

RESEARCH ARTICLE

Seed pretreatment length when producing seeds for restoration may impact seed dormancy in offspring

Marcello De Vitis^{1,2}, Kayri Havens¹, Linda MacKechnie³, Jacob Zeldin^{1,4}, Andrea T. Kramer^{1,4,5}**Abstract**

Introduction: Seed dormancy regulates germination timing to improve seedling survival. Many temperate species produce physiologically dormant seeds, requiring winter conditions (i.e. moist-cold stratification) to overcome dormancy. Exposing seeds to suboptimal stratification lengths for restoration may alter genetic diversity and/or stratification requirements in offspring.

Objectives: We investigate how the moist-cold stratification length seeds are exposed to impacts on germination in offspring and whether variation in seed dormancy is under genetic and/or environmental control, with implications for producing and using germplasm for restoration.

Methods: For one population of each of three *Viola* species, we created two F0 subpopulations using short versus long moist-cold stratification. Resulting plants were grown in a common environment for two generations, with germination proportion and rate quantified under short, long, and no moist-cold stratification treatments. We also assessed the proportion of variation in germination explained by genotype and the interaction between genotype and environment.

Results: Moist-cold stratification length significantly impacted germination in all study species, but transgenerational effects were limited: germination proportion was greater in one species but only when F0 and F1 seeds were exposed to shorter stratification, while germination rate was two to three times slower in another species, but varied by subpopulation. Between 10 and 22% of variation in germination was explained by genotype and its interaction with the environment.

Conclusions: The length of moist-cold stratification seeds with physiological dormancy is exposed to in production may alter how restored populations perform. Species producing seeds with deeper dormancy may be more likely to be impacted.

Implications for Practice: Special consideration should be given to moist-cold stratification length when sowing seeds for restoration to: (1) produce plugs, (2) establish a seed production bed, or (3) restore a species to a site. Species with shallow (vs. deep) seed dormancy may be less impacted by variation in moist-cold stratification length imposed during the germplasm production process. When restoring species with physiological seed dormancy, it is important to ensure seeds are exposed to sufficient moist-cold stratification to overcome dormancy in most seeds. This will help maximize germination, maintain genetic diversity, and produce plants whose offspring are more likely to germinate under natural winter conditions.

Key words: ecological restoration, moist-cold stratification, physiological dormancy, plant propagation, seed germination, seed production, species reintroduction, wild violets

Introduction

Seed dormancy is one of the first traits expressed in a plant's life cycle: it regulates germination timing, detecting favorable environmental conditions to improve the likelihood of seedling survival and reproduction (Baskin & Baskin 2014). For example, 63% of temperate grassland forbs produce seeds with some level of physiological dormancy, requiring moist-cold winter conditions to overcome dormancy and germinate when warm, wet spring conditions are most favorable for survival (Baskin & Baskin 2014, 2022). The length of moist-cold stratification required to break dormancy varies among and within species and populations (Meyer & Allen 1999; Hoyle et al. 2015; ten Brink et al. 2020) and is an important consideration for practitioners when sowing seeds at a restoration site (Pedrini & Dixon 2020). This variation in dormancy is driven by a complex combination of genetic and environmental factors (Baskin & Baskin 2014), and in wild populations it is an effective strategy to help ensure germination and survival across a range of conditions (Fenner & Thompson 2004; Long et al. 2015).

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Globally, a growing need for genetically diverse native plant materials (seeds and plants) for ecological restoration is driving demand for large-scale native plant production efforts (De Vitis et al. 2017; León-Lobos et al. 2020; McCormick et al. 2021). Overcoming dormancy when germinating seeds is one of many steps in the plant production process where important genetic diversity can be altered or lost (Basey et al. 2015; Espeland et al. 2017). For example, in temperate violet species that require moist-cold stratification to germinate, genetic diversity was altered when seeds were exposed to different stratification lengths: two species had lower genetic diversity and one species had higher genetic diversity in produced plants when a shorter stratification length was applied, compared to an optimal length (Diaz-Martin et al. 2023). To ensure restored populations have sufficient genetic diversity to survive and adapt to changing conditions over time (Espeland et al. 2017; Leger et al. 2021), it is important to understand how to overcome dormancy in production settings while ensuring produced materials are best able to thrive at restoration sites.

Understanding how variation in seed dormancy is influenced by genetic and environmental factors can inform efforts to minimize loss of diversity at the germination stage of plant production (Diaz-Martin et al. 2023). The environmental factors (e.g. temperature and photoperiod) that seeds are exposed to during storage, dormancy-breaking, and germination can impact the conditions required to overcome dormancy (i.e. phenotypic plasticity: Baskin & Baskin 2014; Donohue 2009; Penfield & MacGregor 2017). Additionally, the environmental conditions that parents are exposed to during seed development and maturation can impact seed dormancy in their offspring (and following generations: i.e. transgenerational plasticity: Lu et al. 2016; Herman & Sultan 2011). If variation in dormancy is driven primarily by plasticity to environmental factors, production practices related to breaking seed dormancy are less likely to shift or reduce genetic variation, but they may still impact how a seed lot performs in a restoration. However, variation in seed dormancy also often has a genetic component (e.g. it is heritable, and so passed directly from parents to offspring; Meyer & Allen 1999). For example, in the annual species *Amaranthus tuberculatus*, 71–90% of variation in deep dormancy and 33–58% of variation in stratification-mediated dormancy alleviation was related to genetic variation (Leon et al. 2006). Plasticity of germination responses to environmental factors can also have a genetic component (Vu et al. 2015). When variation in seed dormancy has a genetic component, production practices that do not fully break the dormancy of all seeds may shift or reduce genetic variation in the produced native plant material.

Genetic and environmental factors also interact to influence seed dormancy and the conditions required to overcome it (Burghardt et al. 2016; Espeland et al. 2016). Thus, using optimal dormancy-breaking conditions (e.g. moist-cold stratification for many temperate species) that maximize the germination of viable seeds will minimize possible losses or shifts in genetic diversity through the production process. However, optimal germination conditions are unknown for many priority restoration species (e.g. only 49% of 1122 important grassland restoration species in Europe had known germination

conditions: Ladouceur et al. 2018) and can vary greatly among and within populations of the same species (Meyer & Allen 1999; Baskin & Baskin 2014). These challenges are magnified in a rapidly changing climate, where germination will shift in response to changes in precipitation and temperature because of seed dormancy and germination requirements: for example, in response to warmer winters some species that predominantly germinate in the spring may shift germination to the autumn or earlier in the spring (Flanigan et al. 2020; Walck & Hidayati 2022).

We investigated how populations of three congeneric temperate perennial forb species with physiological seed dormancy respond to different moist-cold stratification lengths over two generations. Specifically, we imposed two treatments (short and long moist-cold stratification) on one population per species to create two subpopulations. After assessing genetic diversity in these subpopulations (Diaz-Martin et al. 2023), we grew 10 maternal lines per species and subpopulation under common environmental conditions, grew a second generation of four plants for each maternal line per subpopulation using the same short and long treatments, and then exposed seeds harvested from them to three different germination treatments (no stratification, short stratification, and long stratification) to understand how germination responses varied by treatment and the proportion of variation explained by genotype and the interaction between genotype and environment. Results provide insight into how plant production practices can impact dormancy of seeds used in restoration, as well as how seed dormancy may be impacted as climate change shortens winter length.

Methods

Study Species

We investigated three violets growing primarily in remnant prairie habitats of the Chicago region: *Viola lanceolata* L. (LAN: lance-leaved violet), *V. pedatifida* G. Don (PED: prairie violet), and *V. sagittata* Aiton (SAG: arrow-leaved violet) (Wilhelm & Rericha 2017). All species produce open-pollinated (chasmogamous) flowers in the spring and self-pollinating (cleistogamous) flowers through the growing season when conditions are favorable. They possess a dual seed-dispersal strategy, known as diplochory: first, mature seed capsules explosively dehisce, ejecting seeds away from the maternal plant; then, ants often transport seeds to their nests because of the presence of fleshy elaiosomes on the outside of seeds. Ants eat the elaiosome and leave the seed to germinate in the nest's low-competitive, nutrient-rich environment (Wilhelm & Rericha 2017). Seeds have varying degrees of non-deep or intermediate physiological dormancy, requiring cold-moist stratification for several weeks to germinate. The length of moist-cold stratification required for germination varies by species and seed source (Kilgore et al. 2022; De Vitis et al. 2022: *V. pedatifida* and *V. sagittata* generally have deeper dormancy than *V. lanceolata*) and appears to have at least some genetic control (Diaz-Martin et al. 2023).

All three species are classified as conservative (coefficients of conservatism [*C*-values] are 7, 9, 5, for *V. lanceolata*, *V. pedatifida*, and *V. sagittata*, respectively; Wilhelm & Rericha 2017) and generally declining in the Midwest United States due to habitat loss and degradation. The decline of these species and their habitat has impacted associated species, including the Regal Fritillary butterfly (*Speyeria idalia*), whose caterpillars are known to feed exclusively on violet species (Debinski & Kelly 1998; Selby 2007), and other insects that rely on these species for early-spring floral resources. Despite being an important species for prairie restoration efforts, seeds of violet species are often unavailable from native seed suppliers (White et al. 2018), likely due to challenges associated with harvesting seeds of low-growing, explosively dehiscing seed capsules, as well as low germination rates in many settings (Kilgore et al. 2022). To overcome these challenges, small-scale nursery production has been used to produce seeds for restoration of all three species. This includes collecting and germinating wild-collected seeds to grow plants in a nursery setting (potted plants in a greenhouse or plants grown in-ground in a production bed) and then harvesting seeds from plants over multiple years for use in restoration efforts.

Seed Sourcing

Founder generation (F0) seeds of *V. lanceolata* and *V. sagittata* were sourced in 2019 from plants grown in a nursery bed at The Nature Conservancy's Kankakee Sands Native Plant Nursery (Morocco, IN). Here, violet beds were established around 2015, using seeds collected from four local native populations for both species. Seeds from the beds were harvested and used to grow plants in a greenhouse, and then these plants were transferred outdoors but kept in pots. Every year, for each species, seeds are harvested from both chasmogamous and cleistogamous fruits and mixed, cold-moist stratified for 60 days at 4°C and then used for next year's plant propagation or direct restoration seeding. In addition to harvesting seeds, each year the nursery staff do a small amount of wild collecting to maintain genetic diversity of the greenhouse population. Because source populations and maternal lines are not tracked during production practices, the diversity of the sourced seed sample is unknown.

F0 seeds of *V. pedatifida* were sourced in 2019 from an in-ground production bed at the Lake County Forest Preserve District's native seed nursery in Grayslake, Illinois. This bed was established from greenhouse-produced plants grown from wild-collected seed sourced from a single population in Lake County. When plants were collected, seeds had been grown for one generation in the production bed, with little-to-no noticeable attrition or recruitment.

Seed Germination and Plant Propagation (F0)

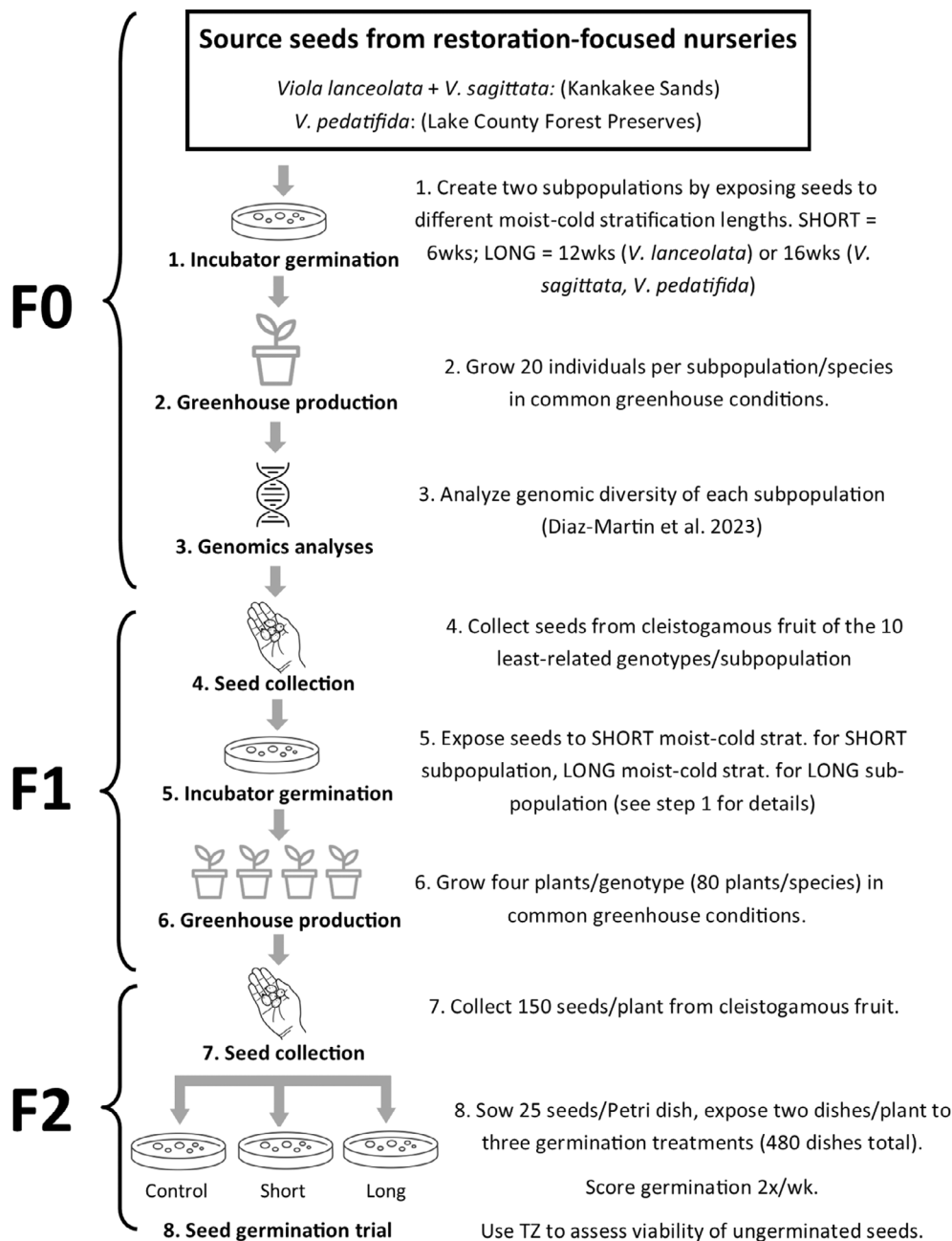
F0 seeds were stored at 5°C at the Chicago Botanic Garden until July 2019, when 100 randomly selected seeds of each species were surface sterilized (1% v/v sodium hypochlorite [bleach] solution for 2 minutes followed by two rinses in sterile water) and sown in 90 mm Petri dishes (see full methods in Fig. 1).

While the viability of each seedlot was not tested, prior research showed maximum germination in seeds produced at these locations ranges from 57% (SAG) to 87% (LAN) (Kilgore et al. 2022). Seeds were sown in Petri dishes on 1.5% agar, sealed with parafilm, and exposed to either SHORT or LONG moist-cold stratification at 0–3°C. Specifically, SHORT stratification was 6 weeks for all species, while LONG stratification was 12 weeks for *V. lanceolata* and 16 weeks for *V. sagittata* and *V. pedatifida*. Stratification lengths were determined to ensure low but sufficient germination in SHORT stratification, and to maximize germination in LONG stratification; we used optimal lengths that varied by species based on conversations with practitioners and Kilgore et al. (2022).

At the end of the cold stratification treatment, Petri dishes were transferred to an incubator set at 25/15°C (alternating day/night temperatures) with a 12/12 photoperiod, and germination was checked every other day. For the SHORT subpopulations, we randomly selected 20 seeds that had germinated (presence of radicle) within 2 weeks after transfer to the incubator. For the LONG subpopulations, we randomly selected 20 seeds that germinated after being transferred to the incubator, excluding a few seeds that had germinated during stratification. The seedlings were planted in individual plug tray cells with soil and kept in a greenhouse with mist (day temperature of 21°C and night temperature of 18°C, with supplemental lighting from 6:00 to 22:00 hours to provide a long-day photoperiod and mist running 3 seconds every 20 minutes from 4:00 to 23:00 hours). After circa 2 weeks, following the emergence of true leaves, the seedlings were transplanted into individual pots of 7.6 × 7.6 cm and transferred to a second greenhouse without mist (day temperature of 19°C and night temperature of 17°C with supplemental lighting 6:00 to 22:00 hours). Plants were watered every one to 2 days as needed and grown until seed production. A total of 120 individuals were cultivated (hereafter called genotypes, for three species × two subpopulations [short and long] × 20 individuals).

Fresh leaf material was collected from 13 to 14 individuals per subpopulation, and the kinship level was investigated within each subpopulation through a restriction site-associated DNA sequencing (RADseq) technique (Diaz-Martin et al. 2023). Sequencing showed that genetic differentiation between subpopulations was relatively low (Nei's pairwise *F_{st}* = 0.070 for *V. pedatifida*, 0.083 for *V. sagittata*, and 0.068 for *V. lanceolata*), but there were significant shifts in genetic diversity between subpopulations of each species, with greater genetic diversity in the SHORT subpopulation for *V. lanceolata* and the LONG subpopulation for *V. sagittata* and *V. pedatifida*. Based on the results of this analysis, we selected the 10 least genetically related individuals within each group to ensure the work was performed with genetically distinct maternal lines. After this selection step, a total of 60 genotypes (10 genotypes per subpopulation per species) were used to create the F1 generation.

All plants were grown under the same greenhouse conditions until seed production. Mature (F1) seeds were collected from cleistogamous fruits in spring 2020. Since the study species exhibit a ballistic dispersal, to allow for seed collection at the time of natural dispersal and to avoid seed losses, randomly selected capsules were bagged with tea bags while still



From The Noun Project. Hand = Gan Khoon Lay, plant pot = Rahmat Hidayat;
 DNA = Megan Mitchell. From Vecteezy: stockgiu = petri dish.

Figure 1. Eight steps taken to produce all three generations of seeds used in this study, starting with incubator germination for generation F0 and ending in a seed germination trial with generation F2.

closed. Bags were checked every 3 days and seeds were collected when the capsule inside was open and seeds had been released. After collection, seeds were stored at room conditions ($20 \pm 2^\circ\text{C}$, 50% relative humidity [RH]) for an average of 28 days, then transferred to a dehydration chamber (15°C , 15% RH) and stored for an average time of 51 days until they

were surface sterilized and sown in Petri dishes following the procedure previously described.

For all three species, seeds produced by SHORT genotypes were only exposed to the SHORT moist-cold stratification treatment (6 weeks for all species), while seeds produced by LONG genotypes were only exposed to the LONG moist-cold

stratification treatment (12 weeks for *V. lanceolata*, 16 weeks for *V. pedatifida*, *V. sagittata*). At the end of the stratification periods, in November 2020, seeds were all moved to an incubator ($T: 25/15^{\circ}\text{C}$; 12/12 photoperiod) and checked once a week for germination. Upon germination, the seedling selection process was repeated, but this time for each genotype we randomly selected four seedlings and transferred them to the greenhouse where we cultivated them until F2 seed production, repeating the steps and conditions previously described. A total of 240 individuals (four plants per genotype per subpopulation per species) were cultivated. In *V. pedatifida*, two genotypes in the LONG subpopulation were excluded due to the death of some plants during cultivation. F2 seed collection and storage followed the procedure described above. To start the final germination experiments all together, storage time varied between 0 and 102 days across species, subpopulations, and genotypes, depending on when the seeds of a single plant were ready for collection (Fig. S1).

Germination Trials (F2)

For four plants of each genotype across all subpopulations and species, seeds from different fruits of the same individual were combined until a quota of 150 was reached. When more than 150 seeds were available, 150 were randomly selected from the pool. Seeds were surface sterilized and sown in Petri dishes following the procedure described above. For each individual plant, six replicates of 25 seeds each were sown, and then two plates were exposed to each of three different treatments: (1) control—seeds were immediately incubated at $25/15^{\circ}\text{C}$ with a 12/12 photoperiod; (2) short—seeds were exposed to the same short cold stratification period used for the short subpopulation (see above for species-specific lengths), followed by incubation at the same conditions as control; (3) long—seeds were exposed to the same long cold stratification period used for the long subpopulation (see above for species-specific lengths), followed by incubation in control conditions. In total, the experiment consisted of 480 Petri dishes of each species, each with 25 seeds (2 Petri dishes \times 3 germination treatments \times 4 plants/genotype \times 10 genotypes \times 2 subpopulations). *Viola pedatifida* was the exception, with only eight genotypes for LONG subpopulation and a total of 432 Petri dishes, each with 25 seeds. Germination (visible radicle emergence) was scored twice a week for 8 weeks, with the removal of germinated seeds from the Petri dishes. At the end of the experiment, a tetrazolium test

(Miller 2010) was performed on the ungerminated seeds to assess seed viability and calculate viability-adjusted germination. Specifically, non-viable seeds were removed from the total seed count and subtracted from the pool of non-germinated seeds.

Statistical Analysis

All statistical analyses were performed in R (v. 4.4.1, R Core Team 2024). To evaluate the effects of subpopulation (SHORT or LONG) and cold stratification treatment (control, short stratification, or long stratification) on germination success in F2 seeds, we built random intercept generalized linear mixed effect models (GLMM) with binomial distributions using the “*lme4*” package (Bates et al. 2015). We modeled viability-adjusted germination at the end of the experiment as a binomial response of successful germination to failed germination, with subpopulation identity, stratification treatment, and their interaction as fixed effects and genotype as a random effect. Models were fit separately for each of the three *Viola* species, and the fixed effect terms were evaluated with Type-II analysis of variance (ANOVA) using Wald Chi-square tests from the “*car*” package (Fox & Weisberg 2019). Following the identification of significant fixed effects, we applied pairwise comparisons of group means with Tukey Honest Significant Difference (HSD) tests at $p \leq 0.05$. To estimate the contribution of the random effect of genotype to variation in germination probability, we estimated the intraclass correlation coefficient (ICC) using the *icc* function in the *performance* package (Nakagawa et al. 2017; Lüdtke et al. 2021) as an estimate of the proportion of variation explained by genotype and the interaction between genotype and environment (Vahsen et al. 2023).

To understand how subpopulation and cold stratification treatment influenced germination trajectories over time, we fit dose–response models with a three-parameter log-logistic function using the *drc* package (Onofri et al. 2022). For all models, we constrained the higher asymptote parameter in all models to ≤ 1 as germination cannot exceed 100%. We estimated dose–response curves for each subpopulation and stratification treatment. Dose–response models were fit separately for each of the three *Viola* species. Maternal line identity was not included in the dose–response models.

Results

Across all three *Viola* species, stratification treatment significantly explained variation in viability-adjusted germination proportion

Table 1. Results of the generalized linear mixed effect models (GLMM) with binomial distributions. Type II Anova with Wald chi-square tests is presented with chi-square, degrees of freedom (*df*), and *p* values shown for the effects of subpopulation (short or long), moist-cold stratification treatment (control, short stratification, or long stratification), and their interaction on germination success of three *Viola* species.

	<i>Viola lanceolata</i>			<i>V. pedatifida</i>			<i>V. sagittata</i>		
	χ^2	df	<i>p</i>	χ^2	df	<i>p</i>	χ^2	df	<i>p</i>
Subpopulation	3.79	1	0.052	0.01	1	0.980	1.83	1	0.176
Treatment	169.55	2	<0.001	2321.50	2	<0.001	1051.82	2	<0.001
Subpopulation:Treatment	4.53	2	0.104	30.47	2	<0.001	215.38	2	<0.001

Note: *p* values below 0.05 are bolded.

(Tables 1 & S1). Generally, within species and subpopulations, seeds exposed to long cold stratification showed the highest germination proportion, while those that were not exposed to any cold stratification (control) had the lowest germination proportion (Fig. 2). These results were strongly supported by Tukey HSD contrasts in *Viola pedatifida*, with germination proportions of approximately 0.75 in the long stratification treatments and less than 0.10 in the control treatments across both subpopulations. Within both subpopulations of *V. lanceolata*, seeds in the long stratification treatment germinated to higher proportions than seeds in the control and short stratification treatments. The main effect of subpopulation was a marginally significant predictor of germination in *V. lanceolata* (Table 2), however, results of the Tukey HSD tests did not identify significant differences between the subpopulations within any of the stratification treatments. Subpopulation was not a significant predictor of germination in *V. pedatifida* and *V. sagittata*.

Differences in germination response between the SHORT and LONG subpopulations were most pronounced in *V. sagittata*, where the SHORT subpopulation germinated to higher proportions in the control and short treatments relative to the LONG subpopulation, though the contrasts in means were not statistically significant after correction for multiple comparisons with the Tukey HSD test. The subpopulation \times stratification treatment interaction term ($\chi^2 = 215.38$, $p < 0.001$) was significant in the *V. sagittata* model. Here, we observed the expected pattern of low germination in the control treatment, high germination under the long stratification treatment, and intermediate values in the short stratification treatment among the LONG subpopulation. However, in the SHORT subpopulation, we observed similar germination proportions among the short and long stratification treatments and germination

proportions in the control treatment nearly as high as those in the short stratification treatment of the LONG subpopulation (Fig. 2).

The adjusted ICC, which identifies differences attributed to genotypes and interactions between genotypes and the environment, explained approximately 20% of the variation in germination proportion in *V. lanceolata* (adjusted ICC = 0.22) and *V. sagittata* (adjusted ICC = 0.19), but a comparatively lower amount of variation in *V. pedatifida* (adjusted ICC = 0.10).

The timing of seed germination (evaluated by model estimated time to 50% germination; Table 2; Fig. 3) was similar for the SHORT and LONG subpopulations of *V. lanceolata* but varied substantially among stratification treatments: seeds exposed to long cold stratification were fastest to germinate (approximately 1.5 weeks) and slowest in control conditions (approximately 3.5–4 weeks). A similar pattern was found for *V. sagittata*, with no meaningful differences between subpopulations, and faster germination in the long cold stratification treatment (approximately 1.7 weeks) than the short treatment (approximately 3 weeks), with the control showing the longest germination at approximately 5.4–6.4 weeks for short and long subpopulations, respectively. For *V. pedatifida*, seeds in the control treatment took longest to reach 50% germination regardless of subpopulation (approximately 5.2 weeks for the LONG subpopulation, 5.9 weeks in the SHORT). However, in the short and long stratification treatments, the two subpopulations germinated at different rates depending on the stratification treatment. In short stratification, the SHORT subpopulation reached 50% germination much slower (approximately 5.9 weeks) than the LONG subpopulation (approximately 2.1 weeks), while in long stratification the LONG subpopulation reached 50% germination 2 weeks faster than the SHORT (2.6 vs. 4.9 weeks).

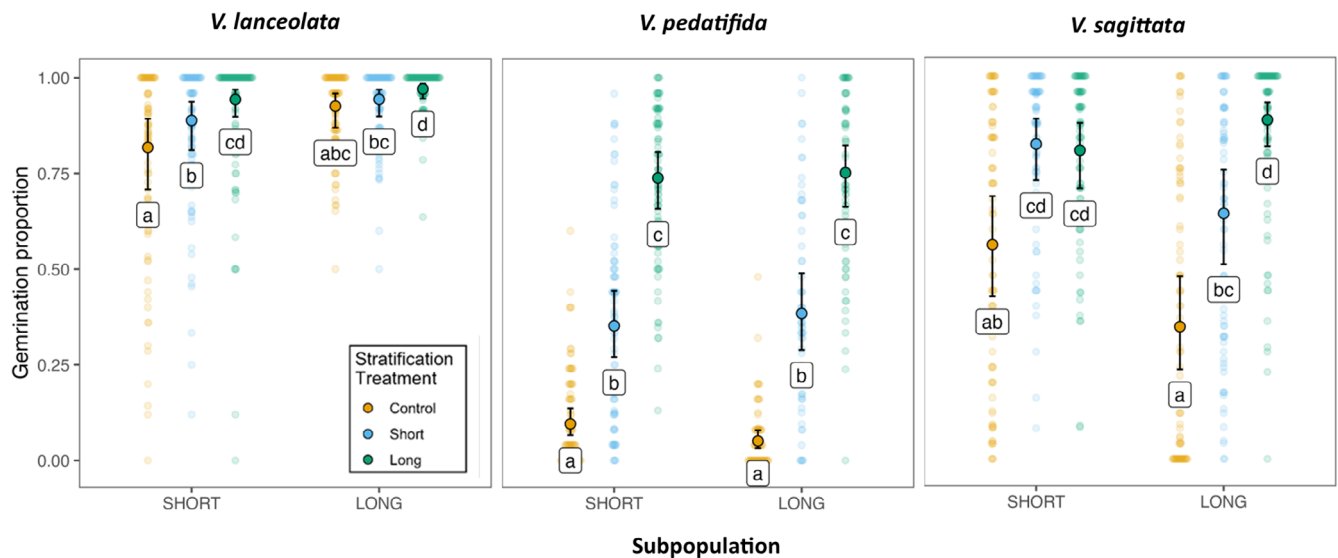


Figure 2. Estimated marginal mean germination proportions of short and long subpopulations for each of three violet species (*Viola lanceolata*; *V. pedatifida*; and *V. sagittata*), after seeds were exposed to three pre-treatments: control = no moist-cold stratification; short = moist-cold stratification for 6 weeks (all species); long = 12 weeks (*V. lanceolata*) or 16 weeks (*V. pedatifida* and *V. sagittata*) moist-cold stratification. Model predictions are presented with 95% CIs as well as raw germination proportions (semi-transparent points). Letter displays show results of Tukey Honest Significant Difference (HSD) tests of pairwise comparisons of group means, where estimates sharing the same letter indicate a lack of significant difference at $p \leq 0.05$.

Table 2. Dose–response curve model coefficients for seed germination in two subpopulations (SHORT and LONG) of three *Viola* species after three pre-treatments: control = no moist-cold stratification; short = moist-cold stratification for 6 weeks (all species); long = 12 weeks (*Viola lanceolata*) or 16 weeks (*V. pedatifida* and *V. sagittata*) moist-cold stratification. Parameters are defined as follows: d is the upper asymptote (maximum germination proportion), e is the inflection point of the curve (interpreted as ED50 or time to 50% germination), and b is the slope at the inflection point. Values reported are coefficient estimates with standard errors in parentheses.

Subpopulation	Treatment	d	e	b
<i>V. lanceolata</i>	SHORT			
	Control	0.79 (0.01)	3.52 (0.03)	−5.17 (0.14)
	Short	0.86 (0.01)	1.70 (0.02)	−6.21 (0.18)
	Long	0.92 (0.01)	1.55 (0.02)	−9.12 (0.29)
	LONG			
	Control	0.92 (0.01)	4.03 (0.03)	−6.14 (0.14)
SHORT	Short	0.93 (0.01)	1.79 (0.02)	−5.17 (0.13)
	Long	0.96 (<0.01)	1.54 (0.01)	−8.23 (0.22)
<i>V. pedatifida</i>	SHORT			
	Control	0.12 (0.01)	5.93 (0.16)	−5.45 (0.44)
	Short	0.45 (0.02)	5.88 (0.18)	−3.47 (0.18)
	Long	0.78 (0.01)	2.62 (0.07)	−2.11 (0.06)
	LONG			
	Control	0.07 (0.01)	5.26 (0.21)	−5.13 (0.58)
SHORT	Short	0.51 (0.03)	2.05 (0.07)	−2.00 (0.11)
	Long	0.79 (0.01)	4.94 (0.32)	−1.76 (0.07)
<i>V. sagittata</i>	SHORT			
	Control	0.62 (0.01)	6.42 (0.07)	−6.40 (0.23)
	Short	1.00 (0.02)	5.21 (0.13)	−2.40 (0.07)
	Long	0.89 (0.02)	1.92 (0.09)	−1.35 (0.06)
	LONG			
	Control	0.39 (0.01)	5.43 (0.09)	−4.95 (0.22)
SHORT	Short	0.68 (0.02)	3.39 (0.12)	−2.07 (0.07)
	Long	0.85 (0.01)	1.70 (0.03)	−3.14 (0.08)

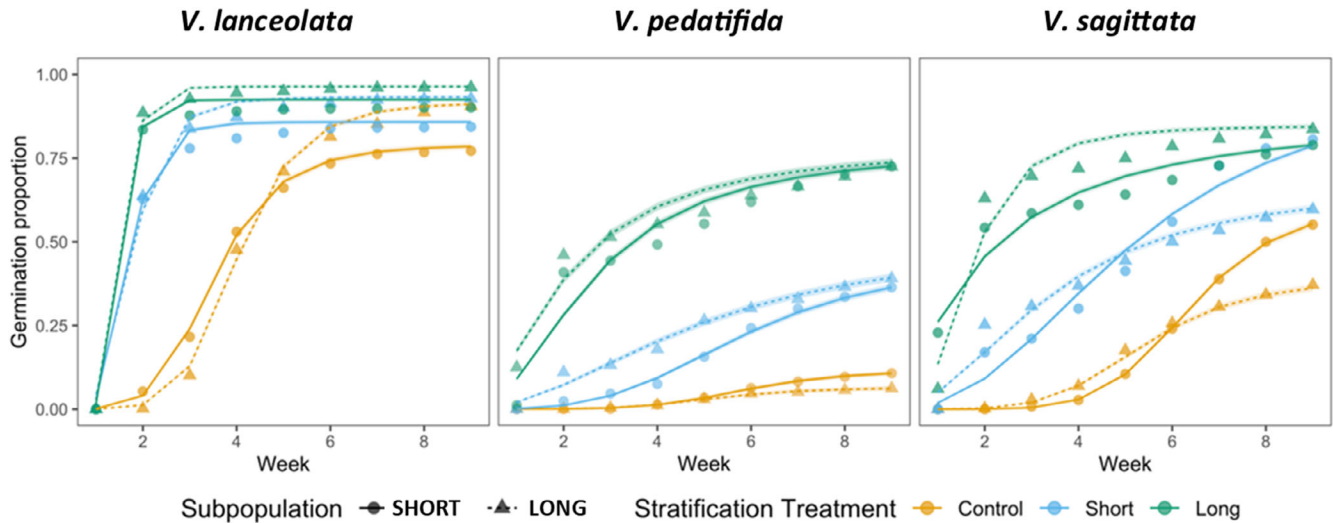


Figure 3. Dose–response curve models for seed germination of two seed subpopulations (short and long) of three *Viola* species (*Viola lanceolata*; *V. pedatifida*; and *V. sagittata*) after three pre-treatments: control = no moist-cold stratification; short = moist-cold stratification for 6 weeks (all species); long = 12 weeks (*V. lanceolata*) or 16 weeks (*V. pedatifida* and *V. sagittata*). Lines represent the predictions of the log-logistic function of the dose–response curves, with shaded areas indicating the standard errors. Points represent the observed mean germination proportion for each subpopulation and stratification treatment at each time point.

Discussion

We created two artificial subpopulations from the same restoration-ready source population (F0) by exposing seeds to two moist-cold stratification lengths (short and long) for three

Viola study species. We then grew their offspring (F1) in common greenhouse conditions using the same moist-cold stratification lengths and tested the seed dormancy and germination rate of their offspring (F2). We found that moist-cold stratification

length significantly impacted germination proportion and rate in all three study species, while the stratification conditions F0 and F1 seeds were exposed to only impacted germination proportion in *Viola sagittata* and germination rate in *V. pedatifida*. These two species also had deeper seed dormancy relative to *V. lanceolata*, and greater shifts in genetic makeup of F0 subpopulations with different moist-cold stratification length exposure (Diaz-Martin et al. 2023). While other studies have identified altered or decreased seed dormancy during the process of cultivation for restoration (Schröder & Prasse 2013; Ensslin et al. 2018; Pizza et al. 2021), our results suggest a combination of genetic shifts as well as transgenerational plasticity related to moist-cold stratification length exposure may explain these shifts in seed dormancy. At least for some species, especially those with deep physiological seed dormancy, the length of moist-cold stratification used to produce seeds for restoration can impact when seeds will germinate at a restored site.

Genetic Contributions to Seed Dormancy

Some of the variation we identified among subpopulations across stratification treatments for *V. sagittata* (germination proportion) and *V. pedatifida* (germination rate) may be a result of genetic differences between subpopulations, as identified in our F0 generation by Diaz-Martin et al. (2023). While we did not test whether our F2 population had the same genetic makeup as our F0, we used only seeds produced by cleistogamous (self-fertilized) fruit and maintained offspring from the same 10 genotypes per subpopulation to minimize genetic shifts across generations. Our findings show that 10–22% of the variation in germination in the F2 generation was explained by genotype and interactions between genotype and environment, further reinforcing results in Diaz-Martin et al. (2023) that seed dormancy has a genetic component in our study species, and that exposure to different moist-cold stratification lengths can shift the genetic diversity of offspring.

Other studies investigating heritability in seed dormancy in perennial forb species generally show lower heritable variation in seed dormancy than we found: 0–3% in *Lychnis flos-cuculi* (Biere 1991) and 7% in *Lobelia inflata* (Simons & Johnston 2006). However, these studies measured only the effect of genotype, not the interaction of genotype and environment (i.e. they measured narrow-sense heritability), and so would be expected to find lower heritability. Interestingly, examples of measured genetic contributions to variation in seed dormancy in annual plants have all been higher, regardless of whether the interaction of genotype and environment was removed (e.g. *Amaranthus tuberculatus* [33–90%]; Leon et al. 2006) or not (*Arabidopsis thaliana* [84–97%]; Debieu et al. 2013; *Bromus tectorum* [68–93%]; Meyer & Allen 1999). This may be because seed dormancy and germination are under stronger selection in annual relative to perennial species, as annuals rely more heavily on seedling recruitment to maintain viable populations (Vico et al. 2016). Understanding the genetic basis of seed dormancy is important, and more research is needed to understand variation among species so steps can be taken to ensure

important genetic diversity is not lost at the many stages of plant production for restoration (Basey et al. 2015).

Impact of Moist-Cold Stratification Length on Seed Dormancy in Future Generations

While the subpopulation did not impact seed dormancy in two of our three species, seeds of *V. sagittata* had more consistent and higher germination when exposed to any length of stratification in the SHORT compared to the LONG subpopulation. This may be driven by transgenerational plasticity resulting from differences in the moist-cold stratification length for F0 and F1 seeds. We are aware of only one study (Lu et al. 2016) investigating how moist-cold stratification conditions impact transgenerational plasticity. Specifically, Lu et al. (2016) showed that in a cold desert annual (*Isatis violascens*), seeds produced by maternal plants that germinated with no cold stratification (autumn-germination) had greater dormancy than seeds produced by maternal plants that germinated following moist-cold stratification imposed by winter conditions (spring-germination). Because these were field-grown plants, the authors concluded that cold stress on rosettes of autumn-germinating plants likely drove increased dormancy in the seeds they produced. For our study, environmental conditions were the same for all generations of both subpopulations of all three study species, with only moist-cold stratification length varying between subpopulations. While not all seeds were produced at the same time, the length of seed production and storage conditions following harvest were similar for each species. Future studies should investigate the potential for across-generation plastic responses to seed dormancy-breaking conditions while holding genetic variation constant to understand how much our results were driven by the small genetic differences we know existed between the two subpopulations in F0 (Diaz-Martin et al. 2023).

Additionally, transgenerational plasticity may be driving variation in the rate of germination in seeds of *V. pedatifida*. Seeds in the SHORT subpopulation germinated almost three times faster than the LONG subpopulation when exposed to short stratification conditions, while under long stratification conditions the LONG subpopulation germinated almost two times faster than the SHORT subpopulation. Shifts in the rate of germination like this have the potential to impact the timing of species arrival at a restoration site (i.e. priority effects), and research has shown that even small alterations in arrival time can impact both community composition and ecosystem functions (Weidlich et al. 2021). Arrival time may be especially important when there is a soil seed bank of weedy or invasive species at a restoration site, as a delay in germination of sown seed may allow seeds in the soil seed bank to have a competitive advantage. However, rapid germination may also expose more seeds to killing events like late frost (Bianchi et al. 2019), illustrating the complexity of understanding how shifts in germination proportion and rate may impact restoration outcomes for any sown species.

Seeds with lower dormancy are more likely to germinate as soon as they receive warm, moist conditions at a restoration site. A loss of dormancy may have negative impacts on the fitness of restored populations, altering when they germinate and lowering the fraction of seeds that can form a soil seed bank (Hoyle

et al. 2014; Espeland et al. 2017; Ensslin et al. 2018). Whether or how these shifts in dormancy impact restoration outcomes depends on the importance of the soil seed bank for the population's long-term survival. While soil seed banks can have an unexpectedly large role in determining how a population will grow or decline over time (Nguyen et al. 2019), it is unclear the extent to which our species rely on the soil seed bank. In general, longer-lived perennial species are less likely to form soil seed banks than annual species (Fenner & Thompson 2004), but at least some perennial species do (Tellier et al. 2011). Further research is needed to understand how shifts in seed dormancy may impact restoration outcomes via changes in whether or how species form soil seed banks.

The ability of early germinating seedlings to survive and successfully reproduce also affects how shifts in seed dormancy impact the fitness of a restored population (Simons & Johnston 2006; Flanigan et al. 2020). For example, if winters are mild, early germinating seedlings may survive in larger numbers and/or grow to a larger size and produce more viable seeds for the next generation than late-germinating seeds (Gremer et al. 2020). However, if harsh winters or late-season cold snaps kill early emerging seedlings, restoration outcomes for that species may be negatively impacted. Research on the likelihood of survival for seedlings germinating at different times across the season is needed to fully understand the impacts of seed dormancy changes on the survival of restored populations.

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Supporting Information

The following information may be found in the online version of this article:

Figure S1. Dry after ripening (DAR) time (days) for seeds harvested from plants in SHORT and LONG subpopulations of the three study species.

Table S1. Estimated marginal mean germination proportions from generalized linear mixed effect models of cumulative germination in three violet species.