COLD STRATIFICATION OF *SALVIA AZUREA* VAR. *GRANDIFLORA* BENTH. (LAMIACEAE) SEEDS TO BREAK DORMANCY

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ABSTRACT

Seed dormancy and its maintenance rely on a range of environmental signals and cues such as light, temperature, soil pH, and moisture. A significant contributor to the cycle of dormancy and germination is temperature. *Salvia azurea* var. *grandiflora* Benth. is an Oklahoma native perennial that produces blue flowers with one seed per flower. Seeds were collected from four sites in Oklahoma and Cleveland Counties, OK, and randomly assigned to one of three treatment groups: no cold stratification, 3-week cold stratification, and 6-week cold stratification. Seeds were monitored daily, and seed germination date was recorded for analysis. Survival analysis indicated there was a significant correlation between the amount of time in a cold stratification environment and number of seeds that germinated. Seeds in the 6-week cold stratification treatment group exhibited increased germination compared to the other two treatment groups.

INTRODUCTION

Environmental temperature and climate variability are dominant influences for many species' life-history traits (Bernareggi et al. 2016; Fernandez-Pascual 2019). Plant reproductive phases, such as seed germination and seedling establishment and survival, are more sensitive to variation in climatic conditions (Bernareggi et al. 2016; Nonogaki 2017) than other phases of the plant life cycle. Seed dormancy is an adaptation that allows for persistence of a population during seed dispersal or impact by climatic conditions, including temperature extremes and drought (Baskin and Baskin 2001; Messick and Hoagland 2018). This mechanism is dominated by an intricate balance between the phytohormones gibberellins and abscisic acid (Footitt et al. 2014; Nonogaki 2014; Hradilova et al. 2019). Balance continues to be maintained through cohorts of genes that regulate the hormones' perception, sensitivity and metabolism through the use of complex signaling pathways necessary for seed dormancy and the control of germination (Footitt et al. 2014; Nonogaki 2017; Tudela-Isanta et al. 2017; Fernandez-Pascual 2019). Cold affects dormancy levels and cycling by affecting levels of abscisic acid and making environmental temperature an important signal for numerous plant species (Footit et al. 2014; Nonogaki 2014), and has been studied in molecular detail in relation to dormancy induction and cycling control by hormones. Warmer temperatures (above 15°C) are generally related to lower levels of dormancy (Footit et al. 2014), whereas cooler temperatures (10-15°C) induce higher dormancy levels in seeds and are correlated with strongly enhanced abscisic acid levels.

Seeds may exhibit physiological dormancy, physical dormancy, or a combination of both (Baskin and Baskin 2001). The most observed type of dormancy, physiological dormancy, is regulated mainly by the relative levels of hormones that either inhibit or promote germination. It produces seeds with distinct and valuable advantages. Physiological dormancy ensures germination will not occur in an adverse climatic environment, although short periods of favorable conditions may present themselves (Carrera-Castano et al. 2020). It also provides for maximum dispersal of seeds, which decreases competition between the parent plant and offspring (Baskin and Baskin 2001; Carrera-Castano et al. 2020).

Physiologically dormant seeds also exhibit dormancy cycling, which is the alleviation and re-induction of dormancy (Baskin and Baskin 2001; Long et al. 2015) in response to changing environmental signals and cues. Physical dormancy is when a seed coat is impermeable to water and gases needed for germination. Physically dormant seeds are unable to exhibit dormancy cycling because the change in seed coat permeability cannot be reversed (Baskin and Baskin 2001; Long et al. 2015).

Salvia azurea var. *grandiflora* Benth. (pitcher sage)*,* a member of the Lamiaceae (Mint Family), is a native perennial with a distribution range from the eastern U.S. to New Mexico and Colorado (Figure 1).
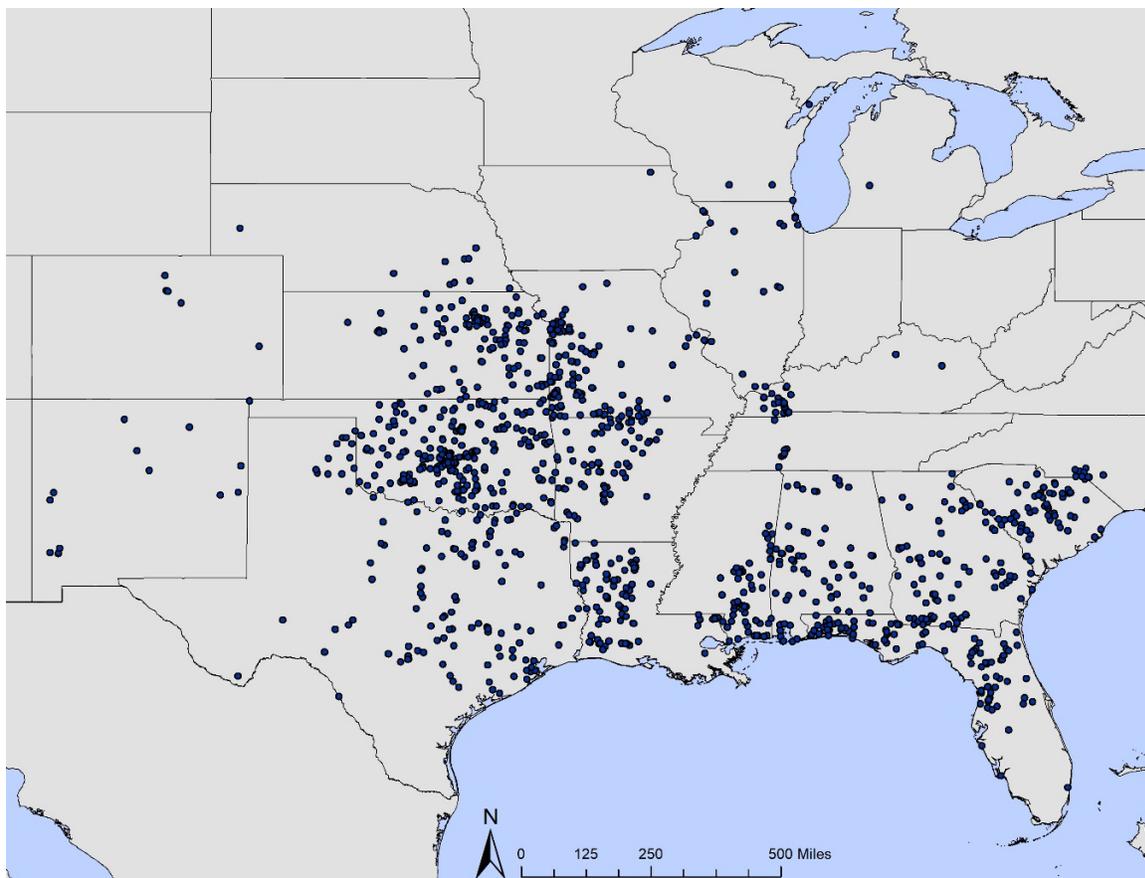

Figure 1 *Salvia azurea* var. *grandiflora* distribution range in the United States.

It reaches 50-150 cm in height with leaves averaging 3-7 cm long and 1-2.5 cm wide (McGregor et al. 1986). *Salvia azurea* var. *grandiflora* produces blue flowers (occasionally white), from July through October (McGregor et al. 1986), with one seed per flower. It is an important resource for generalist pollinators, including monarch butterflies (Figure 2), which use *S. azurea* var *grandiflora* as a nectar source during their fall migration (Xerces Society for Invertebrate Conservation 2021). One specialist bee, *Tetraloniella cressoniana* (blue sage bee), has coevolved with *S. azurea* var. *grandiflora* and depends on the nectar of *S. azurea* var. *grandiflora* as its sole source of nutrition (LaBerge 2001; Schuette 2016).

Understanding the seed germination requirements of *S. azurea* var. *grandiflora* is important to study because the data have the potential to contribute to our knowledge of climate change and its effect on environmental temperature and the cycle of dormancy. The goal of this study was to determine the relative length of cold stratification required to produce an increased rate of germination for *S. azurea* var. *grandiflora*. My null hypothesis was that cold stratification would have no effect on germination probability.

Figure 2 *Salvia azurea* var. *grandiflora* in northwestern Oklahoma with monarchs nectar feeding. Photo credit: Amy Buthod 2018, Oklahoma Biological Survey, Norman, OK.

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METHODS

Salvia azurea var. *grandiflora* seeds were collected from four sites (Figure 3; Table 1) located using the Oklahoma Vascular Plant Database (OVPD; Hoagland et al. 2021). A total of 270 seeds were obtained from among these sites, with the number of plants from each site varying dependent upon availability. Then seeds were pooled and randomly assigned to one of three treatment groups: no cold stratification (no cold), three weeks of cold stratification at 4°C (3-week cold), and six weeks of cold stratification at 4°C (6-week cold). Each treatment consisted of three replicates each having 30 seeds per petri dish for a total of 90 seeds per treatment. The seeds were placed in standard (90 mm x 15 mm) petri dishes containing a thin layer of vermiculite and moistened with deionized water.

This helped to maintain consistent moisture levels and aided in controlling potential mold growth. The no cold treatment group was placed on a lab bench at room temperature under lights set for 12 hours on and 12 hours off. The 6-week cold treatment group was placed in a standard refrigerator for the stratification period. Three weeks into this stratification period, the 3-week treatment group was placed in the same cold stratification environment.

Table 1. Latitude and longitude of sample sites.

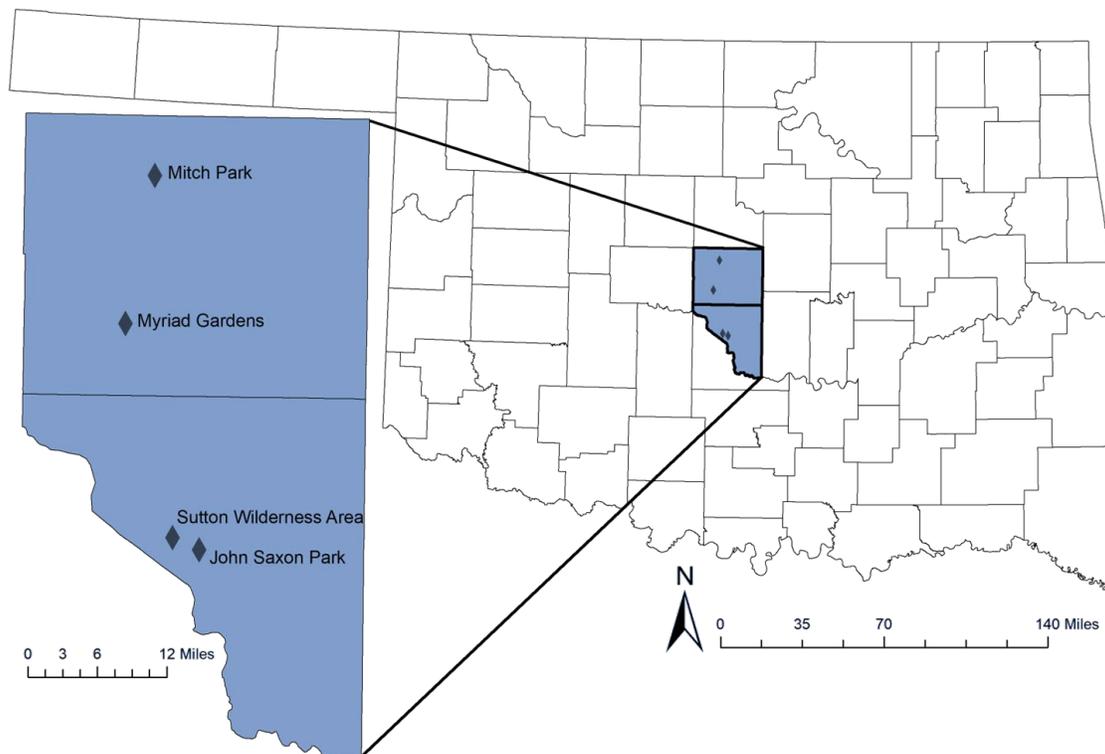| Collection Site	Latitude	Longitude
Mitch Park	35.65085	-97.47227
Myriad Gardens	35.46651	-97.5145
John Saxon Park	35.18675	-97.39542
Sutton Wilderness	35.20141	-97.43688

Figure 3 Collection sites in Oklahoma and Cleveland counties, OK.

Prior to cold stratification, only the no cold treatment group was exposed to light, but because the seeds were covered with vermiculite, there might have been little exposure. Upon removal from cold stratification, all three treatment groups received 12 hours of light and 12 hours of dark. Moisture levels of all the treatment groups were monitored every day to ensure continued proper moisture maintenance and seeds were lightly misted as needed. Observations of each treatment group were made at that time and any germination was recorded. Seeds were considered to have germinated upon emergence of the radicle from the seed. All three treatment groups were observed every day for six weeks to allow for latent germination of seeds that still appeared to be dormant. Germinated seeds were carefully removed, transplanted, and allowed to grow.

Data analysis consisted of survival analysis, as this test is a powerful methodology for analyzing seed germination (McNair et al. 2012; Manso et al. 2013; Messick and Hoagland 2018; Sanchez-Toledano et al. 2018). Although initially intended to estimate the survival of patients in medical studies, survival analysis can be applied to seed germination studies, as it effectively estimates the failure rate of the seed to survive as a seed (McNair et al. 2012; Manso et al. 2013; Messick and Hoagland 2018; Sanchez-Toledano et al. 2018). In other words, it estimates the probability that a seed will not germinate. The probability of not germinating can then be converted to the probability of germinating by simply subtracting the probability estimate of not germinating from one (Messick and Hoagland 2018; Romano and Stevanato 2020).

Survival analysis consists of Kaplan-Meier survival curve estimation which is then compared using the semiparametric Cox proportional hazards (PH) model. The Cox PH model estimates the proportional hazard for germination to *not* occur. As most, if not all, seed germination data involving cold stratification treatments violate the proportional hazard ratio assumption, a stratified Cox PH model is run instead (McNair et al. 2012; Messick and Hoagland 2018; Romano and Stevanato 2020), where the data are then split into their respective cold stratification treatments and the stratified model is run to compare treatments and a hazard ratio is obtained (McNair et al. 2012). A hazard ratio of 1.00 indicates there is an equal probability of germination between treatments, while a hazard ratio greater than 1.00 indicates the first treatment in the comparison has that many times greater probability of germinating (McNair et al. 2012; Messick and Hoagland 2018).

Survival analysis was run using the survival package (v. 2.44-1.1; Therneau 2019) in R version 3.6.1 (R Core Team 2019). Kaplan-Meier survival estimates were calculated and converted into germination curves. A stratified Cox PH model was then run to obtain the hazard ratios between treatments. Additionally, we tested for potential "tray effects" between each petri dish within a treatment by adding frailty to the stratified Cox PH models (McNair et al. 2012; Messick and Hoagland 2018).

RESULTS

In the no cold treatment, no seeds germinated, and all 90 seeds exhibited continued dormancy for the duration of the study. Relative to the other two treatment groups, the additional light to which this group was exposed did not promote germination as all seeds in the no cold stratification group-maintained dormancy. The 3-week cold treatment had a total of 14 seeds (15.5%) that germinated, while the 6-week cold treatment had a total of 54 (60%) germination events. The Kaplan-Meier survival estimates (Figure 4) showed increased germination probability for the 3-week and 6-week cold, with the 6-week
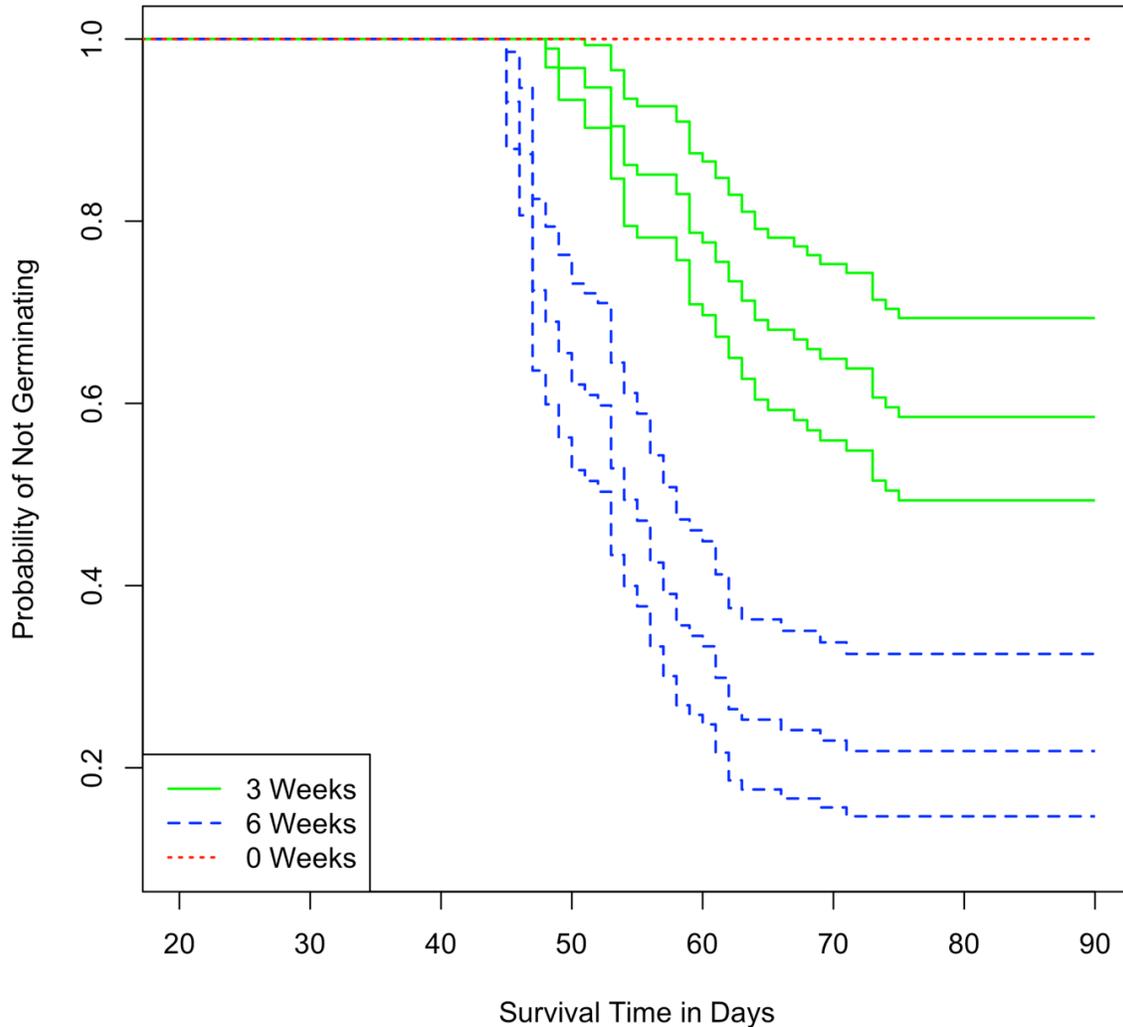
Figure 4 Kaplan-Meier survival time (as a seed) estimates by stratification treatment with 95% confidence intervals.

cold having the highest probability of germination. The converted Kaplan-Meier estimates are presented as germination curves in Figure 5. The stratified Cox PH model results, comparing the 3-week cold to the no cold were not significant, indicating there was no difference in the likelihood of germination within the allotted time interval between these two treatments, even though germination events did not occur in the no cold treatment. The stratified Cox PH models indicated that 6-week cold was 3.4 times more likely (as indicated by the hazard ratio of 3.4; p-value < 0.001) than the 3 week cold to germinate within that given time interval. This indicated that there was a significant correlation between length of time exposed to a cold stratification treatment and the probability of germination to occur. Frailty analysis results also showed a significant difference between cold stratification treatments (p-value < 0.001) but no significant effect (p-value = 1.00) on germination with regards to petri dish designation.
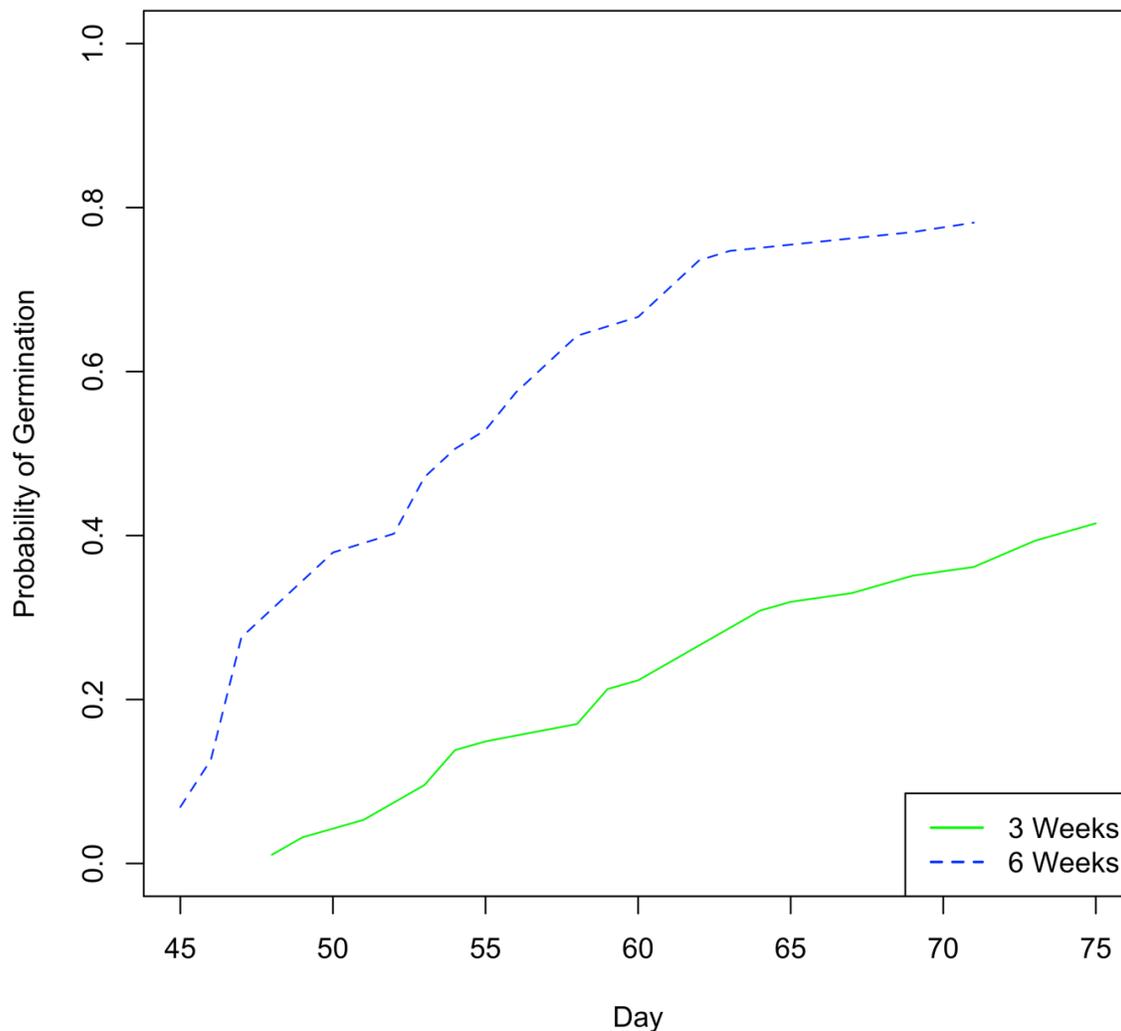
Figure 5 Germination curves by stratification treatment, calculated as one minus the Kaplan-Meier estimate. No cold treatment not shown, as zero seeds germinated.

DISCUSSION AND CONCLUSIONS

Salvia azurea var. *grandiflora* seeds germinated in larger numbers after a 6-week cold stratification period compared to the other treatments indicating that a period of cold stratification is necessary to obtain higher germination. The 6-week cold stratification treatment group responded to induced environmental cues in accordance with natural environmental cues it would receive outdoors in Oklahoma, its native cold-stratification environment. From approximately December to February

(6-10 weeks), the average temperature in central Oklahoma ranges from 4.2°C to 5.1°C (Oklahoma Climatological Survey 2021), which correlates to the length of time and temperature stratification implemented in this study. I reject my null hypothesis that cold stratification would have no effect on germination.

Seed dormancy is a remarkable evolutionary adaptation to help promote species *continuation* and survival. The ability of a species to persist temporally and spatially, particularly throughout unfavorable climatic conditions, helps to ensure their continuation. Climate change

can have an immense influence on plant regeneration, seed dormancy and cycling, survival, and diversity, changing natural biological ecosystems throughout our planet (Chhettri and Rawal 2017). Germination defines the intricate, high-risk transition phase between seed, radicle emergence and seedling establishment, during which environmental temperatures strongly influence germination success (Chhetri and Rawal 2017; Hradilova et al. 2019). Climate change can alter environmental signals including temperature, soil moisture and composition, radiation and humidity that may prevent, hinder or enhance the release of dormancy and implementation of the germination process (Bernareggie et al. 2016; Chhettri and Rawal 2017). This could in turn present a risk to species fecundity (reproductive success).

Specialist pollinators such as *Tetraloniella cressoniana* (blue sage bee), that have evolved to inhabit a *single* niche in the local ecosystem and rely on one species of flora for survival could be adversely affected by an alteration in the natural seed dormancy and release cycle. This could result in a seed germination phenological shift which would ultimately affect species continuation, composition and diversity (Footitt et al. 2014; Bernareggi et al. 2016; Chhetri and Rawal 2017). A phenological shift in seed germination could alter flowering time (Mo et al. 2017) which could adversely affect Monarch butterflies in their annual migration, as they rely on numerous flower sources for nectar feeding during that time. As climate change reshapes our global environment, the ability of plant species to persist will depend on their seeds' ability to adapt to continuing environmental fluctuation in local and global temperature.

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