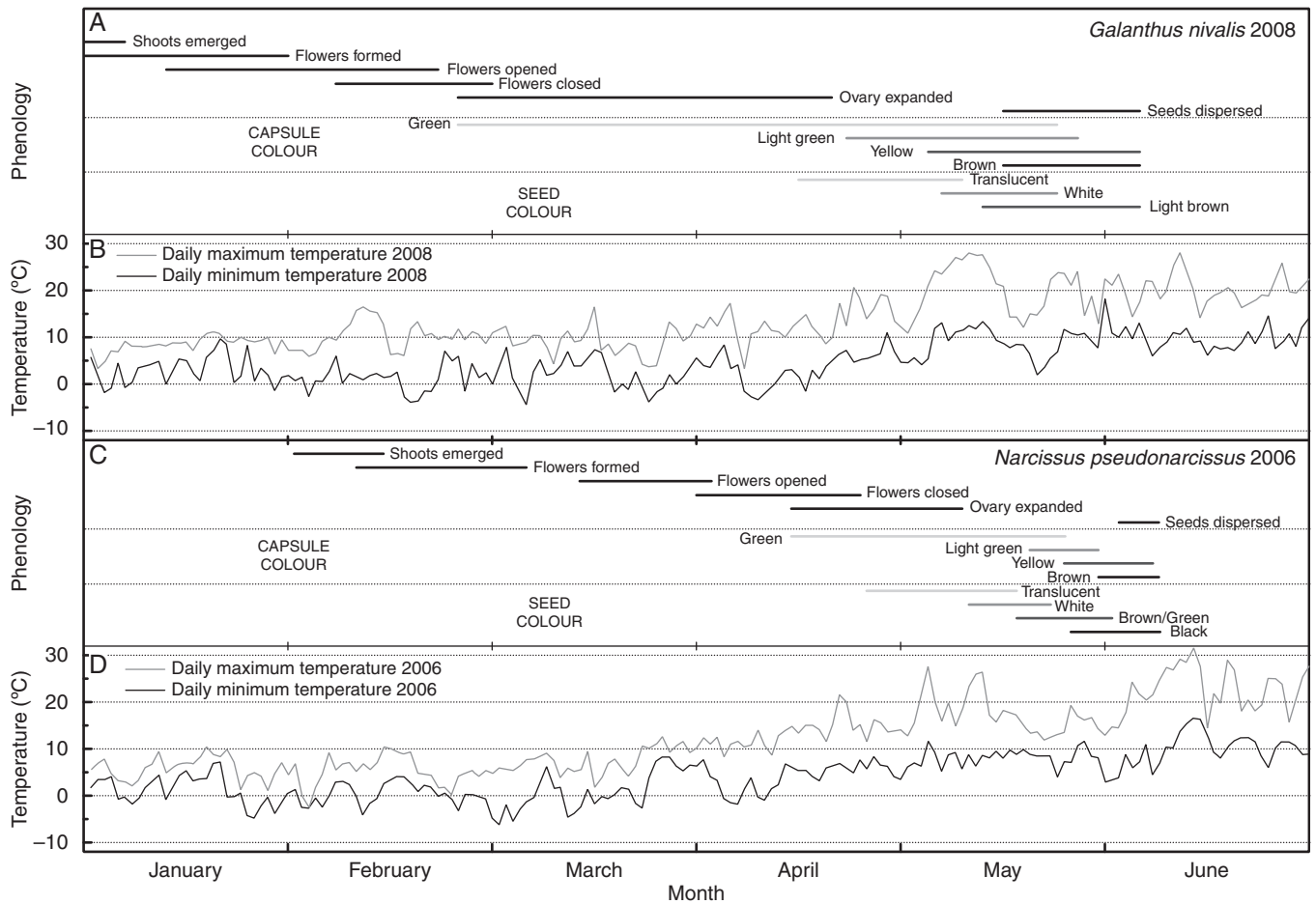


FIG. 1. Phenology of flowering, fruit and seed development of (A) *Galanthus nivalis* (2008) and (C) *Narcissus pseudonarcissus* (2006), and maximum and minimum daily temperatures at Wakehurst Place in West Sussex in (B) 2008 and (D) 2006, respectively.

#### Seed weight and moisture content determination

Seed moisture content (and also fresh and dry weight) was determined gravimetrically according to the low-constant-temperature-oven method (ISTA, 2005) for 482 seeds from 47 capsules, 380 seeds from 39 capsules, and 347 seeds from 189 capsules for *N. pseudonarcissus* 2006, *N. pseudonarcissus* 2009, and *G. nivalis* 2008, respectively. Fresh weight was obtained by weighing individual seeds on a seven-place balance (Mettler Toledo, Beaumont Leys, UK). Seeds were then dried for  $17 \pm 1$  h at  $103 \pm 2$  °C in a Genlab 100 L ventilated oven (Jencons-PLS, Forest Row, UK), and reweighed to determine dry weight. Moisture content was calculated as a percentage of fresh seed mass.

#### Embryo length and viability

Dried seeds were suspended over water (100 % RH) for 24 h to rehydrate before placing onto 1 % distilled water agar for 24 h to imbibe. Rehydrated and fresh seeds were dissected longitudinally near the mid-section (avoiding damaging the embryo), using a Cobra light microscope (Vision Engineering, Woking, UK) and placed into a 1 % aqueous solution of tetrazolium chloride (ISTA, 2005) for 72 h at 30 °C in

the dark [method for the genus *Amaryllis*, modified from Moore (1985)]. Seeds in which the entire embryo and endosperm stained a uniform bright or dark red colour were scored as viable. After staining, seeds were dissected to record embryo length using a Nikon SMZ645 dissecting microscope with a C-W10X/22 micrometer (Southern Microscopes, Maidstone, UK): 167 embryos from *N. pseudonarcissus* 2006, 412 embryos from *N. pseudonarcissus* 2009, and 117 embryos from *G. nivalis* 2008 seeds were measured.

#### Germination testing

Fresh or dried seeds (one seed per well) were sown on 1 % distilled water agar (Microbiological Agar Granules, Merck, Darmstadt, Germany) in 10 mm Nunc multiwell dishes (Scientific Laboratory Supplies Ltd), which allowed individual seeds to be monitored. *Narcissus pseudonarcissus* seeds collected in 2006 were placed in cooled incubators (LMS Ltd, Sevenoaks, UK) at 25/10 °C for 84 d then moved to 15/5 °C for 84 d (8/16 h light/dark photoperiod). Improved germination conditions were later determined and so, for *G. nivalis* seeds collected in 2008 and *N. pseudonarcissus* seeds collected in 2009, dishes were double-wrapped in aluminium foil, sealed in clear plastic bags and placed for 56 d at each of, in



sequence, 25, 20, 15 and 10 °C (with fresh agar every 56 d). In total, 612 dried *N. pseudonarcissus* (2006), 374 fresh and 356 dried *N. pseudonarcissus* (2009) and 351 fresh and 297 dried *G. nivalis* (2008) seeds were tested for germination. Seeds dried before germination testing were placed at 15 % RH and 15 °C for 28 d, attaining a mean moisture content of  $7.1 \pm 0.13$  % (s.e.) for *G. nivalis* and  $6.9 \pm 0.33$  % (s.e.) for *N. pseudonarcissus*. Seeds were assessed for germination at regular intervals under a dim, safe, green light comprising three 15–20 W cool white fluorescent tubes covered by three layers of no. 39 (primary green) Cinemoid as described in [Probert and Smith \(1986\)](#). Seeds were scored as germinated when the radicle was at least 2 mm long. Seeds that had not germinated at the end of a test were dissected to determine whether they remained firm (and so assumed viable).

#### Post-shedding germination testing and embryo elongation

Germination tests ( $n = 50$  seeds per species and temperature) were set up on fresh seeds from naturally dehiscent capsules. Poorly developed seeds were excluded from samples. Seeds were placed into Nunc multiwell dishes containing 1 % distilled water agar; *N. pseudonarcissus* seeds collected in 2006 were placed at three alternating (8/16 h) temperatures (10/0, 15/5 or 25/10 °C) and *N. pseudonarcissus* (2007) and *G. nivalis* (2007) seeds at six constant temperatures (0, 5, 10, 15, 20 or 25 °C). *Narcissus pseudonarcissus* seeds were exposed to light (8/16 h light/dark cycle), while seeds of *G. nivalis* were kept in the dark. Selection of these environments, and those above for germination tests, were based on developing experience with these species in the Millennium Seed Bank (data not shown). Germination was scored as above.

Duplicate samples were placed onto agar and into identical conditions to assess post-shedding embryo elongation. At each sampling (initially every 30 d, subsequently less frequently), ten seeds were randomly removed for embryo length measurements and the remaining seeds placed onto fresh agar and returned to the incubators. Seeds were dissected and placed into tetrazolium to assess viability (as described above). Only non-germinated, viable seeds were assessed for embryo length.

Additional seeds incubated in the dark at 20 °C for 84 d and then moved to either 10 °C (*N. pseudonarcissus* 2007) or 15 °C (*G. nivalis* 2007) were checked every 1–3 d for germination. Embryo lengths of germinated seeds ( $n = 20$  seeds per species) were measured as described above as soon as the radicle was first observed. The germination embryo length measurement did not include the section of radicle that protruded beyond the seed coat.

#### Data analysis

All analyses were done in Genstat for Windows Eleventh Edition (VSN International Ltd, Hemel Hempstead, UK). Seed dry weight, fresh weight and moisture content were initially regressed against each of the days after flowering, thermal time from flower opening and sample day (calculated by setting the last sample date, which occurred within the period during which most seed was being dispersed, to zero) to determine which of these explanatory variables best

accounted for the variation observed in seed development. Sample day was selected as the explanatory variable that enabled full and representative presentation of the results.

Variance increased with seed development. Logarithmic transformation of individual capsule means of seed dry weight, fresh weight and moisture content satisfied the requirement for normality and equal variance (with the exception of moisture content for *N. pseudonarcissus*). Regressions were weighted by the number of observations for capsule means. Given the expected patterns of seed development, linear and split-line regressions (dry weight), linear and quadratic regressions (fresh weight), and linear regressions (moisture content) were fitted to capsule means.

Pre-shedding embryo lengths of fresh and fully rehydrated dried seeds were not significantly different in either *G. nivalis* 2008 (Mann–Whitney U-test,  $P = 0.137$ ) or *N. pseudonarcissus* 2009 (Mann–Whitney U-test,  $P = 0.138$ ). Embryo lengths for both cohorts were thus combined for analysis.

Linear, split-line or quadratic regressions were fitted to embryo length measurements against post-shedding duration at each temperature. Multiple linear regression analysis was performed on natural log-transformed post-shedding embryo length data to determine the relative importance of duration of incubation and temperature on elongation.

## RESULTS

### Phenology

Development from shoot emergence to seed dispersal occupied the first 5 months of the calendar year for both species (Fig. 1). Not surprisingly, given the cool temperatures, the duration between flower formation and ovary expansion or capsule swelling (i.e. seed formation) was considerable, especially in *G. nivalis*. In contrast, subsequent fruit ripening and seed development was more rapid (Fig. 1). Capsule colour progressed from green through light green then yellow to brown as seeds matured in both *G. nivalis* and *N. pseudonarcissus*. In *G. nivalis*, individual flower life span was much reduced in later-opening flowers. There were no differences, otherwise, in seed development among different flowering cohorts. By mid-May, some seeds within capsules of both species had aborted. The number of seeds per capsule was substantially greater in *N. pseudonarcissus* than in *G. nivalis* (Table 1).

### Dry mass accumulation, moisture content and embryo elongation during seed development

As expected, seed moisture content declined while seed fresh weight, dry weight and embryo length increased during seed development (Fig. 2), with considerable variability in each. Seeds were shed at the comparatively high moisture content of 59–67 % (Fig. 2A–C). Seed filling (Fig. 2D–I, Table 1) and embryo elongation (Fig. 2J–L) continued until, or almost until, seed dispersal. When dispersed, the seeds had not equilibrated with ambient RH [ $75.1$  % ( $\pm 0.47$  s.e.)], as freshly collected seeds of *G. nivalis* and *N. pseudonarcissus* held at 75 % RH and 15 °C dried within 14 d to 22 and 17 % moisture content (Table 1), respectively.

TABLE 1. Developing seeds per capsule, seed moisture content (MC) on shedding or after a further 14 d at 75 % RH and 15 °C (simulating the environment into which seeds are shed), and dry weight at shedding in *Narcissus pseudonarcissus* and *Galanthus nivalis* (where N is the number of capsules and n is the number of seeds; all values are means  $\pm$  s.e.)

Species and year	No. of seeds per capsule	MC (%) on shedding	MC (%) after 14 d at 75 % RH, 15 °C	Dry weight (mg)
<i>N. pseudonarcissus</i> 2006	43 $\pm$ 4.2 (N = 33)	66.8 $\pm$ 1.96 (n = 50)	16.7 $\pm$ 0.48 (n = 3 $\times$ 20)	4.10 $\pm$ 0.199 (n = 50)
<i>N. pseudonarcissus</i> 2009	45 $\pm$ 2.8 (N = 46)	59.1 $\pm$ 1.35 (n = 58)	–	6.00 $\pm$ 0.247 (n = 58)
<i>G. nivalis</i> 2008	6.2 $\pm$ 0.34 (N = 260)	60.1 $\pm$ 1.65 (n = 12)	22.0 $\pm$ 0.73 (n = 10)	7.08 $\pm$ 0.696 (n = 12)

#### Acquisition of ability to germinate and tolerate rapid desiccation

In *N. pseudonarcissus* (2009), some seeds were able to germinate 19 d before dispersal, when mean seed moisture content was 84 % (Fig. 3A). Ability to germinate increased progressively thereafter, reaching 88 % when moisture content had declined to 66 %, 3 d before most seeds were dispersed. The ability to germinate was first detected in fresh *G. nivalis* seeds somewhat earlier, 29 d prior to dispersal, when mean seed moisture content was 77 % (Fig. 3B). It then increased gradually to a maximum of 79 % when moisture content had declined to 67 %, 12 d before dispersal, with a subsequent slight decline at dispersal (Fig. 3B).

The proportion of seeds that were fully formed (i.e. solid) on dissection (for embryo measurement) was positively associated with that which germinated immediately following collection in parallel tests for both *G. nivalis* 2008 ( $r = 0.91$ ,  $P = 0.001$ ) and *N. pseudonarcissus* 2009 ( $r = 0.93$ ,  $P = 0.004$ ). The seed populations at dispersal were not homogeneous in either species, with some seeds failing to accumulate dry matter, possibly the result of seed abortion. This observation tallies with the substantial proportion of non-viable seeds at dispersal (e.g. open bars in Fig. 3A, B at the final sample day).

The development of desiccation tolerance lagged behind that of germinability (Fig. 3). Desiccation tolerance (here the ability to germinate following rapid drying at 15 % RH) was first observed in *N. pseudonarcissus* 12 d (2009) and 13 d (2006) before dispersal when moisture content was 73–78 %, about 7 d after germinability was first detected. Fewer than half the seeds at dispersal were viable after rapid drying (Fig. 3C, D). Desiccation tolerance was not detected in *G. nivalis* until seed moisture content had declined to 63 %, 23 d after the ability to germinate was first observed and only 6 d before dispersal (Fig. 3E). A maximum of 36 % of seeds close to dispersal germinated following rapid desiccation, with 44 % potentially viable.

#### Capsule and seed characteristics

In *N. pseudonarcissus*, fewer than half of fresh seeds from green capsules were potentially germinable (Fig. 4A). Most seeds in light green capsules were germinable, with a greater proportion of non-viable seeds on dissection in yellow and brown capsules (Fig. 4A). A similar pattern of increasing then decreasing germinability with capsule colour progression occurred in *G. nivalis*, but with maximum germinability in seeds from yellow capsules (Fig. 4B).

Less than 1 % of seeds from green capsules were desiccation tolerant in *N. pseudonarcissus*, but seeds from light green and yellow capsules were progressively better able to withstand rapid desiccation (Fig. 4A). *Narcissus pseudonarcissus* seeds from yellow and brown capsules showed similar desiccation tolerance (Fig. 4A). No *G. nivalis* seeds from green or light green capsules tolerated desiccation; however, a small fraction from yellow capsules and a marginally larger fraction from brown capsules survived drying (Fig. 4B).

#### Post-shedding embryo elongation

At dispersal, embryos of *N. pseudonarcissus* were longer than those of *G. nivalis* (Figs 2J–L and 5), but broadly comparable amongst years within species, ranging from 1.1 to 1.4 mm in *N. pseudonarcissus* and from 0.6 to 0.7 mm in *G. nivalis*. Considerable embryo elongation (almost doubling in *N. pseudonarcissus* and more than trebling in *G. nivalis*) occurred post-shedding in hydrated seeds over prolonged periods *in vitro* at different temperatures (Fig. 5A–C). Rates of embryo elongation were ten times slower in *N. pseudonarcissus* and three times slower in *G. nivalis* than pre-shedding elongation (at the equivalent temperature).

Embryo elongation was temperature dependent: embryos in seeds of both species held at warmer temperatures ( $\geq 15$  °C) elongated more rapidly than those in cooler ( $< 15$  °C) conditions (Fig. 5A–C). Multiple regression analysis for both species at constant temperatures indicated that temperature ( $P < 0.001$ ) and incubation period ( $P < 0.001$ ) both affected embryo length, as did their interaction ( $P < 0.001$ ). The models explained a greater proportion of the variance for *G. nivalis* ( $R^2 = 0.68$ ,  $P < 0.001$ ) than *N. pseudonarcissus* ( $R^2 = 0.36$ ,  $P < 0.001$ ), with temperature more influential, especially in *G. nivalis*. The germination embryo length was similar in both species, at 2.10–2.36 mm (Fig. 5B, C). In warmer temperatures ( $\geq 10$  °C in *N. pseudonarcissus* and  $\geq 15$  °C in *G. nivalis*), the mean germination embryo length was eventually exceeded or attained, while embryos at cooler temperatures elongated slowly throughout the investigation without reaching the germination embryo length. Embryo elongation in *N. pseudonarcissus* appeared to cease once the germination embryo length was reached; in *G. nivalis*, elongation continued, albeit more slowly.

Temperature regimes in which more rapid elongation was detected were also those at which germination occurred more readily (Fig. 5D–F). The greatest germination was observed in *N. pseudonarcissus* at 25/10 °C (2006) and 15 °C (2007)

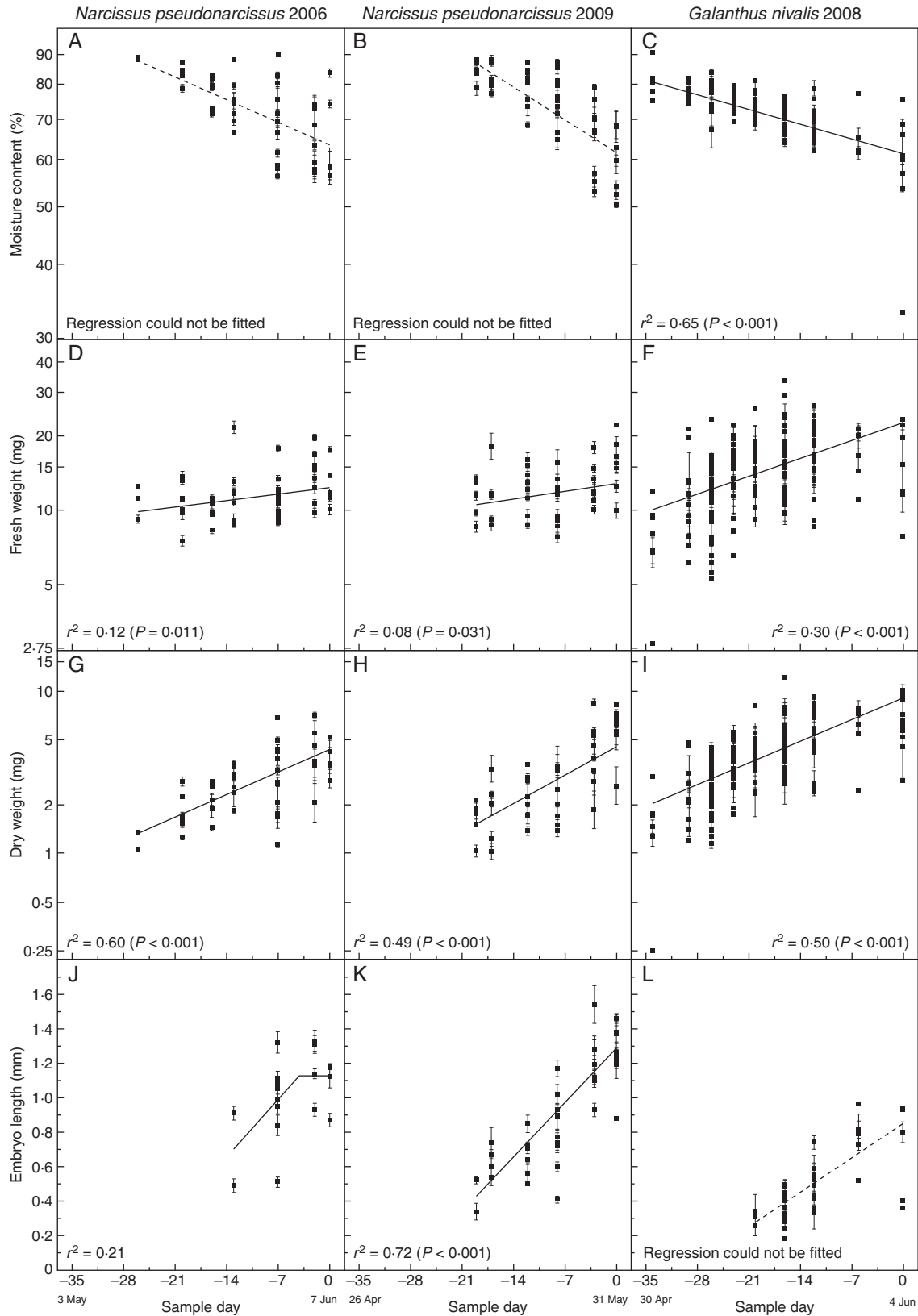


FIG. 2. (A–C) Mean moisture content (logarithmic scale), (D–F) fresh weight (logarithmic scale), (G–I) dry weight (logarithmic scale), and (J–L) embryo length of seeds ( $\pm$  s.e.) from individual capsules during seed development plotted against sample day (where seed dispersal occurred mainly on day zero). A total of 482 seeds from 47 capsules (*Narcissus pseudonarcissus* 2006), 380 seeds from 39 capsules (*N. pseudonarcissus* 2009) and 347 seeds from 189 capsules (*Galanthus nivalis* 2008) were sampled. All results, including outliers, are shown. For linear regression analysis (C–I, K) and split-line regression analysis (J), one outlier was removed from the *N. pseudonarcissus* 2006 data set, two from the *N. pseudonarcissus* 2009 data set and three from the *G. nivalis* 2008 data set. In (A), (B) and (L), trend lines are shown where regression analysis was not possible.



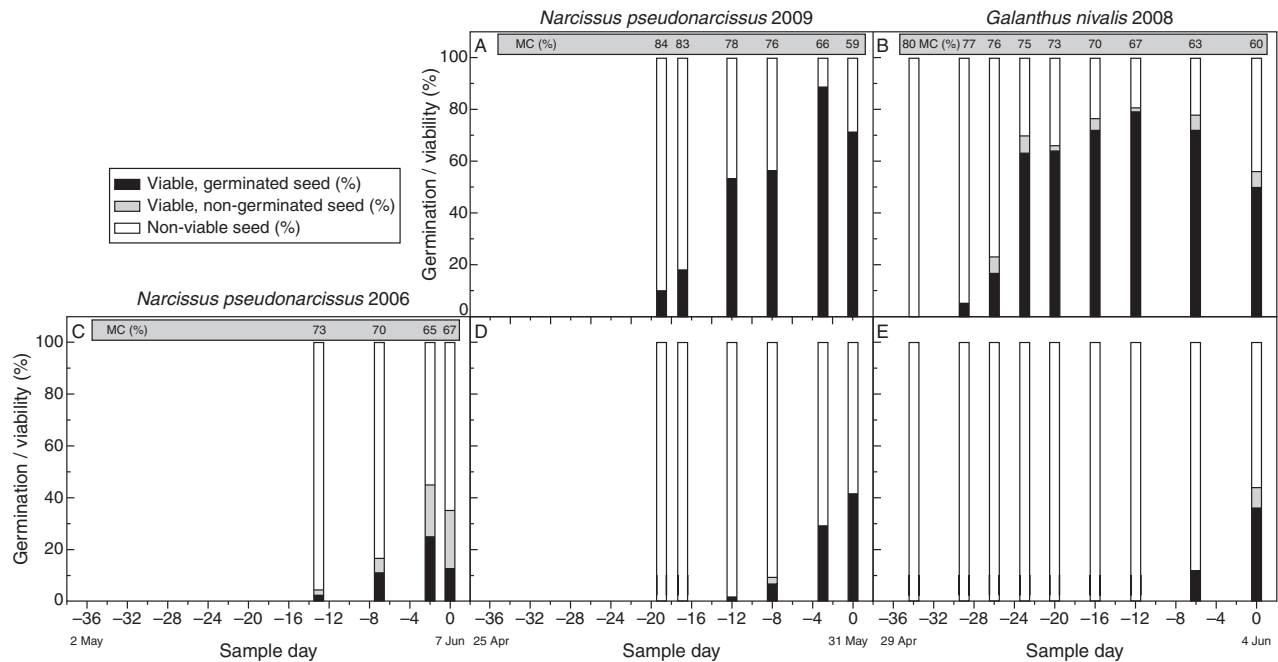


FIG. 3. Changes in viability and ability to germinate during seed development of (A) fresh *N. pseudonarcissus* 2009 ( $n = 374$ ), (B) fresh *G. nivalis* 2008 ( $n = 351$ ), (C) dried *N. pseudonarcissus* 2006 ( $n = 612$ ), (D) dried *N. pseudonarcissus* 2009 ( $n = 356$ ) and (E) dried *G. nivalis* 2008 ( $n = 297$ ) seeds. Mean moisture content (MC, fresh weight basis) is shown for seeds from the same set of capsules. Seeds were sampled from 12 May 2009 (day -19) until observed natural seed dispersal on 31 May 2009 (day 0) for *N. pseudonarcissus* (A, D), from 25 May 2006 (day -13) until 7 June 2006 (day 0) for *N. pseudonarcissus* (C) and from 1 May 2008 (day -34) until 4 June 2008 (day 0) for *G. nivalis* (B, E). Seeds were incubated for a total of 224 d at decreasing constant temperatures of 25–20–15–10 °C (56 d each) in the dark.

and in *G. nivalis* at 20 °C, starting around the same time as germination embryo length was reached. Germination occurred to a lesser extent at slightly cooler temperatures (10 °C in *N. pseudonarcissus* and 15 °C in *G. nivalis*) (Fig. 5E, F), commencing before embryos at these temperatures had reached the mean germination embryo length (Fig. 5B, C).

## DISCUSSION

The hypothesis that seeds are shed when they are mature is rejected for both of these moist temperate woodland species: seed dispersal occurred whilst seeds were immature and considerable embryo development occurred after shedding. At dispersal, there was no strong evidence of prior cessation of dry weight accumulation (Fig. 2G–I) in seeds of *G. nivalis* and *N. pseudonarcissus*. Furthermore, maturation drying was absent (Fig. 2D–F), i.e. no loss in fresh weight, and similarly seeds had not equilibrated with ambient RH prior to shedding (Table 1). Instead, most of the seed development period comprised seed filling, with seeds being shed at high moisture content (>59 %) into the environment (Fig. 2A–C, Table 1), similar to that of the temperate woodland herb *Anemone nemorosa* [66 % moisture content, fresh weight basis, 1.97 g (g d. wt)<sup>-1</sup>] dispersed in late spring (Ali *et al.*, 2007). This pattern is unusual compared with most non-fleshy orthodox seeded species, which typically undergo maturation drying before dispersal (Ellis and Pieta-Filho, 1992; Hay and Probert, 1995; Samarah *et al.*, 2004), equilibrating with the RH of the environment after maximum dry weight is attained

(Probert and Hay, 2000), or compared with fleshy fruited orthodox species where seeds continue to mature *in planta* for several weeks after seed filling until fruit drop and seeds are shed at high moisture contents (Welbaum and Bradford, 1989; Berry and Bewley, 1991; Demir and Ellis, 1992).

Seeds are shed at the end of May (Fig. 1) into moist, shady habitats (Church, 1908), with continuous hydration in the soil seed bank probable (Fenner and Thompson, 2005). Indeed, species from damp temperate habitats may not have been exposed to the selection pressure of seed able to withstand considerable desiccation before germination (Ali *et al.*, 2007). It is therefore reasonable to assume that the linear embryos of *G. nivalis* and *N. pseudonarcissus*, which elongated during seed development (Fig. 2) and continued to develop *in vitro* for a considerable period of time post-shedding (Fig. 5), may do so similarly in the natural environment, albeit at a slower rate. Several other temperate plant species, including *A. nemorosa* (Ali *et al.*, 2007; Mondoni *et al.*, 2008), *A. ranunculoides* (Mondoni *et al.*, 2009) and *Scilla bifolia* (Vandelook and van Assche, 2008) possess embryos that grow continuously within the seed following dispersal without an extended period of developmental arrest.

Temperature was the most influential factor in post-shedding embryo elongation, with more rapid elongation rates at warmer temperatures (Fig. 5A–C), similar to embryo elongation patterns reported in *Daucus carota* (Gray *et al.*, 1988), *N. hispanicus* (Copete *et al.*, 2011) and by other authors also for *N. pseudonarcissus* (Vandelook and van Assche, 2008) seeds. Germination was delayed and sporadic (Fig. 5D–F). Several authors have suggested a minimum embryo length

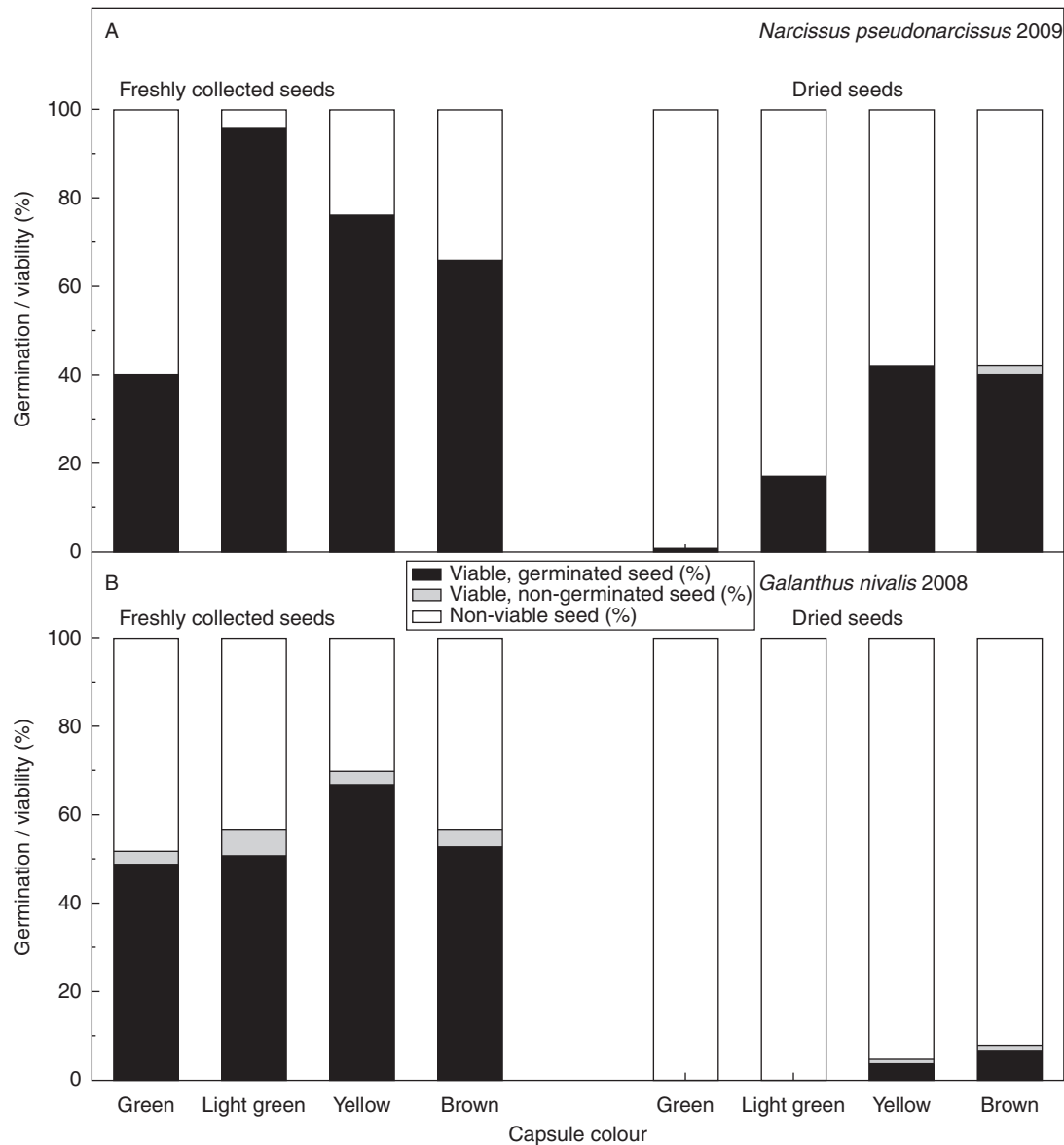


FIG. 4. The ability of fresh and dried seeds to germinate from different coloured capsules sampled during seed development in (A) *Narcissus pseudonarcissus* 2009 ( $n = 374$  for fresh and  $n = 356$  for dried seeds) and (B) *Galanthus nivalis* 2008 ( $n = 351$  for fresh and  $n = 297$  for dried seeds). This analysis is drawn from the same overall data set as that in Fig. 3; for further details, see Fig. 3.

for germination in species where embryos must elongate before radicle emergence (van der Toorn and Karssen, 1992; Hidayati *et al.*, 2000; Kondo *et al.*, 2004; Copete *et al.*, 2011). Although the mean embryo length at germination was determined for *N. pseudonarcissus* (Fig. 5B) and *G. nivalis* (Fig. 5C) from move-along germination experiments (warm followed by cool temperatures as may be experienced in the natural environment), attainment of this embryo length neither guaranteed, nor was required for, germination. Little germination occurred in seeds at 25 °C (Fig. 5E, F), in which the apparent germination embryo length was attained or exceeded, while germination at cooler temperatures (10 °C in *N. pseudonarcissus* and 15 °C in *G. nivalis*) commenced in some seeds before the germination embryo length was

reached (Fig. 5B, C). Hence, while embryo elongation is necessary, the notion of a critical embryo length for germination in these species is questionable. This is in agreement with Karlsson *et al.* (2003), who showed that the embryo length at which *Argemone ochroleuca* germinated differed with temperature.

As embryos elongated before radicle emergence (Fig. 5A–C) and the latter did not occur within 30 d of dispersal (Fig. 5D–F), seeds may be said to possess morphophysiological dormancy (Baskin and Baskin, 1998, 2004). An increasing number of studies suggest, however, that if embryo elongation occurs continuously from dispersal to radicle emergence, seeds should be classified as non-dormant (e.g. Ali *et al.*, 2007; Mondoni *et al.*, 2008; Carasso *et al.*, 2011).

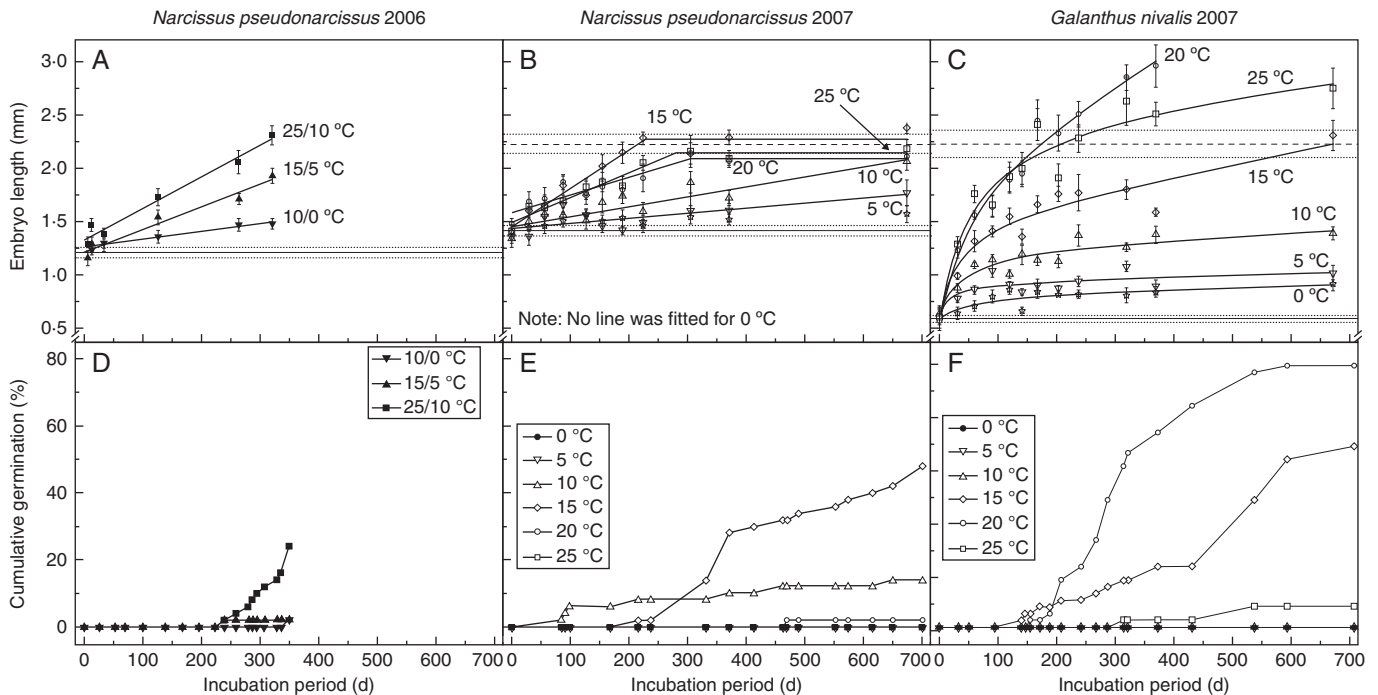


FIG. 5. Embryo length (mean  $\pm$  s.e.,  $n = 10$  per temperature) of viable, non-germinated (A) *Narcissus pseudonarcissus* 2006, (B) *N. pseudonarcissus* 2007 and (C) *Galanthus nivalis* 2007 seeds, and cumulative germination ( $n = 50$ ) (D–F, respectively) following incubation at alternating or constant temperatures in the light (8/16 h light/dark photoperiod) for *N. pseudonarcissus* or darkness for *G. nivalis*. Solid horizontal lines ( $\pm$  s.e., dotted lines) in A–C are mean embryo length at shedding. For comparison, mean embryo length (horizontal broken line,  $\pm$  s.e., dotted line) of seeds subsequently able to germinate at 15 °C (*G. nivalis* 2007) and 10 °C (*N. pseudonarcissus* 2007) following incubation at 20 °C for 84 d, are shown. No regression could be fitted for embryo length at 0 °C in *N. pseudonarcissus*.

Similarly, the classification of species with shoot ('epicotyl') dormancy, where delayed emergence is a result of continuous slow shoot development at cold temperatures rather than developmental arrest, has been challenged (Vandelook and van Assche, 2008). Alternative definitions of dormancy include '... the temporary suspension of visible growth of any plant structure containing a meristem' (Lang, 1987) and '... an arrest in development of seed embryos ... in conditions otherwise suited for growth' (Taylorson and Hendricks, 1977). Under these definitions, seeds in which embryo elongation continues after shedding would be considered non-dormant.

Substantial variation in dry weight, fresh weight and moisture content during *G. nivalis* and *N. pseudonarcissus* seed development (Fig. 2A–I) is not unique. In a recent study on *Trifolium ambiguum*, seed-to-seed variability in final dry weight and mass maturity was considerable (Hay et al., 2010). A reason for this variability during seed development may be that flowering occurs during the cold part of the year, especially in *G. nivalis*. Not only do flowers of *G. nivalis* open over an extended 7 week period (Fig. 1), but individual flowers may stay open for up to 8 weeks (Church, 1908), with the potential for pollination to occur throughout this period.

During *G. nivalis* and *N. pseudonarcissus* seed development, the ability to germinate and to withstand rapid drying increased as seeds matured, reaching a maximum just prior to seed release, with the former being acquired before the latter (Fig. 3), similar to that of other species such as *Digitalis purpurea* (Hay and Probert, 1995) and *Acer*

*platanoides* (Hong and Ellis, 1990). The ability to tolerate rapid drying developed relatively late in seed development, especially in *G. nivalis*, and in less than half of the seed population in both species (Fig. 3C–E).

The slight decline in germination of fresh seeds before dispersal from more mature capsules (Figs 3A, B and 4) may have been confounded by dormancy induction in a proportion of seeds. An alternative and more plausible explanation for this, as well as the low incidence of desiccation tolerance at dispersal (Figs 3C–E and 4), however, may be the premature breaking of the connection of the seed to the placenta, or perhaps selective abortion, before germinability and desiccation tolerance, respectively, were acquired. Ovule abortion can take place at any stage before seeds reach maturity (Lersten, 2004). Substantial seed abortion can occur in both *N. pseudonarcissus* (Balatková et al., 1977) and *G. nivalis* (Budnikov and Kricsfalusy, 1994). Hence, the considerable variation within capsules (Fig. 2) may in part be due to seed abortion.

The ability of seeds to tolerate desiccation may not be essential in these species *in situ*, particularly in *G. nivalis*, as it is highly unlikely that seeds would experience conditions in their natural environment that would result in rapid drying to low moisture contents; yet the latter is recommended for *ex situ* seed conservation (FAO/IPGRI, 1994). As most seeds were unable to germinate following this desiccation procedure at the point of natural dispersal (Fig. 3C–E), conventional collection and drying protocols may not be suitable for producing high quality seed collections of these species. That desiccation

tolerance increases right up until seed dispersal in both species highlights the importance of not collecting seeds until evidence of seed maturity is observed (Hay and Smith, 2003), such as yellow or brown capsule colour (Fig. 4) and natural dispersal (e.g. capsules starting to split).

Given that some seeds of both species withstand desiccation to approx. 6–7 % (Fig. 3C–E), it is unlikely that they possess intermediate (Ellis *et al.*, 1990) or recalcitrant storage behaviour, but neither do we have firm evidence that they are orthodox. Their poor seed germination and storability record in the Millennium Seed Bank may be due to failure of a large proportion of seeds to complete maturation *in planta* and therefore become fully desiccation tolerant at the time of seed collection (shedding), especially in *G. nivalis* (Fig. 3). Improvement of desiccation tolerance (and also longevity) in immature or prematurely collected seeds may be achieved by slow drying rates or delayed drying, which is thought to allow the continuation of natural ripening events (Hay and Probert, 1995, 2011; Hay *et al.*, 1997; Lima *et al.*, 1998; Probert *et al.*, 2007). Delayed drying improved the ability of seeds of *A. nemorosa* to survive drying as embryos developed to the desiccation-tolerant heart shape (Ali *et al.*, 2007). However, such procedures cannot benefit seeds that aborted early in their development.

The similarity of this evidence across several species suggests a distinct typology of seed development in early spring-flowering temperate woodland understorey plants which matches their ecological niche, whereby seeds are dispersed close to the end of seed filling without substantial developmental arrest or significant maturation drying (i.e. whilst immature) and with continued seed development occurring naturally on the woodland floor or in the soil seed bank under the tree canopy. In *ex situ* conservation practice, therefore, fruits of these species may need to be placed in similar, simulated environments after collection. Investigation of the post-collection sequence of environments that enable high ability to germinate is the subject of further research.

#### ACKNOWLEDGEMENTS

We thank Darren Murray (VSN International Ltd) for advice on development regression analyses, John Adams and Nicola Keogh for laboratory support, David Hardman and Stephen Robinson for permission to collect seeds, and Robin Probert for helpful discussions. This work was supported by the Millennium Commission, The Wellcome Trust and Orange plc. The Royal Botanic Gardens, Kew receives grant-in-aid from Defra, UK.

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