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Impacts of anthropogenic change on plant reproduction and fitness

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To the Graduate Council:

I am submitting herewith a thesis written by Alexandra S. Faidiga entitled "Impacts of anthropogenic change on plant reproduction and fitness." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.

Susan Kalisz, Major Professor

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Impacts of anthropogenic change on plant reproduction and fitness

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DEDICATION

I would like to dedicate this thesis to my mother, Susan Faidiga, whose unwavering love and support made it possible for me to pursue my graduate degree and any other goal I set out to accomplish.

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ABSTRACT

Humans are altering natural systems around the globe in myriad ways. For plant species, such anthropogenic changes have resulted in increasingly fragmented populations, desynchronized interactions with mutualists, and shifted geographic ranges, among other effects. However, despite numerous examples of human impacts on plant populations, the consequences of these changes on plant reproduction remain poorly understood. In my thesis, I investigate the impacts of two forms of anthropogenic change–habitat disturbance and climate warming–on plant reproduction and fitness. I take two distinct approaches to address questions posed at local and regional scales.

In Chapter I, I ask how inbreeding depression varies across the life cycle of the critically imperiled California endemic species, *Collinsia corymbosa* (Plantaginaceae). I show that, consistent with other primarily outcrossing species, inbreeding depression in *C. corymbosa* is most pronounced late in life history, specifically during the female reproductive phase of the life cycle. Inbred plants demonstrated significantly lower rates of autonomous autogamy (δ [delta] = 0.448) and flower production (δ [delta] = 0.225), limiting the ability of this species to set seed in the absence of pollinators.

In Chapter II, I ask whether flowering and fruiting dates have advanced for 14 spring-flowering plant species in the Blue Ridge and Ridge & Valley ecoregions of eastern Tennessee over the past century. Additionally, I investigate how phenological sensitivity to spring temperature varies between ecoregions. Utilizing phenological observations sourced from 1000+ digitized herbarium specimens, I show that the sensitivity of flowering phenology to spring temperature at the community level varies between the Ridge & Valley and Blue Ridge (2.7 and 1.3 days earlier per degree Celsius warming, respectively). Further, I show that, while the flowering phenology of the majority of species investigated is sensitive to spring temperature in both ecoregions, flowering and fruiting dates have not significantly advanced in recent decades.

Overall, I found variation in plant reproductive responses to anthropogenic change at the maternal family, population, species, community, and regional levels. Together, my research demonstrates that assessing reproduction and fitness at these multiple scales provides nuanced insights into the adaptive capacity and ultimate persistence of species in the Anthropocene.

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Abstract

Inbreeding depression, the reduction in fitness of selfed progeny relative to outcrossed progeny, is one of the primary drivers of plant mating system evolution and can be influenced by a number of factors including population size, outcrossing rates, and inter-annual variability in pollinator visitation. Endemic species, which have limited geographic distributions, specialized habitat preferences, and can maintain small population sizes, are especially at risk for reduced genetic diversity and subsequent inbreeding depression when their habitats are disturbed or fragmented. Evaluating the timing and magnitude of inbreeding depression in rare and endemic species is critical to understanding the fitness consequences of reduced genetic diversity and the ultimate capacity of these species to adapt in response to disturbance and climate change. In this study, I ask how the magnitude of inbreeding depression varies across the life cycle of the critically imperiled California endemic plant *Collinsia corymbosa* Herder (Round-Headed Chinese Houses; Plantaginaceae), a species whose only confirmed extant population is currently threatened by disturbance. To quantify inbreeding depression across the life cycle, I compared seedling, vegetative, female reproductive, and male reproductive traits between inbred and outbred plants generated using a hand pollination experiment in the greenhouse. I show that inbred plants set significantly fewer fruits autonomously, produce fewer flowers, and have lower total biomass than outbred plants. Inbreeding depression in this primarily outcrossing species (t_m = 0.79 ± 0.1 SE) was most pronounced in the female reproductive phase of the life cycle, with inbred plants demonstrating significantly lower rates of autonomous autogamy (δ = 0.448) and flower production (δ = 0.225). My results highlight that, because endemic species can vary in the timing and magnitude of inbreeding depression across the life cycle, understanding in which life stages inbreeding depression is most strongly manifested can help to direct conservation efforts for these species where they are needed most. Due to the reduced capacity for autonomous autogamy in inbred *C. corymbosa* plants, future work assessing the influence of disturbance on pollinator visitation within this species' only confirmed extant population will be critical for its persistence.

Introduction

The mating systems of species play critical roles in their response to local ecological and environmental conditions. Mating system determines not only how genetically variable offspring are, but also a species' ability to set seed in the absence of pollinators or mates (e.g., Kalisz et al. 2004, Moeller 2006, Eckert et al. 2010). Despite the fact that inbreeding depression (ID), the reduction in fitness of selfed progeny relative to outcrossed progeny, is expected to generally disfavor selfing (Lande and Schemske 1985), nearly half of the plant species quantified for outcrossing rates display mixed mating systems, where individuals produce both outcrossed and selfed progeny (Goodwillie et al. 2005, Vogler and Kalisz 2001). Although ID has long been known to negatively affect progeny fitness (e.g., Darwin 1876, Charlesworth and Charlesworth 1987, Husband and Schemske 1996, Keller and Waller 2002, Armbruster and Reed 2005), selfing may be maintained in mixed-mating species when the negative fitness consequences of ID for maternal plants are offset by reproductive assurance in the case of insufficient outcross pollen receipt (Lloyd 1992, Kalisz et al. 2004, Moeller 2006). Thus, the particular mating system of a plant population (i.e. selfing, mixed mating, or outcrossing) depends on historical mating patterns that influence the expression of ID as well as the ecological conditions experienced by that population.

Quantifying the magnitude of ID in a plant population can, in part, help to understand how its mating system may continue to evolve in the future. Classical theory predicts that a population will maintain outcrossing if ID > 0.5 due to reduced fitness of selfed progeny or evolve toward selfing when ID < 0.5 due to the automatic transmission advantage of self-fertilization (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Further, the timing of the expression of ID during an individual's lifespan provides more useful insight into the population's mating system history than the magnitude for any one trait alone. In their meta-analysis, Angeloni et al. (2011) found that the magnitude of ID does not differ between self-compatible and self-incompatible species, but that the difference in the magnitude of ID among life history stages was significant in self-compatible species and non-significant in self-incompatible species. Their results also aligned with those of other studies finding that the majority of ID is expressed in later life stages (i.e., growth and flower production) for primarily selfing species while primarily outcrossing species express the majority of ID either early (i.e., seed production) or late (Husband and Schemske 1996, Winn et al. 2011). This is likely due to strong selection against mutations at early life stages that are purged in highly selfing species but are maintained with some level of outcrossing (Husband and Schemske 1996). Additionally, selfing taxa are thought to efficiently purge deleterious recessive alleles over time (Husband and Schemske 1996, Barrett and Charlesworth 1991, but see Winn et al. 2011), although substantial ID may be maintained in selfing taxa if recessive mutations are only mildly deleterious and therefore subject to drift and fixation (Husband and Schemske 1996, Keller and Waller 2002, Lohr & Haag 2015). Thus, if a population experiences changes in the environment (e.g., habitat fragmentation) that affect the amount of inbreeding within a population, the timing of the resulting change in ID within a plant's life cycle could have different demographic consequences for the population depending on its mating system.

To assess the magnitude of ID within plant populations, researchers typically compare the mean fitness of individuals that experienced different degrees of inbreeding in nature or have been artificially inbred through experimental crosses in controlled conditions. The results of such studies have shown that ID affects a multitude of individual plant traits, including reduced fruit (Kephart et al. 1999, Borba et al. 2001) and seed set (Schemske 1983, Kephart et al. 1999, Severns 2003, Glaettli and Goudet 2006), lower germination and growth rates, and overall reproductive success (e.g. Wolfe 1993, Mayer et al. 1996, Kephart et al. 1999, Galloway and Etterson 2007, Collin et al. 2009) of inbred relative to outcrossed progeny. However, given that the timing of the expression of ID may depend on a population's mating system (Kalisz 1989, Husband and Schemske 1996, Winn et al. 2011), accurate estimation of ID requires fitness measurements across an entire plant's lifespan.

Importantly, while most ID studies have demonstrated low female reproductive success of inbred progeny (e.g. Kalisz 1989, Jóhannsson et al. 1998, Kephart et al. 1999, Hayes et al. 2005, Galloway and Etterson 2007, Mena-Ali et al. 2008), far fewer studies have quantified ID for male fitness traits in plants (reviewed in Losdat et al. 2014). Male fitness is an important component of total reproductive success because pollen traits can significantly affect the number of seeds sired by an individual (Snow and Spira 1991, Jóhannsson et al. 1998, Melser et al. 1999). Further, a substantial portion of a plant's genome is expressed in pollen grains (Mulcahy et al. 1996), and if deleterious mutations are present in the genome, total plant fitness could be reduced even if ID in female traits is relatively low (Mulcahy 1979) because low fruit or seed set may be due to low pollen fitness. In the few species tested for ID in male traits, inbreeding decreased male fitness through pollen quantity (Carr and Dudash 1995, Hayes et al. 2005),

pollen viability (Mayer et al. 1996, Dudash et al. 1997), and pollen tube growth (Snow and Spira 1991, Aizen et al. 1990, Jóhannsson et al. 1998, Melser et al. 1999, Hayes et al. 2005, but see Pélabon et al. 2016 for a discussion of the role of pollen competition in selection). Therefore, to accurately assess the consequences of ID on reproductive success across the life cycle, male fitness needs to be taken into account.

Endemic plant species may be especially sensitive to environmental disturbances that can increase ID and as such are valuable systems in which to investigate ID. Endemic species by definition have limited geographic distributions, specialized habitat preferences (Kruckeberg and Rabinowitz 1985), and can maintain small population sizes (Magurran and Dornelas 2010, Mace et al. 2010, Butchart et al. 2010). Further, many endemic taxa are species of increasing conservation concern and often occur in "biodiversity hotspots," areas of both high endemism and high vulnerability to disturbance (Myers et al. 2000). Habitats of endemic species commonly face encroachment associated with human disturbance and development (Cincotta et al. 2000), resulting in habitat loss (Wilcove et al. 1998, Brooks et al. 2002, Dirnböck et al. 2011) and habitat fragmentation that leads to reduced population sizes (Fischer and Stöcklin 1997, Wilcove et al. 1998) that can drive coincident changes in pollinator visitation rates (Spigler 2009). In addition, altered environmental conditions driven by global climate change (Loarie et al. 2009, Hughes et al. 2003, Thomas et al. 2004, Malcom et al. 2006) can further destabilize endemic populations. Together, these factors call into question how the mating systems of endemic plant species are shaped by ID (Spigler et al. 2010, Spigler et al. 2017) and how they may change in the future in the face of anthropogenic threats.

ID may be commonly expressed in endemic plant populations due to their limited size and isolation (Karron 1987, Paschke et al. 2002). Further, ID in small or highly fragmented endemic populations may increase due to higher rates of mating with relatives (i.e., biparental inbreeding, Ellstrand and Elam 1993), increased heterospecific pollen transfer, a decline in pollinator visitation to small populations that drives increased selfing (Spigler et al. 2010, Knight et al. 2005, Spigler 2018), or combinations of these forces. Likewise, genetic variation may already be reduced in populations of rare or endemic species due to either drift and founder effects, or strong directional selection toward phenotypes best fit to a limited number of habitats (Karron 1987, Aguilar et al. 2008). Because of the demographic (Cosset et al. 2019, Leimu et al. 2010) and genetic (Jump & Peñuelas 2006, Keller & Largiader 2003, Aguilar et al. 2008) impacts of habitat changes associated with human disturbance, the timing and magnitude of ID is likely to influence the long-term persistence of many endemic plant populations. Further, ID itself may feedback on the expression of the mating system and be environment-dependent (Cheptou & Donohue 2010, Cheptou & Schoen 2002). Thus, given the complex interplay among a species' mating system, ID, and drift, understanding the mating system and the magnitude of ID in populations of endemic species is crucial for their conservation in the face of anthropogenic threats.

Here I present results from a study of ID in the California endemic *Collinsia corymbosa* (Plantaginaceae). I investigate the effects of mating system and ID on plant traits across the life cycle including measures of both male and female fitness by comparing offspring derived from both self and outcross pollinations. Specifically, for the narrow endemic *C. corymbosa* I ask:

1) What is the degree of selfing in the wild?
2) To what extent does inbreeding depression manifest across seedling, vegetative, and both female and male reproductive life stages?

3) Does inbreeding depression affect the ability of this species to produce offspring through selfing?

I predict that *C. corymbosa*'s fitness will be critically influenced by the interplay of the mating system, inbreeding depression, and the fitness differences of pollen produced by selfed or outcross pollination events.

Methods

Study system

Collinsia corymbosa Herder (Round-Headed Chinese Houses; Plantaginaceae), is a winter annual that is a narrow endemic native to the California coast (Fig. 1.1 in Appendix I). The California Department of Fish and Game assigns *C. corymbosa* the S1 "Critically Imperiled" ranking at the state level on its Special Plants, Bryophytes, and Lichens List, and it is rare or endangered in California (California Natural Diversity Database 2020). Additionally, as of 2016, this species is known from only 11 populations in five counties in California, and is presumed extirpated from San Francisco County (California Native Plant Society 2020). Confirmed extant populations have a highly restricted distribution: they occur only on coastal dunes with the majority of recent (<20 years) observations occurring in a single location (B. Baldwin, personal communication; Fig. 1.2).

Flowers of *C. corymbosa* are arranged in a compact inflorescence (Fig. 1.1 inset) and flowering at the study site, dunes north of Mill Creek, Mendocino County, CA, occurs from April to June. The bilaterally symmetrical flowers are large compared to those of other species in the genus *Collinsia* (14-22 mm; Randle et al. 2009), borne in whorls with 5 to 20 simultaneously open flowers, and are primarily visited by bees (S. Kalisz, pers. obs.). Each flower can produce up to 12 seeds (S. Kalisz, unpublished data). Like other members of the genus *Collinsia*, the flowers of *C. corymbosa* are hermaphroditic, self-compatible, and protandrous, but are capable of high rates autonomous fruit set in the absence of pollinators (83% of *C. corymbosa* flowers set fruit autonomously in a growth chamber experiment; Kalisz et al. 2012).

Seeds were collected in individual coin envelopes from 29 naturally-pollinated individuals from two large adjacent areas occupied by *C. corymbosa* on dunes north of Mill Creek, Mendocino County, CA in the spring of 2004. Seeds were transported to the lab and stored at room temperature. In November 2006, seeds were planted in Sunshine germination mix™, maintained in growth chambers under 18°C/1°C, 10-h light/14-h dark day/night conditions in a Percival chamber, and the soil was kept uniformly moist to induce germination. After the first true leaves developed (early 2007), all individuals were transplanted to 2 ½" pots containing Fafard™ potting mix and placed in the greenhouse. A subset of plants from 18 of the 29 maternal families were harvested to estimate the mating system of the population. The remaining plants grew to flowering and were used in the experiments described below.

Mating system estimation

Aboveground tissue was harvested from 5-10 seedlings/family for 18 seed families and stored individually at -80 °C. To extract DNA, samples were individually placed in liquid nitrogen, ground, and processed using the CTAB DNA extraction protocol adapted from Doyle & Doyle (1987). Progeny were genotyped using four fluorescently tagged microsatellite markers developed for *Collinsia verna* (*A131, A134, B116, B105*) following the published protocols

(Dunn et al. 2005). Multiplex fragment analyses (Applied Biosystem 3730) and allele calling (Genemapper software, Applied Biosystems, Carlsbad, CA, USA) were used to determine the multi-locus genotypes of each individual progeny.

To calculate the average outcrossing rate of the population, multi-locus genotype data were analyzed with the maximum-likelihood program MLTR (Ritland 1990) with Newton-Raphson iteration. Confidence intervals (95%) were calculated as the interval from the 2.5- to 97.5- percentile of the distribution of 1000 replicate bootstrap estimates of the multi-locus outcrossing rate estimates (t_m), with the progeny (family) array as the unit of resampling.

Seedling and vegetative traits

One individual from each of the 29 maternal families grown in the growth chamber as described above was used to produce selfed and outcrossed progeny for inclusion in the ID experiment. On each of these plants, flowers on the second flowering whorl or higher were assigned to self or outcross treatments, marked with color coding fabric paint, and emasculated. After 5-6 days (to ensure stigmatic receptivity), hand pollinations of the emasculated flowers were then performed with either self-pollen (taken from another flower of the same plant) or outcross-pollen (a mixture of pollen taken from flowers of 4-5 other plants). The resulting fruits and seeds were then collected when mature. Hereafter, I refer to a "maternal family" as the seeds and the resulting plants of a single individual included in this crossing program.

Seeds from each fruit per maternal family were counted and individually weighed, and in 2007, seeds were planted and maintained to germination in a Percival chamber as described above. Two to eight selfed (average = 3.9) and two to eight outcrossed progeny (average = 4.1) were randomly chosen from each maternal family for inclusion in the study. In total, 23 of the 29 maternal families had sufficient progeny to be included in the study. To estimate inbreeding depression in early life stages, germination dates were scored three times each week after planting. These resulting seedlings were transplanted into 2 ½" pots containing Fafard ™ potting mix and transferred to the greenhouse for the remainder of the study.

To assess developmental instability predicted to accompany inbreeding (Kalisz 1989, Sherry and Lord 1996, Clarke 1995), trichome density and leaf asymmetry were measured on a single, most recently fully expanded leaf from the third vegetative whorl of each plant. For each leaf collected, the total number of trichomes was counted across the upper surface and a photograph of the leaf was taken using the Optimus imaging program. Total leaf area and leaf area to the left or right of the midrib were then calculated from this image. Trichome density was calculated as the number of trichomes on the upper leaf surface divided by total leaf area, and leaf asymmetry as the absolute value of the difference between the leaf area to the left vs. right of the midrib. At the conclusion of the experiment, plants were collected, dried, and weighed in order to measure total above ground biomass.

Floral traits

To estimate ID in floral traits, plants were scored weekly for the appearance of flower buds and counted the total number of flowers produced by each plant. To estimate autonomous fruit set per plant, 10 flowers along the main stem of each plant were scored for fruit set as a binary trait (1 = fruit, 0 = no fruit) starting at the 4th floral whorl. Because plants were grown in a pollinator-free greenhouse, any seeds produced were considered to be the product of autonomous selfing. Of the 10 fruits scored for fruit set, mature fruits were collected and the number of seeds per fruit were counted. I used the average seeds per fruit from these 10 flowers as an estimate of

average autonomous seed set per fruit for each individual. I then calculated total autonomous seed set per individual as: (average autonomous seed set per fruit * total flower production).

Male fitness: Pollen germination and pollen tube growth

To estimate the effect of ID and flower age on the male fitness metric of pollen germinability, percent pollen germination was quantified from selfed and outcrossed progeny using 20 outcross and 18 selfed plants randomly chosen from the study group. From each plant, one newly dehisced anther and one old anther (>7 days old) that was fully dehisced and whose pollen had fallen into the keel of the flower were collected. Pollen from each anther was then spread across Petri plates containing BKM growth medium (Kearns and Inouye 1993) and incubated at 22°C. Percent pollen germination was recorded for each individual 15-, 30-, and 120-minutes post-spread by assessing 50-100 pollen grains along a continuous transect across the middle of the plate under a light microscope. Each pollen grain on the transect was scored as 'germinated' (having a discernible pollen tube) or 'not germinated' (no pollen tube), and plates were returned to the incubator between sampling points. Percent pollen germinated was calculated as: (# pollen grains with pollen tubes)/(total # pollen grains assessed).

To quantify the effects of ID, flower age, and maternal and paternal cross type on pollen tube growth, hand-pollinations were performed using pollen from selfed and outcrossed plants and pollen tube growth in the style was measured. Because rates of callose plug formation are correlated with pollen tube growth rate (Snow and Spira 1991) and can differ between self and outcrossed pollinations (Lush and Clarke 1997, Tupy 1959), inter-callose plug (ICP) distances between sequential callose plugs were measured as a proxy for pollen tube growth rate. First, at least two flowers on each of 46 plants (24 outcross, 22 selfed) were emasculated to prevent autonomous self-fertilization. When the stigmas of these pollen recipient 'maternal' plants were mature, they were then pollinated with pollen from one of four pollen cross types: (1) self-pollen from a recently opened flower, (2) outcross-pollen from a recently opened flower, (3) self-pollen an older flower (>7 days old), (4) outcross-pollen from an older flower. Outcross-pollen was collected from one of 40 pollen donor 'paternal' plants (20 outcross, 20 selfed) while self-pollen was collected from flowers of the same plant (103 hand-pollinations total). Four hours after each hand pollination, styles were collected and fixed in 70% ethanol. Styles were then digested in 1M NaOH for two hours, placed on microscope slides with 0.5% aniline blue, squashed, and examined under a UV light microscope. The first four ICP distances from the stigma end of the style were measured for each pollen tube to calculate an average ICP distance, and the minimum and maximum ICP distance was recorded for each maternal plant and cross type.

Data analysis

I performed all statistical analyses in R (R Core Team 2019). To analyze the effects of ID on plant traits across the seedling, vegetative, flowering, and late life stages of the *C. corymbosa* life cycle, I compared the performance of selfed and outcrossed plants using mixed effects models with family as a random effect and cross type as a fixed effect. The significance of cross type in explaining fitness differences between selfed and outcrossed plants at all life stages (except for initial seed mass) was analyzed using ANCOVA with initial seed mass as a covariate. I checked all variables for ANCOVA assumptions before including them in my analyses and transformed them where appropriate. For variables with non-normal error structures, I utilized generalized linear mixed effects models using the lme4 package (Bates et al. 2014). When analyzing days to germination, days to flowering, and number of flowers, I used a Poisson error

structure with a log link function. I modeled autonomous fruiting ability as a binomial variable where 1 represented the condition that a plant set at least one fruit out of ten, and 0 represented the condition that a plant set zero fruits out of ten. Then, I subset the data to include only plants that set at least one seed out of ten fruits to model fruit set, autonomous seed set and estimated total autonomous seed production as normally distributed random variables. To test for the presence of family-level variation in inbreeding depression in individual plant traits ('VIFLID', Kelly 2005), I took a model comparison approach by performing X^2 tests on reduced and full models where reduced models included maternal family as a random intercept effect and full models included family as a random intercept effect and cross type as a random slope effect. To summarize the magnitude of ID across the life cycle, I calculated ID as $\delta = 1-(W_s/W_o)$ where (W_s = fitness of selfed individual, W_o = fitness of outcrossed individual). Due to differential survival, not all traits were measured on all plants, so I constructed and analyzed separate models for each plant trait.

To analyze the effects of ID on the male fitness trait of pollen germination, I used a split-plot ANOVA with pollen age and time as within-subject factors and cross type as a between-subject factor. I treated time as a factor with three levels: 15-, 30-, and 120-minutes. Post-hoc tests were performed to compare pollen germination of selfed and outcrossed plants at each time point and pollen germination at each time point within each cross type with Bonferroni adjustment for multiple comparisons. For the male fitness trait of pollen growth measured as ICP distance with time, I log-transformed ICP data and analyzed it as a normally distributed random variable as described above with both maternal and paternal family as random effects. Pearson Product-Moment Correlations were calculated between pollen traits and floral traits using the cor.test function.

Results

Mating system estimation

The outcrossing rate (t_m) determined from multi-locus genotype data was 0.79 ± 0.1 SE, indicating that for the sampled year, this *C. corymbosa* population primarily set seed via outcrossing, and lies at the upper border between mixed mating species and highly outcrossing (Goodwillie et al. 2005; Winn et al. 2011).

Inbreeding depression in plant traits across the life cycle

Seeds produced from self-pollination of plants raised from field-collected seeds had significantly lower mass than those produced from outcross pollination ($F_{1,30.3} = 4.19$, p = 0.05). Model comparison revealed family-level variation in this trait; the model that allowed both the slope and intercept to vary by family explained more variation in the data than the model that allowed the intercept alone to vary ($X^2 = 79.8$, df = 1, $p = 2.2 \times 10^{-16}$; Table 1.1). Germination rate was low overall (16% on average) regardless of cross type or seed mass. Cross type had no effect on the seedling trait of germination success or any of the three vegetative traits of trichome density, leaf area, or leaf asymmetry (Table 1.2). Selfed plants had significantly lower total biomass than outcrossed plants at the end of the experiment ($F_{1,179.4} = 7.87$, p =0.006; Table 1.2). Selfed plants produced significantly fewer flowers ($X^2 = 27.75$, df = 1, $p = 3.59 \times 10^{-7}$) and had lower autonomous fruiting ability ($X^2 = 13.74$, df = 1, $p = 2.10 \times 10^{-4}$) than outcrossed plants. For the plants that did set fruit, however, there was no significant difference in autonomous seed

set, fruit set, or estimated total autonomous seed production between selfed and outcrossed plants (Table 1.2).

Maternal cross type had a significant effect on pollen germination ($F = 10.10$, $p = 2.00 \times 10^{-3}$; Table 1.3); however, post-hoc tests with Bonferroni adjustment for multiple comparisons revealed that pollen germination was not significantly different between selfed and outcrossed plants at any time point at the significance level of $p = 0.006$. Percent pollen germination increased similarly with time ($F = 117.2_{2,211}$, $p = 5.83 \times 10^{-35}$; Fig. 1.3) and with younger pollen (pollen that was < 7 days old; $F = 6.24_{1,211}$, $p = 0.013$; Table 1.3) for both selfed plants and outcrossed plants. On average, germination of new pollen was 8.6% greater than germination of old pollen (means = 51.5% ± 2.36 SE for new pollen and 42.9% ± 2.77 for old pollen). For ICP distances of pollen on plant stigmas after hand pollinations, neither maternal cross type, paternal cross type, nor pollination cross type had a significant effect on any ICP distance metric (average, minimum or maximum), but paternal cross type did have a marginally significant effect on minimum ICP distance ($F_{1,95.5} = 3.47$, $p = 0.07$; Table 1.3), with self-pollen growing more rapidly than outcrossed pollen. I found no significant correlations between male fitness metrics of pollen germination and ICP distance and seed or fruit set. However, correlation analysis was limited due to the large number of plants that failed to set any fruits (Fig. 1.4), and the fact that I did not measure male fitness traits on all plants, reducing my sample size and my power to detect correlations between male fitness and female reproductive success.

Overall, the magnitude of ID was greatest in autonomous fruiting ability ($\delta = 0.448$) and flower production ($\delta = 0.225$), resulting in selfed plants setting significantly fewer fruits (and therefore fewer seeds per plant) and having lower estimated total autonomous seed set overall. ID was lowest in seedling and vegetative traits ($-0.003 > \delta > 0.210$ for seedling and vegetative traits together) and greatest in female reproductive traits ($-0.027 > \delta > 0.448$; Fig. 1.5). Although selfed seeds were significantly lighter than outcrossed seeds, the magnitude of the difference between selfed and outcrossed plants for initial seed mass was much smaller than that for later life traits (see Fig. 1.6).

Discussion

The population of *Collinsia corymbosa* investigated is primarily outcrossing and varied in both the timing and the magnitude of inbreeding depression (ID) across the life cycle. My results support those of past studies (Husband and Schemske 1996, Angeloni et al. 2011) that find the greatest magnitude of ID in mixed mating and primarily outcrossing species to be expressed in seed/fruit production and reproductive effort (number of flowers). However, despite the fact that *C. corymbosa* is capable of autogamy, and has been found in a past study to be an efficient autonomous selfer based on fruit set (0.83 ± 0.01 SE, Kalisz et al. 2012), I found that both outcrossed and inbred plants in my study expressed extremely low autonomous fruit set (outcrossed plants = 0.14 ± 0.02 SE, selfed plants = 0.08 ± 0.01 SE). This disparity between studies may relate to differences in growth conditions and which fruits were sampled. Kalisz et. al. (2012) grew flowering plants in a growth chamber and limited fruit collection to the third, fourth and fifth floral whorls on the main stem of the plant, while plants in this study were grown to flowering in a greenhouse and 10 fruits were randomly selected above the fourth floral whorl. Since flowers on the upper flowering whorls of *Collinsia* species often fail to set seed putatively due to resource limitation, this difference in the location of the fruits sampled could contribute to the differences in results between studies. Further, my study has a greater sample size than that used in Kalisz et al. 2012 (233 plants measured for autonomous fruit set in this study compared

to 8 in Kalisz et al. 2012). Regardless of this disparity, my results demonstrate that inbreeding clearly reduced the ability of plants to produce offspring through autonomous selfing. Although autonomous seed set did not differ between selfed and outcrossed plants when they did set fruit, and selfed and outcrossed seeds did not differ in their germination rates, inbreeding significantly reduced the likelihood that a plant would set at least one fruit out of the ten measured. Further, inefficient selfing based on fruit set was not offset by high flower production; selfed plants also produced 23% fewer flowers on average than outcrossed plants. Together, these two factors acted synergistically to greatly reduce the total estimated autonomous seed set of selfed plants; this trait demonstrated the greatest ID among all traits ($\delta = 0.568$).

If *C. corymbosa* plants in the wild also express poor selfing ability, it may help explain this species' relatively high outcrossing rate in the field ($t = 0.79 \pm 0.1$ SE). The outcrossing rate of this species is on the lower end of functionally outcrossing ($t > 0.8$, Goodwillie et al. 2005) but is on the high end for the genus; most *Collinsia* species fall within the range of outcrossing rates that defines mixed mating ($0.2 < t_m < 0.8$; Kalisz et al. 2012). Given the poor selfing ability of *C. corymbosa* plants found in this study, the outcrossing rate observed in this population could reflect higher abortion rates of selfed fruits and seeds if maternal plants preferentially provision outcrossed progeny or low effectiveness of self-fertilization. Additionally, the outcrossing rate estimated here is greater than that estimated for seeds collected in a different year from the same *C. corymbosa* population ($t_m = 0.6$, Kalisz et al. 2012), indicating that this population may experience annual fluctuations in pollinator availability or visitation rates. All *Collinsia* species are capable of autonomous self-pollination when the style elongates and places the stigma in the vicinity of dehisced anther sacs at the front of the keel petal (developmental protandry; Kalisz et al. 2012, Kalisz et al. 1999). Thus, the outcrossing rate of *C. corymbosa* in my study likely reflects the outcross pollen deposition by pollinators before self-pollination was achieved.

In contrast to the result that ID decreased female fitness through reduced autonomous autogamy, the result that ID in male fitness traits was less pronounced was surprising. Pollen tube growth rates did not differ among cross types. I anticipated lower pollen performance of selfed plants given that the majority of ID studies including male fitness traits have shown reduced pollen performance with inbreeding (reviewed in Losdat et al. 2014). However, while ID in pollen tube growth is expected under conditions of pollen competition, directional selection for traits that enhance pollen competition may not be strong in populations that typically experience pollen limitation of seed production (Mulcahy & Mulcahy 1987). Although I did not measure pollen limitation of *C. corymbosa* in the field, pollen limitation is widespread in natural plant populations (Knight et al. 2005) and likely contributes to the maintenance of selfing in mixed mating species such as *C. corymbosa* (Baker 1955, Stebbins 1974, Ashman et al. 2004). Further, while the population examined in this study was relatively large (~1300 plants), it is an isolated population. Because fragmentation (Cunningham 2000, Eckert et al. 2010) is predicted to decrease pollinator visitation and therefore increase inbreeding for mixed-mating plants (Spigler et al. 2010), it is likely that the total fitness of individuals in this population is more strongly limited by outcross pollen receipt than by the competitive ability of pollen. Thus, male traits influencing pollen competition may be less important to overall fitness than traits that influence pollinator attraction in *C. corymbosa*.

Pollen quantity and quality may be more important than pollen competition in populations where seed production is typically limited by outcross pollen receipt because conspecific pollen grains on the stigma are lower than the number of available ovules per flower. Therefore, faster growing pollen tubes would not increase fitness when pollen competition is

absent. Surprisingly, while I found that pollen from selfed plants had greater germinability overall at each time point, and the difference between selfed and outcrossed plants decreased with time until 120 minutes when germination was approximately equal. It is unclear why selfed plants may have higher pollen germination rates, and there are no studies to my knowledge showing a similar pattern. However, in a study that compared germinability of pollen produced over the floral lifespan in a primarily selfing and primarily outcrossing *Collinsia* sister species pair, Malagon et al. (2019) found that the selfing species' pollen germinability declined only slightly with age (17%) compared to the dramatic decline in germinability of the outcrossing species' pollen (88%). Because outcrossing species allocate more resources to larger, showier, and often more numerous flowers than primarily selfing species (Goodwillie et al. 2010), the male fitness of these outcrossing species may be more resource-limited than that of selfing species. Although Malagon et al. (2019) compared two species with different mating systems and my study compared inbred and outbred plants of the same species, resource allocation to male and female reproduction could play a role in the performance of more highly inbred individuals within a single year. The selfed plants in my study produced significantly fewer flowers than outcrossed plants, so it is possible that they allocated more reproductive resources to male traits, resulting in greater pollen germinability. Additional studies that disentangle the interplay of mating system, pollen limitation, and inbreeding depression in resource allocation to male and female function are needed.

The mating system of *C. corymbosa* and the resulting expression of ID across the life cycle may play a role in limiting its distribution. Species that are efficient at autonomous selfing are thought to be better adapted for colonizing new habitats because they possess the ability to set seed in the absence of conspecific mates (Baker 1955, Stebbins 1957, Grossenbacher et al. 2014; but see Pannell et al. 2015). This pattern holds true in the genus *Collinsia*: smaller-flowered species that are more effective autonomous selfers occupy larger ranges and more abiotic niches than their larger-flowered, primarily outcrossing sister species (Randle et al. 2009; Grant and Kalisz 2019). This pattern is also seen for the more selfing sister species of *C. corymbosa*, *C. barstiifolia,* which occupies a significantly larger range and habitat types than *C. corymbosa* (Randle et al. 2009; Grant and Kalisz 2019). However, given the extremely limited distribution of *C. corymbosa* compared to all other *Collinsia* species, factors other than mating system are likely at play. The range of *C. corymbosa* is likely limited by habitat specialization: extant *C. corymbosa* populations are restricted to coastal dunes. Thus, colonization in this species may be limited by its primarily outcrossing mating system, limited seed dispersal, and the narrow range of suitable habitats that it can establish and grow to flowering in post-dispersal.

Conclusion

My study points to negative effects of ID in this narrow endemic annual species, *C. corymbosa*. Decreased pollinator service in the wild will likely result in significant ID in *C. corymbosa*. The historic range of *C. corymbosa* has been reduced by human development to few remaining populations, and it is current locations, on dunes along beaches of the Pacific Ocean, are threatened by foot traffic (California Native Plant Society 2020). The further reduction of already small population sizes could increase ID because mildly deleterious alleles are more likely to drift to fixation in smaller populations (Barrett and Kohn 1991). Further, because the genetic diversity of a population influences its ability to evolve in response to changing environments (Knight et al. 2018), inbreeding in this population could reduce its capacity to adapt in response to disturbance and climate change in the future. Although small populations

tend to express lower magnitude ID than large populations (Angeloni et al. 2011) and may be more efficient at purging strongly deleterious alleles, large, primarily outcrossing populations that experience recent reductions in size may experience severe reductions in mean fitness overall despite low inbreeding depression due to increased genetic load (Lohr & Haag 2015). Given the low autonomous fruit set in this species even in outcrossed individuals, pollinator service will be important for this species' persistence. If pollination services decline in the future, the maintenance of genetic diversity within populations could be crucial to the ability of *C. corymbosa* to persist via evolution toward more efficient selfing. If this species cannot effectively compensate for reduced outcrossing (Eckert et al. 2010) because of low overall autonomous selfing ability, as suggested by my results, then this population is likely to decline. Further studies that take population history (e.g. annual size fluctuations) into account when evaluating the relationship between mating system and the expression of ID across the life cycle will be crucial for understanding how other endemic species with few extant populations may be most effectively conserved in the future.

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APPENDIX I

Table 1.1 Results of model comparisons between full and reduced models for the effects of cross type on seedling, vegetative and reproductive traits of *Collinsia corymbosa*. Full models had cross type as a random slope effect and family as a random intercept effect, while reduced models had family as a random intercept effect only. Seed mass was included as a covariate in all models.

	Test statistic for model comparison			
Seedling trait	df	X^2	*p*	Δ*AIC*
Initial seed mass	**2**	**79.83**	**2.2 x 10-16**	**-75.84**
Germination success	2	0.0055	1.00	4.00
Days to germination	2	0.0049	1.00	4.00
Vegetative trait	df	X^2	*p*	Δ*AIC*
Trichome density	2	1.32	0.52	2.69
Leaf area	2	0.89	0.64	3.10
Leaf asymmetry	2	0.00	1.00	4.00
Reproductive trait	df	X^2	*p*	Δ*AIC*
Days to flowering	2	2.49	0.29	1.50
Number of flowers	2	0.55	0.76	3.40
Autonomous fruiting ability	2	0.09	0.96	3.91
Fruit set				
Autonomous seed set	2	4.74	0.09	-0.74
Estimated total autonomous seed production	2	0.49	0.78	3.51
Late life stage trait	df	X^2	*p*	Δ*AIC*
Total biomass	2	1.54	0.46	2.46

Table 1.2 Split-plot analysis of variance for the effects of maternal cross type, pollen age, and time on pollen germination and analysis of covariance for the effects of pollination cross type, maternal and parental cross type, and pollen age on male fitness traits of *Collinsia corymbosa*. Pollen age and time were treated as within-subjects factors and cross type was treated as a between-subjects factor, and time was a factor with three levels: 15, 30, and 120 minutes. Pollination cross type = pollen from the maternal plant (self-pollination) or from another individual (outcross pollination), maternal/paternal cross type = whether the maternal/paternal plant was the product of a self- or outcross-pollination, and pollen age = pollen came from a newly opened flower or an older flower (open for >7 days).

	Pollen trait											
	% Pollen germinated			Average ICP distance			Maximum ICP distance			Minimum ICP distance		
Source of variation	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Pollination cross type	--	--	--	1	1.15	0.29	1	0.18	0.67	1	1.65	0.20
Paternal cross type	--	--	--	1	1.41	0.24	1	1.45	0.23	1	3.47	0.07
Maternal cross type	1	9.45	**2.00×10^{-3}**	1	2.53	0.12	1	2.10	0.15	1	0.97	0.33
Pollen age	1	11.00	**1.00×10^{-3}**	1	1.83	0.18	1	1.58	0.21	1	1.35	0.25
Time	2	106.17	**4.04×10^{-33}**	--	--	--	--	--	--	--	--	--

Table 1.3 Analysis of covariance for effects of cross type on seedling, vegetative and reproductive traits of *Collinsia corymbosa* with initial seed mass as a covariate. Autonomous fruiting ability was modeled as a binary variable where 1 represents the condition that a plant set at least one fruit out of ten randomly sampled fruits, and 0 represents the condition that a plant set zero fruits out of ten. Fruit set, autonomous seed set, and estimated total autonomous seed production were modeled for plants that set at least one fruit out of ten randomly sampled fruits.

	Source of variation				
	cross type				
Seedling trait	df	*F*	X^2	*p F*	*p Chisq*
Initial seed mass	1	4.19	4.21	**0.05**	**0.04**
Germination success	1	--	0.09	--	0.76
Days to germination	1	--	0.67	--	0.41
Vegetative trait	df	*F*	X^2	*p F*	*p Chisq*
Trichome density	1	2.07	2.10	0.15	0.14
Leaf area	1	0.09	0.09	0.77	0.77
Leaf asymmetry	1	0.0009	0.0009	0.98	0.98
Reproductive trait	df	*F*	X^2	*p F*	*p Chisq*
Days to flowering	1	--	3.13	0.08	--
Number of flowers	1	27.75	27.80	**3.59×10^{-7}**	**1.347 x 10-7**
Autonomous fruiting ability	1	--	13.74	**2.10×10^{-4}**	--
Fruit set	1	1355.00	--	0.85	--
Autonomous seed set	1	0.008	0.008	0.93	0.93
Estimated total autonomous seed production	1	1.94	2.00	0.17	0.17
Late life stage trait	df	*F*	X^2	*p F*	*p Chisq*
Total biomass	1	7.87	7.90	**0.006**	**0.005**

Figure 1.1 Coastal California dune habitat of *Collinsia corymbosa* from which seeds were collected at dunes north of Mill Creek, Mendocino County, CA. Inset: *C. corymbosa* grown in greenhouse.

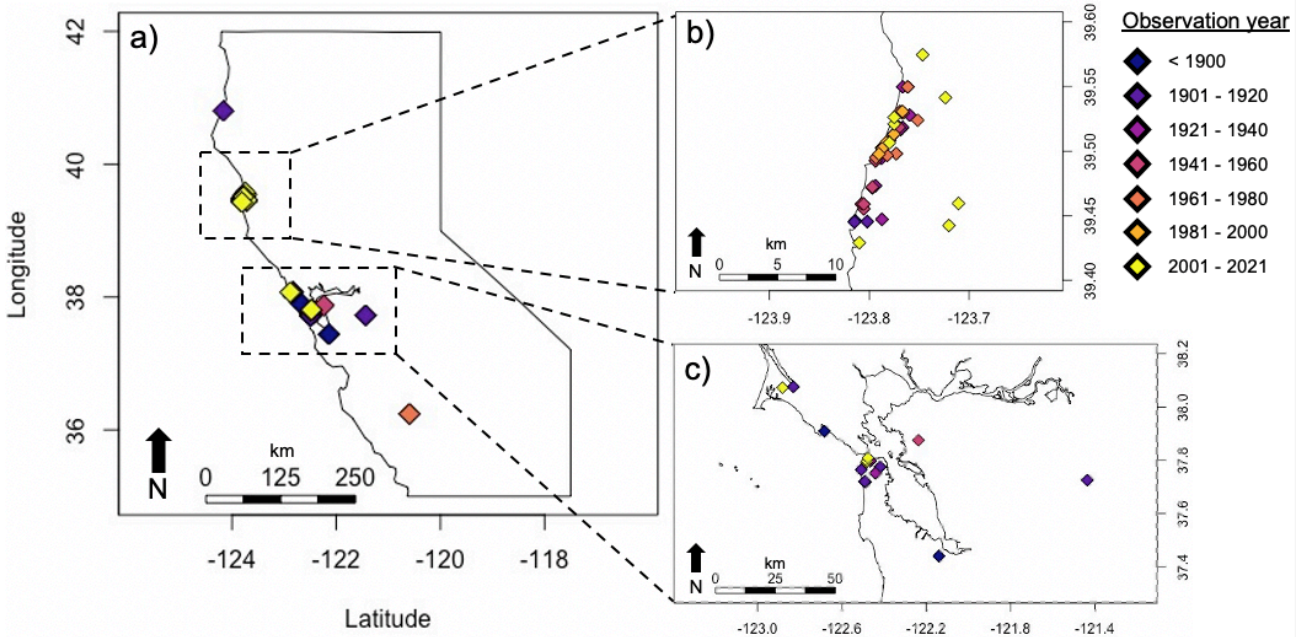

Figure 1.2 Observations of *Collinsia corymbosa* a) across its entire range, b) in and surrounding dunes north of Mill Creek, Mendocino County, CA and c) in and surrounding the San Francisco Bay area, San Francisco County, CA. Observation records were obtained from research-grade iNaturalist observations (iNaturalist 2020) and the University of California, Berkeley Jepson Herbarium (UC).

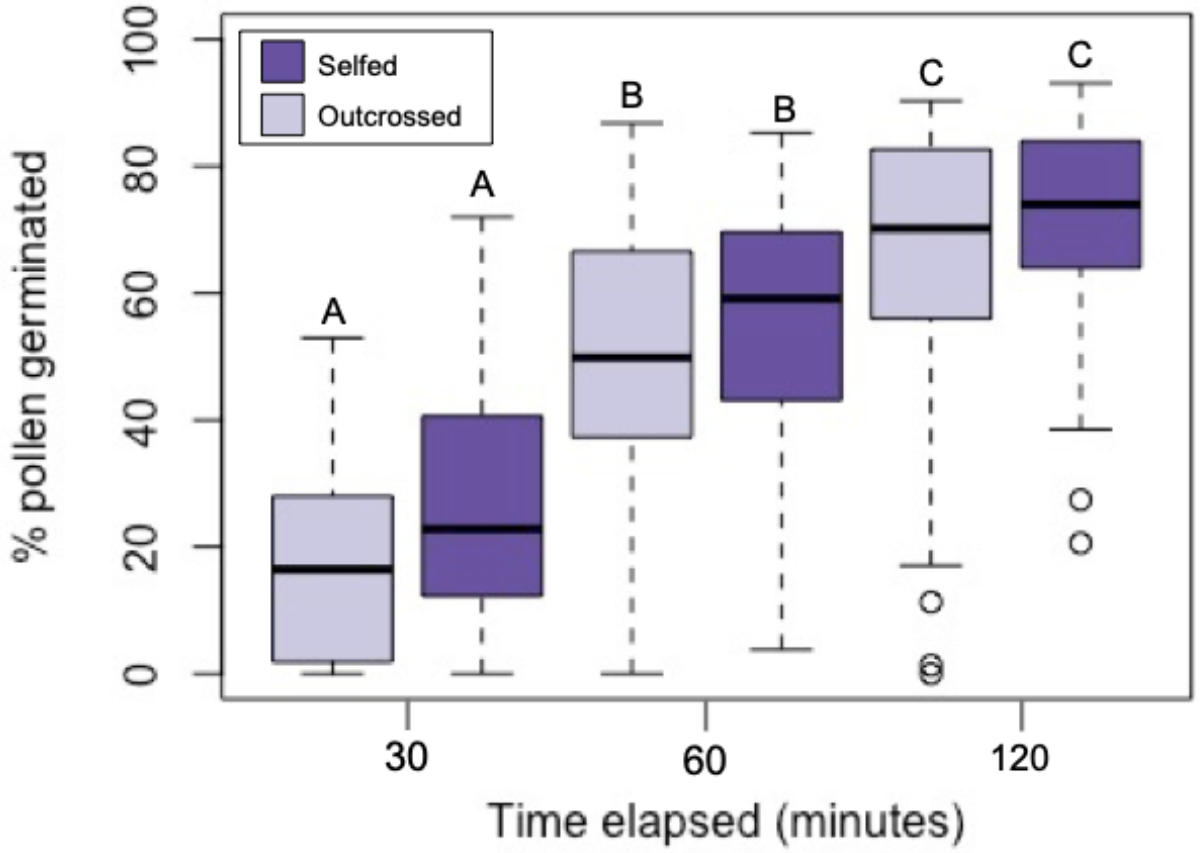

Figure 1.3 Effect of time and cross type on pollen germination rates for pollen from new flowers in selfed and outcrossed *Collinsia corymbosa* plants. Older pollen similarly decreased germination rates for both selfed and outcrossed plants (not pictured). Boxes with different letters are significantly different from one another at the Bonferroni-adjusted significance level of $p < 0.006$.

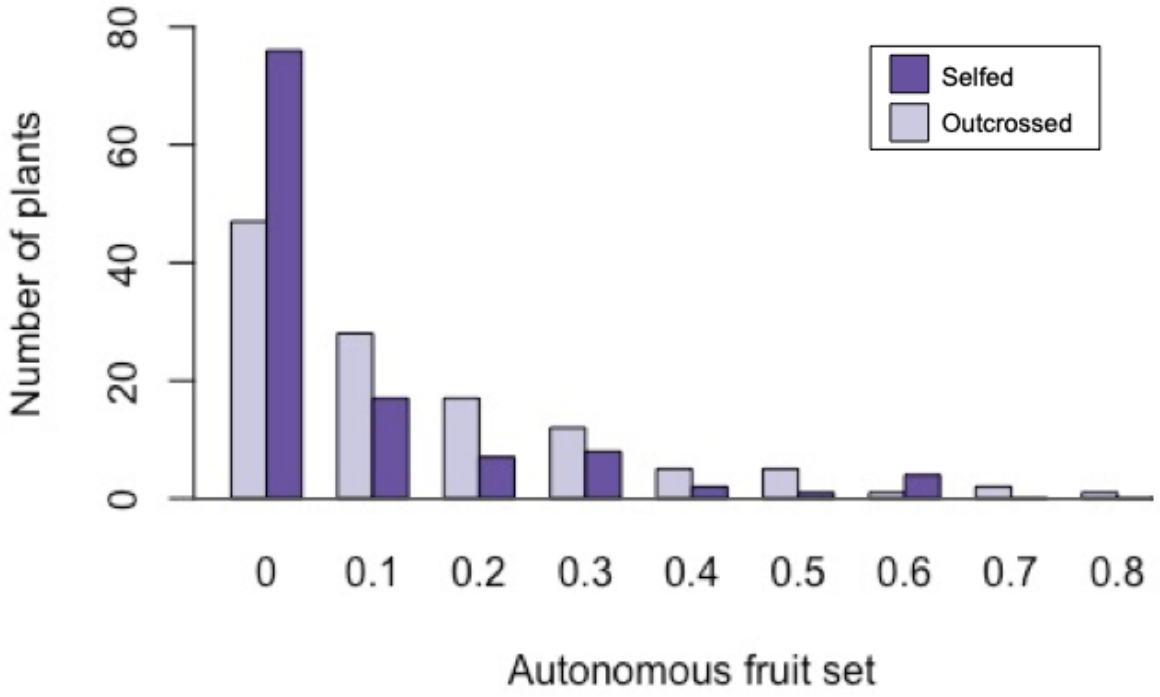

Figure 1.4 Distribution of autonomous fruit set (proportion of fruits set out of ten) of selfed and outcrossed *Collinsia corymbosa* plants.

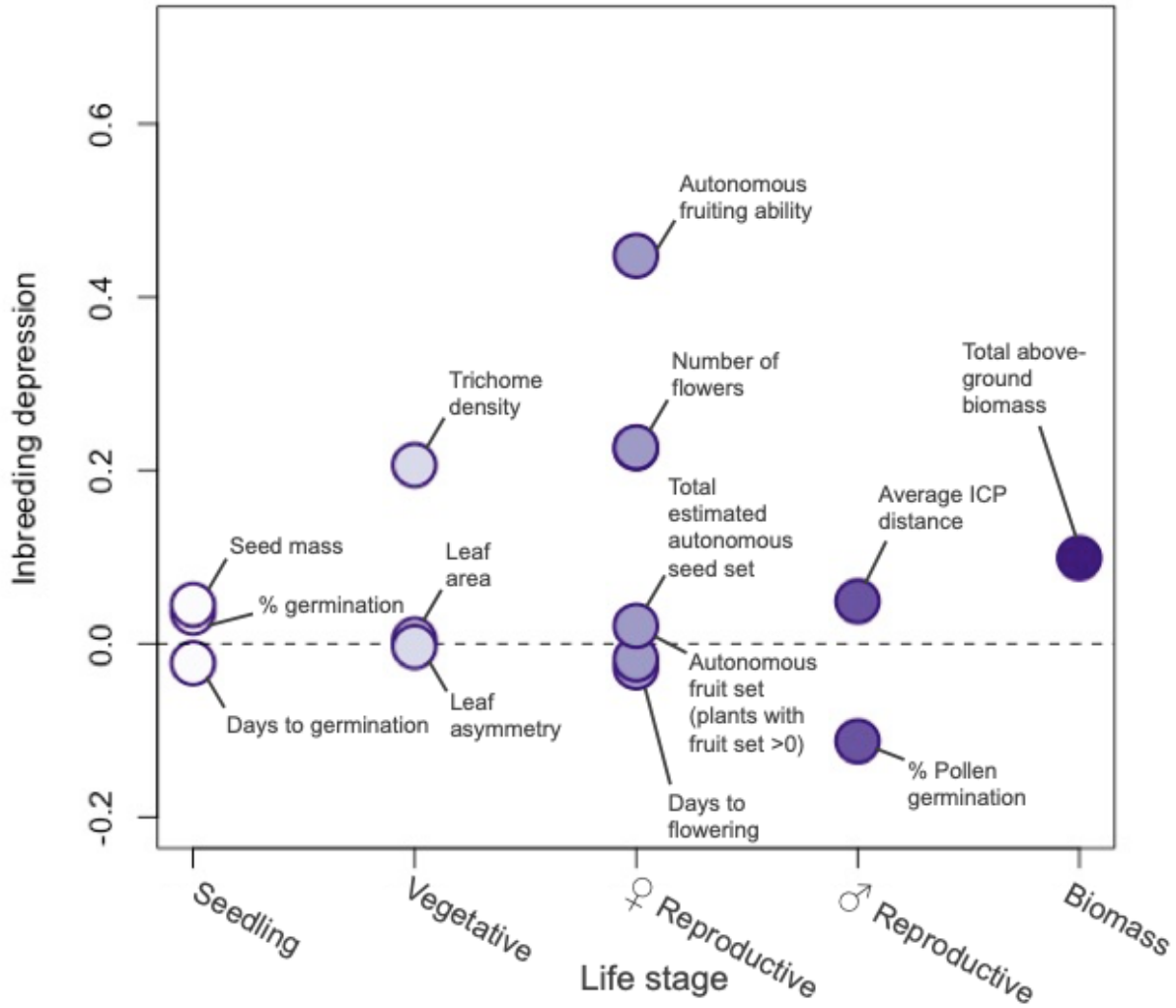

Figure 1.5 Inbreeding depression (ID) across the life cycle of *Collinsia corymbosa*. For each plant trait measured at a given life stage, inbreeding depression was calculated as $\delta = 1-(Ws/Wo)$, where Ws = mean fitness of selfed plants and Wo = mean fitness of outcrossed plants.

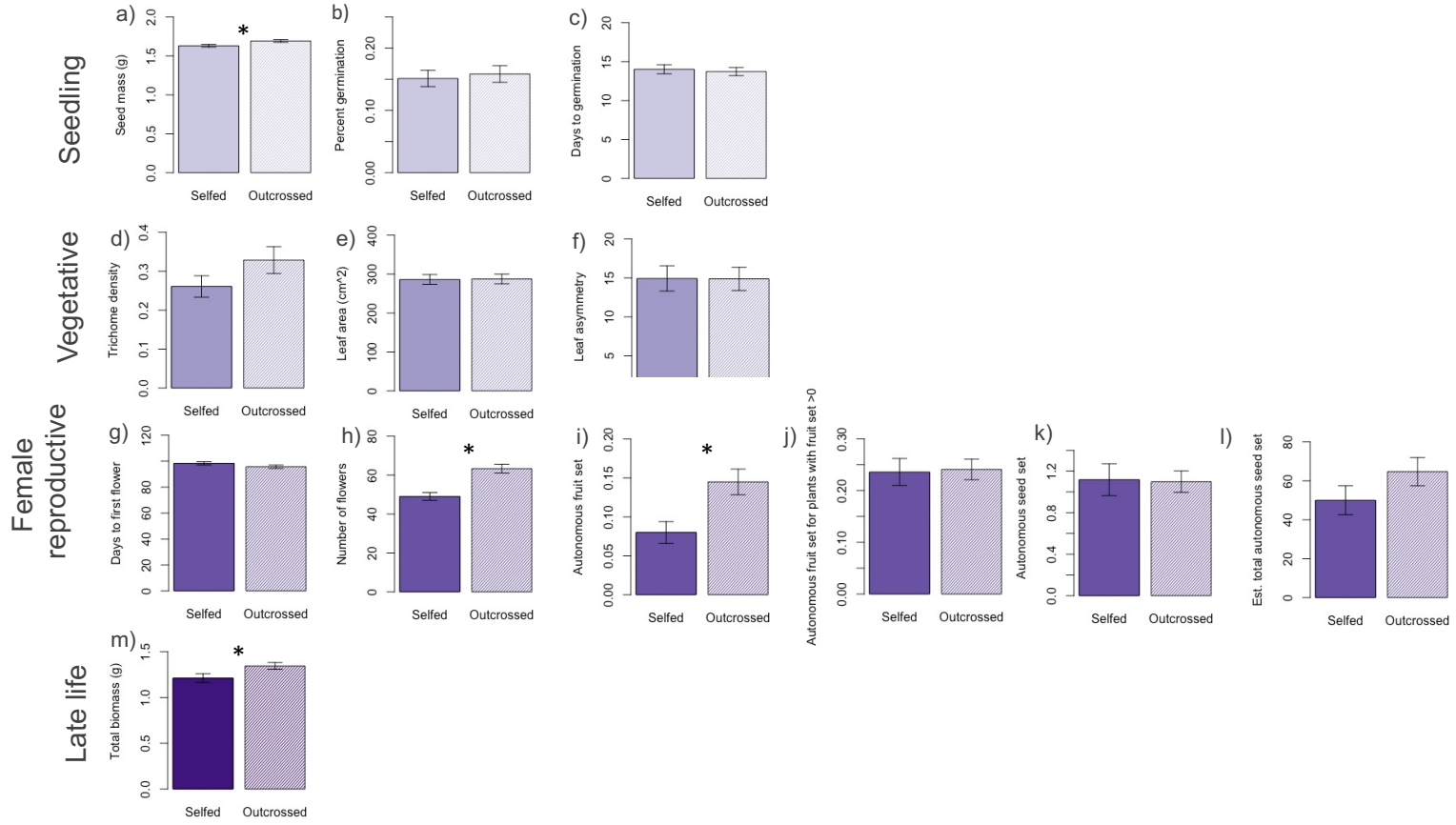

Figure 1.6 Effect of cross type (selfed or outcrossed) on *Collinsia corymbosa* seedling fitness components of a) initial seed mass, b) percent germination, and c) days to germination; vegetative fitness components d) trichome density, e) leaf area and f) leaf asymmetry; female reproductive fitness components g) days to first flower, h) number of flowers, i) autonomous fruit set (10 randomly sampled fruits), j) autonomous fruit set of plants that set at least one fruit out of the ten sampled, k) autonomous seed set of plants that set at least one fruit out of the ten sampled, l) total estimated autonomous seed production (number of flowers x seed set/fruit) for plants that made at least one fruit out of the ten sampled; and late life fitness component m) total biomass. Asterisks represent a significant difference between selfed and outcrossed individuals for a given fitness component ($p < 0.05$). Error bars represent mean ± SE.

CHAPTER II: SHIFTS IN SPRING PHENOLOGY IN RESPONSE TO SPRING TEMPERATURE IN EASTERN TENNESSEE

Abstract

Phenology is a key trait that can determine survival and reproduction, and thus is crucial to the ultimate fitness of organisms. Shifts in plant phenology in response to rising temperatures are one of the clearest indicators of the effects of recent climatic change. In North America, many species in the New England region are currently flowering earlier in the year relative to historical flowering dates in response to rising spring temperatures. However, comparatively fewer studies have examined phenological shifts in the Southeastern United States, a hotspot of biodiversity in North America. Further, the few existing studies of phenology in the Southeastern US do not account for ecoregional variation, a potentially important predictor in an area characterized by great variation in abiotic conditions over relatively small geographic areas throughout the lower Appalachians. Here, I use 10000+ digitized herbarium records along with associated location-specific temperature data to characterize how the phenologies of 14 common spring-flowering plant species in Eastern Tennessee, a hotspot of biodiversity in the Southeastern US, have responded to warming temperatures over the past century. My results illustrate that, at the community level, plants in different ecoregions differ in their sensitivity to temperature, with plants in the Ridge & Valley ecoregion flowering 2.7 days earlier per degree Celsius warming compared to 1.3 days for plants in the Blue Ridge ecoregion. Additionally, I found that the timing of early flowering at the community level is especially sensitive to spring temperature in both ecoregions. Finally, I show that the majority of spring-flowering species in both ecoregions demonstrated phenological sensitivity of flowering to spring temperature, indicating that in warmer years, the majority of species flowered earlier. Despite the demonstrated sensitivity of communities and species to spring temperature, I did not find support for significant shifts in community flowering within eastern Tennessee in recent decades, likely due to increases in mean annual temperature in the southeast being driven primarily by warming summer (rather than spring) temperatures. These results highlight the importance of including ecoregion as a predictor in phenological models to capture potential variation in temperature sensitivity among populations, and suggest that even small increases in temperature can have dramatic effects on species' and communities' phenologies in response to climate in the Southeast.

Introduction

One of the best demonstrated effects of recent climate change are shifts in phenology in response to warming temperatures (Parmesan 2006, Jones and Daehler 2017, Davis et al. 2015). Because the timing of developmental events in spring-flowering plant species is highly sensitive to environmental conditions, these species are excellent indicators of climate change (Polgar & Primack 2011) and have been the focus of several phenological studies (reviewed in Willis et al. 2017). In the understory of temperate forests, spring-flowering plants are adapted to a seasonal climate that includes cyclical fluctuations in temperature and light availability after canopy closure. As a consequence, phenological shifts in these species have the potential to affect plant fitness by altering carbon gain (Heberling et al. 2019), synchrony with pollinators (Kudo and Cooper 2019, Forrest 2015), and the length of the growing season (Meineke et al. 2021).

Long-term observational datasets are considered the "gold standard" of phenological data (Davis et al. 2015) and have been valuable resources for studying phenological trends. Studies utilizing such data sets have revealed patterns in phenological events that are easy to measure and have a history of long-term observation, such as earlier first flowering dates (Piao et al. 2018) and woody plant spring bud break and leaf-out (Panchen et al. 2014) in response to warming temperatures. However, such studies require long-term monitoring efforts and are often

limited in taxonomic or temporal scope (Wolkovich et al. 2014, Park et al. 2018). Herbarium collections have become increasingly popular tools for conducting phenological research over the past decade because they include location-specific historical data that allow for the exploration of long-term trends resulting from climate change (Jones and Daehler 2018). Herbarium specimens provide a snapshot of a species at a given date and place and hold a wealth of information including morphology, the presence of reproductive structures, herbivory, and other traits that cannot be captured with observational data alone. Further, flowering dates estimated from herbarium records are shown to reflect field observations, substantially increase sampling range, and alleviate sampling bias in climatic space when comparing historic and contemporary observational data across climatic conditions (Davis et al. 2015).

Much of the phenological research in North America utilizing herbarium specimens has taken place in cooler environments above 38º latitude, primarily in New England (Bertin 2015, Davis et al. 2015, Willis et al. 2010, Primack et al. 2004, Calinger et al. 2013, Gallinat et al. 2018). These studies show significant advances in flowering phenology over the past century in response to rising temperatures, with the strongest shifts typically occurring in spring flowering species. In contrast, long-term phenological trends in the southeastern US, a hotspot of biodiversity in North America, remain poorly understood. One study in West Virginia shows that the spring ephemerals *Erythronium americanum* and *Dentaria laciniata* have advanced their spring flowering by 0.91 days per decade over the past century (Petrauski et al. 2019); however, this study is limited in that it examined the phenological responses of only two species. Another study analyzing over 19,000 records of spring-, summer- and autumn-flowering species in South Carolina revealed that the earliest flowering species are the most sensitive to increasing March temperatures, but that there have been no long-term advances in spring flowering nor spring temperature over the past century (Park and Schwartz 2015). Thus, more work is needed to understand the importance of spring temperature in driving plant phenology in plant communities of the southeastern US.

One often-overlooked factor that may potentially influence variation in phenological response across broad geographical areas is the ecoregion from which observations are recorded. An ecoregion is an area of relative homogeneity in abiotic and biotic factors, including soils, vegetation, climate, geology, and physiography (Griffith et al. 1997). Ecoregions are defined by a hierarchical system that divides North America into increasingly narrow regions based on these shared features. By ignoring ecoregions, phenological studies could be missing potential information on how sensitivity varies within plant communities and individual species across relatively small geographic areas because climatic change is not geographically uniform.

In this study, I focus on two distinct ecoregions. The easternmost portion of Tennessee is comprised of two ecoregions, the Blue Ridge and the Ridge & Valley, that have different geological formations and experience different climates. The Ridge & Valley (elevation 152–1311 m; annual rainfall 1350 mm; average winter and summer temperatures 2 and 25 °C, respectively; Hart et al. 2008) is characterized by a series of parallel, even-crested ridges and valleys of primarily limestone. The climate is classified as mesothermal with short, mild winters and long, hot summers (Hart et al. 2008). In contrast, The Blue Ridge (elevation 600–1600 m; annual rainfall 1600 mm; average winter and summer temperatures 0.56 and 22 °C, respectively) is characterized by steep slopes and narrow valleys with and montane mesic forests dominated by oak–hickory communities (Surasinghe and Baldwin 2014).

In this study, I address the following questions:

1) Are spring-flowering species in eastern Tennessee presently flowering earlier than they did in the prior half of the 20th century?
2) How phenologically sensitive are spring-flowering species and communities to spring temperature in eastern Tennessee?
3) Does ecoregion explain variation in community- and species-level responses to spring temperature?

Methods

Study species

I chose to focus on spring-flowering plant species because they are known to be particularly sensitive to spring temperature when compared to later flowering species (Fitter and Fitter 2002, Park and Schwartz 2015) and are therefore excellent indicators of climate change (Polgar et al. 2011). To be included in this study, each spring-flowering species had to meet the following criteria: (i) there were at least 50 unique observations available with county-level locality information, (ii) date information included the year, month, and day of collection, (iii) reproductive structures were easily identifiable and distinguishable from one another, and (iv) the number of observations was >14 in both the Blue Ridge and Ridge & Valley Ecoregions. These criteria led me to select a group of fourteen species that span 11 plant families and flower across the range of the spring growing season (Table 2.1).

To obtain specimen data, I made use of digitized herbarium specimens drawn from three sources: 1) The Southeast Regional Network of Expertise and Collections (SERNEC), 2) The University of Tennessee Herbarium (TENN), and 3) The Great Smoky Mountains National Park Herbarium (GSMNP). SERNEC is a consortium of over 233 southeastern herbaria that offers an extensive digitized collection of specimens spanning over 200 years of observations for public download on-line (www.sernecportal.org). Because herbarium specimen locality information is organized by county, I selected 16 counties in eastern Tennessee from which to source herbarium specimens within the Blue Ridge and Ridge & Valley regions for this study (Fig. 2.1 in Appendix II). Counties were chosen such that the sample area within each ecoregion was approximately equal. To obtain specimen records from SERNEC, I submit a query for each species in the list of counties chosen. Because four of the seven counties selected at the easternmost border of Tennessee are nearly equally split between the Blue Ridge and Ridge & Valley ecoregions (Blount, Monroe, Sevier and Cocke; see Fig. 2.1), I obtained specific locality descriptions (e.g. mountain peaks, trail heads, cities, etc.) for specimens in those counties when available and used the GeoLocate web application (www.geo-locate.org) to determine from which region they were collected. If a specimen was located in one of those four counties and did not have specific locality information, it was removed from the data set.

Although studies including elevation as a covariate in phenology models often use the mean elevation of a county (e.g. Park et al. 2019), this metric would not be realistic in my study because elevations within counties split between the Blue Ridge and Ridge & Valley can differ by >1500m (e.g., city of Sevierville ≈ 275m, Clingman's Dome ≈ 2025m). Additionally, the majority of herbarium specimens in this study (especially ones from the earlier half of the 19th century) did not have locality information specific enough to determine a latitude and longitude from which to derive elevation data. Thus, I was not able to include elevation as a covariate in this study.

After removing duplicate, mis-labeled, non-reproductive, or damaged specimens that were unable to be scored, the final data set from SERNEC comprised 1249 specimens. The majority of observations in this study are sourced from SERNEC (85%). To increase my sample size, I also obtained a number of digitized specimens from the Great Smoky Mountains National Park Herbarium (4% of observations) and from a collection of previously undigitized specimens from the University of Tennessee Herbarium that were digitized for this project (15% of observations). In total, the final data set contained 1483 unique observations spanning 141 years.

Phenological data collection

The majority of phenological studies using digitized herbarium specimens use one of two approaches to categorizing reproductive phenology: (1) a binary approach, where a score of 1 or 0 indicates either the presence or absence of flowers on a specimen (e.g. Park and Schwartz 2015, Bertin 2015), or (2) a "relative" approach, where a specimen is considered flowering if a given proportion of flowers are open (typically 50% but up to 75%; e.g. Davis et al., 2015, Primack et al. 2004, Park et al. 2019). While these approaches are sufficient at capturing general phenological trends across broad spatial scales, finer-scale scoring methods that assign a phenophase based on the relative number of buds, flowers, and fruits present on a specimen enable more precise estimates of phenological trends (Pearson 2018). Thus, I defined five "scores" that categorized specimens into phenophases based on the relative proportion of reproductive structures present on a given specimen:

0: No reproductive structures present
1: Early flowering (less than 50% of reproductive structures are open flowers, fruits absent)
2: Peak flowering (greater than 50% of reproductive structures are open flowers, fruits absent or present)
3: Late flowering (less than 50% of reproductive structures are open flowers, fruits present)
4: Fruiting (only fruits present)

To assign phenophases to digitized specimen images, I trained ten undergraduate students working for the University of Tennessee Herbarium to recognize the reproductive structures of each individual species. Students determined the phenophase of individual specimens by counting the total number of reproductive structures (buds, flowers, and fruits) present on a specimen, then assigning a score 0-4 based on the relative number of each reproductive structure. In order to ensure consistency in scoring among students, I created reference sheets that included photos of reproductive structures on digitized herbarium specimens for each species. I then held a one-hour training session detailing our scoring methodology, and had each student score ten random specimens for which I had previously categorized the phenophase. If there were any discrepancies in our categorizations, the student and I met to discuss the proper scoring technique, and they re-scored ten different random specimens until we agreed on the proper categorization. A study by Willis et al. (2017) utilizing crowd sourcing (Amazon's Mechanical Turk service) to hire anonymous workers with no previous botanical experience to score the phenological stage of herbarium specimens showed that, with proper training, non-experts produce the same data quality as expert botanists. Thus, I am confident that the phenological

scores assigned by trained herbarium students reflect the true phenophases of the herbarium specimens in this study.

Climate data

While spring flowering phenology in temperate regions can be influenced by several abiotic factors, including spring temperature (Primack et al. 2004, Miller-Rushing and Primack 2008), snowmelt (Inouye 2008), and precipitation (Matthews and Mazer 2016), short-term records of flowering phenology in the southeastern United States imply that flowering phenology in this region is more closely related to temperature than to precipitation (Abu-Asab et al. 2001, Funderburk and Skeen 1976). Thus, I chose to use spring temperature (March-May) as the primary environmental predictor of spring phenology in this study.

Mean monthly temperatures for each county across the year range of the data set were obtained from NOAA's Global Historical Climatology Network (http:// ncdc.noaa.gov/ghcnm/). For counties split between the Ridge & Valley and Blue Ridge, I selected weather stations located within each ecoregion, and calculated mean spring temperatures separately by ecoregion. Because data were not available for all years in all counties, specimens that were collected in a year for which there were no climate data available were removed from the data set. In total, the final working data set contained 1077 unique observations.

Data analysis

To test for changes in flowering and fruiting phenophases over time, I regressed the year of observation of a given phenophase against the day of the year that it was observed. I then used a Welch's t-test, which is robust to unequal variances and sample sizes (Ruxton 2006), to determine if there was a difference between historical (pre-1970) and recent (1970 and later) flowering or fruiting dates for each species. The year 1970 was chosen as the dividing year for historical and recent observations because climate data suggest that global surface temperatures began to steadily increase around 1970 (Pachauri et al. 2014) and this year has been used as the cut-off between historical and recent phenophase observations in several herbarium-based phenology studies (e.g., Petrauski et al. 2019, Bertin 2015, Abu-Asab et al. 2001). Thus, using the year 1970 to divide historical and recent observations allows me to compare a time frame with a cooler climate and low inter-annual variation to a more recent time frame where average temperatures were increasing and higher on average (Bertin 2015).

I estimated phenological sensitivity to spring temperature (mean temperature over March, April and May) by regressing spring temperature in the county and ecoregion from which a specimen was collected against the Julian day of year of a phenophase. To characterize community-level phenological sensitivity, I binned data for all species together and used ecoregion and spring temperature as fixed effects and the year of observation and species as random effects. Although it would have been ideal to allow the random slope of each species to vary in response to spring temperature, I did not have enough statistical power to do so. Thus, to estimate variation in phenological sensitivity among species, I ran separate models to obtain slope estimates for the linear relationships between spring temperature and the Julian day of year of a phenophase for each individual species in each ecoregion.

Results

Shifts in phenology over time

Focal species showed varied phenological patterns and sensitivity to climate both at the community level and within species among the Ridge & Valley and Blue Ridge ecoregions. Mean flower dates of individual species calculated across all years of observation ranged from late March (e.g., *Hepatica acutiloba* DC. (Sharplobe Hepatica; Ranunculaceae), *Sanguinaria canadensis* L. (Bloodroot; Papaveraceae)) to early May (e.g., *Maianthemum racemosum* (L.) Link (False Solomon's Seal; Ruscaceae)*, Polygonatum biflorum* Walt. Ell. (True Solomon's Seal; Ruscaceae)). The earliest and latest flowering species showed the least amount of variation in mean flowering date among ecoregions, with April-blooming plants flowering earlier in the Ridge & Valley than the Blue Ridge on average (Fig. 2.2). Across the full temporal range of observations, plants in the Ridge & Valley flowered 6.03 days earlier on average than plants in the Blue Ridge region (Welch's-t test; $t_{1040.7} = 5.5$, $p < 0.001$). When divided into historical and recent time frames, plants in the Ridge & Valley flowered 2.16 days earlier post-1970 than they did pre-1970, although this difference was not significant (Welch's t- test; $t_{264.2} = 1.21$ $p = 0.25$, 95% CI: -1.53, 5.85). Plants in the Blue Ridge region showed little change in flowering phenology between historical and recent time frames (Fig. 2.3).

Phenological sensitivity to temperature

At the community level, both spring temperature and ecoregion had significant impacts on flowering phenology (Table 2.1). For every degree Celsius increase in temperature, spring flowering advanced 1.3 days on average across both ecoregions (95% CI: -2.13, -0.41) when analyzing all stages with flowers present together (stages 1-3). Results of models run separately for each ecoregion revealed that plants in the Ridge & Valley ecoregion showed a greater sensitivity to spring temperature, with flowering dates advancing by 2.7 days/ºC on average (95% CI: -4.13, -1.24) compared to 1. days/ºC (95% CI: -2.61, -0.02) in the Blue Ridge. When analyzing early and peak flowering phenophases separately in each region, early spring flowering was more sensitive to spring temperature than peak flowering in both ecoregions, with plants in the Blue Ridge showing greater sensitivity to spring temperature (-6.01 days/ºC , 95% CI: -9.11, -2.89) than those in the Ridge & Valley (-2.29 days/ºC, 95% CI: -4.80, -0.14). However, peak flowering was more sensitive to spring temperature in the Ridge & Valley (-1.73 days/ºC, 95% CI: - 3.17, -0.33) than in the Blue Ridge (-0.95 days/ºC, 95% CI: -2.40, 0.43; Fig. 2.3). Fruiting was not significantly impacted by spring temperature in either ecoregion.
At the species level, sensitivity to temperature varied within species among ecoregions as well. In the Ridge & Valley, the confidence intervals for the slope of the line relating spring temperature to flowering date was negative (indicating advances in flowering phenology) and did not overlap zero for three of fourteen species (*Geranium maculatum* L. (Wild Geranium; Geraniaceae)*, Dentaria diphylla* Michx. (Crinkleroot; Brassicaceae)*,* and *Thalictrum thalictroides* (L.) Eames & B. Boivin (Rue Anemone; Ranunculaceae). In the Blue Ridge, the confidence intervals for all species overlapped zero (Fig. 2.4; although see upper confidence limits for *G. maculatum and D. diphylla*).

Discussion

These results demonstrate that, although spring-flowering species in eastern Tennessee exhibit variation in the magnitudes of their phenological sensitivity to spring temperature (i.e., they flower earlier in warmer springs), mean flowering dates have not advanced over the past century for most species tested. These results are consistent with those of another study in the southeastern US that pooled data from >1700 species in South Carolina across the entire flowering season of spring to autumn (Park and Schwartz 2015). This pattern is likely explained by the fact that, although average annual temperature has been steadily increasing in the study region since the latter half of the 20th century (Fig. 2.6), spring temperature has not changed substantially in the southeastern US over the 20th century as a whole (Costanza et al. 2016). The most appreciable changes in temperature in this region have occurred during the summer (+2ºC since 1980, Costanza et al. 2016), a time of year that is not expected to affect spring flowering. In contrast, Petrauski et al. (2019) found that flowering for early spring species *Erythronium americanum* Ker-Gawl (Dogtooth Violet; Liliaceae) and *Dentaria laciniata* in West Virginia have advanced 0.91 days/decade since 1904. While I did not find evidence of significant shifts in flowering for any individual species, *Dentaria diphylla*, a species in the same genus as *D. laciniata*, was one of three species in the Ridge & Valley region whose mean flowering date had a significant negative response to spring temperature (i.e. flowered earlier in warmer springs). These results make sense in light of the fact that the two *Dentaria* species and *E. americanum* are among some of the earliest plants to flower in the spring and have relatively short flowering periods ("spring ephemerals"). Other studies have found that the earliest flowering species often have the strongest responses to spring temperature (Park and Schwartz 2015), and the results of this study show that early flowering is more sensitive to temperature than peak flowering or fruiting (Fig. 2.4).

Although I did not find evidence of significant advances in flowering date during the latter half of the 20th century, the result that spring-flowering species showed advances in flowering of around 2.5 days/ºC on average in response to warming spring temperatures supports those of other studies in the southeast (Park and Schwartz 2015, Petrauski et al. 2019) and elsewhere (Bertin 2015, Park et al. 2019, Primack et al. 2004, Panchen et al. 2012). Further, individual sensitivities were variable among species on average (Fig. 2.5), suggesting that future phenological responses to continued climatic change will be heterogeneous within communities. Although my ability to estimate the sensitivity of individual species was limited by my sample sizes, my results show that the flowering phenology of the spring-flowering community in both the Blue Ridge and Ridge & Valley were sensitive to spring temperature. However, individuals in the Ridge & Valley appear to show a trend toward greater sensitivity to spring temperature and flowering earlier in recent decades, which could reflect the fact that phenological sensitivity is "riskier" in the Blue Ridge due to greater inter-annual climate variability (Park et al. 2018). In the higher elevation Blue Ridge ecoregion, temperatures tend to be lower (Fig 2.6) and the growing season tends to be shorter, so being highly sensitive to temperature in this ecoregion may present greater risk of frost damage, mismatch with mutualists, and other negative fitness consequences than it does to individuals than in the lower-elevation Ridge & Valley.

Variation in phenological shifts and sensitivity to temperature can be further complicated by intraspecific variation. Indeed, I found that at the species level, individuals in the Ridge & Valley are showing stronger responses to spring temperature than those in the Blue Ridge. Although mean flowering date was not significantly different among historical and recent time periods for either region, species in the Ridge & Valley exhibit a trend toward earlier flowering

in recent decades whereas species in the Blue Ridge do not. Similarly, none of the species examined in the Blue Ridge had phenological sensitivities significantly different from zero, but three species in the Ridge & Valley showed significant advances in flowering with increasing spring temperature. The individual species-level estimates of phenological sensitivity presented in this study, while limited by sample size, are lesser in magnitude compared to those of species in northeastern spring-flowering plant communities (Willis et al. 2010, Primack et al. 2004). These results make sense given that spring warming in the southeast has not been as dramatic as that in the northeast; however, species still demonstrate phenological sensitivity to temperature, indicating the potential for species to be able to adapt to continued climate warming in the future.

Differential responses to climate change across relatively small geographic areas (i.e. within a single county located in two ecoregions) could result in changes to the structure and composition of plant communities, potentially altering gene flow and interactions between plant and animal species such as pollinators or seed dispersers (i.e., phenological mismatch; Miller-Rushing et al. 2010). For example, individuals with greater sensitivities to temperature have been found to be at greater risk of herbivory than less sensitive species (Meineke et al. 2021), and plants with greater sensitivity to snowmelt date than that of the emergence of their pollinators have been found to have reduced seed set in years where snowmelt occurred early (Kudo and Cooper 2019). Thus, the result that the same communities in the Ridge & Valley and Blue Ridge demonstrate different levels of sensitivity to spring temperature and show different magnitudes of phenological shifts in response to climate over the past century imply that the fitness consequences of continued climatic change on plant species will be heterogeneous not only within communities, but across the landscape as well.

Conclusion

The fitness consequences of high phenological sensitivity are complex. In the short term, dynamic phenological tracking of climate via high sensitivity to spring temperature could be potentially risky in cooler and less predictable environments because it puts species at risk of being exposed to freezing temperatures if they flower too early (Park et al. 2019). However, in the long-term, the ability of species' phenologies to track changes in temperature may be necessary for their persistence in light of continued climate warming. Willis et al. (2008) used historical records of the phenology and abundance of 473 spring wildflowers in Massachusetts to assess the relationship between phenological sensitivity to temperature and change in abundance. The authors found that the species that whose flowering times did not effectively track seasonal temperature have greatly declined in abundance over the past 100 years. Given that the majority of species in my study did not show strong sensitivity of flowering phenology to temperature (particularly in the Blue Ridge), species in eastern Tennessee may be at risk for decline or even local extirpation if they are not able to adjust their flowering times in response to predicted, long-term temperature change. This is especially concerning given that the Blue Ridge ecoregion is a center of biodiversity in the eastern United States and contains the greatest floristic diversity in the entire state (US Environmental Protection Agency 1997). My study highlights the importance of considering ecoregion as a predictor in phenological studies, because it allows researchers to account for differences in local abiotic conditions across even relatively small geographical areas that may explain inter- and intraspecific variation in phenological trends across time.

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APPENDIX II

Table 2.1 Number of specimens in each ecoregion and observation time span of focal species. RV = number of specimens in Ridge & Valley ecoregion, BR = number of specimens in Blue Ridge ecoregion.

Code	Species	Common name	Family	Date range	BR	RV
DENDIP	*Dentaria diphylla* Michx.	Crinkleroot	Brassicaceae	1923-2014	44	40
DENLAC	*Dentaria laciniata* Muhl. Ex Willd.	Cutleaf Toothwort	Brassicaceae	1933-2019	26	47
ERYAME	*Erythronium americanum* Ker-Gawl.	Dogtooth Violet	Liliaceae	1925-2010	19	37
GERMAC	*Geranium maculatum* L.	Wild Geranium	Geraniaceae	1934-2012	48	37
HEPACU	*Hepatica acutiloba* DC.	Sharplobe Hepatica	Ranunculaceae	1934-2019	36	14
MAIRAC	*Maianthemum racemosum* (L.) Link.	False Solomon's Seal	Ruscaceae	1931-2019	33	42
PODPEL	*Podophyllum peltatum* L.	Mayapple	Berberidaceae	1925-2015	32	36
POLBIF	*Polygonatum biflorum* (Walt.) Ell.	True Solomon's Seal	Ruscaceae	1925-2012	40	36
SANCAN	*Sanguinaria canadensis* L.	Bloodroot	Papaveraceae	1919-2019	39	30
THATHA	*Thalictrum thalictroides* (L.) Eames & B. Boivin	Rue Anemone	Ranunculaceae	1925-2003	41	36
TIACOR	*Tiarella cordifolia* L.	Allegheny Foamflower	Saxifragaceae	1925-2016	79	52
TRILUT	*Trillium luteum* (Muhl.) Harbison	Yellow Trillium	Melanthiaceae	1914-2015	40	40
UVUGRA	*Uvularia grandiflora* Sm.	Largeflower Bellwort	Colchicaceae	1925-2019	9	27
VIOSOR	*Viola sororia* Willd.	Common Blue Violet	Violaceae	1928-2017	79	38

Table 2.2 Results of mixed-effects models analyzing the relationship between spring temperature, ecoregion, and Julian day of flowering. Full models were analyzed using pooled data from both ecoregions, and Ridge & Valley and Blue Ridge models were analyzed using data from each respective ecoregion.

Phenological stage	**Ecoregion**	X^2	*df*	*p*
All flowering stages (flowers present)	*Both ecoregions*			
	Avg. spring temperature	8.4	1	**3.6 x 10-3**
	Ecoregion	7.4	1	**6.7 x 10-3**
	Avg. spring temperature * ecoregion			N.S.
	Ridge & Valley			
	Avg. spring temperature	14.3	1	**1.5 x 10-4**
	Blue Ridge			
	Avg. spring temperature	4.2	1	**0.04**
Early flowering (>50% of reproductive structures are open flowers, fruits absent)	*Both ecoregions*			
	Avg. spring temperature	16.0	1	**6.3 x 10-5**
	Ecoregion	0.1	1	0.74
	Avg. spring temperature * ecoregion	4.1	1	**0.04**
	Ridge & Valley			
	Avg. spring temperature	3.5	1	0.06
	Blue Ridge			
	Avg. spring temperature	14.9	1	**1.1 x 10-4**
Peak flowering (> 50% of reproductive structures are open flowers, fruits absent or present	*Both ecoregions*			
	Avg. spring temperature	2.7	1	0.1
	Ecoregion	9.1	1	**2.5 x 10-3**
	Avg. spring temperature * ecoregion			N.S.
	Ridge & Valley			
	Avg. spring temperature	6.0	1	**0.01**
	Blue Ridge			
	Avg. spring temperature	1.9	1	0.17

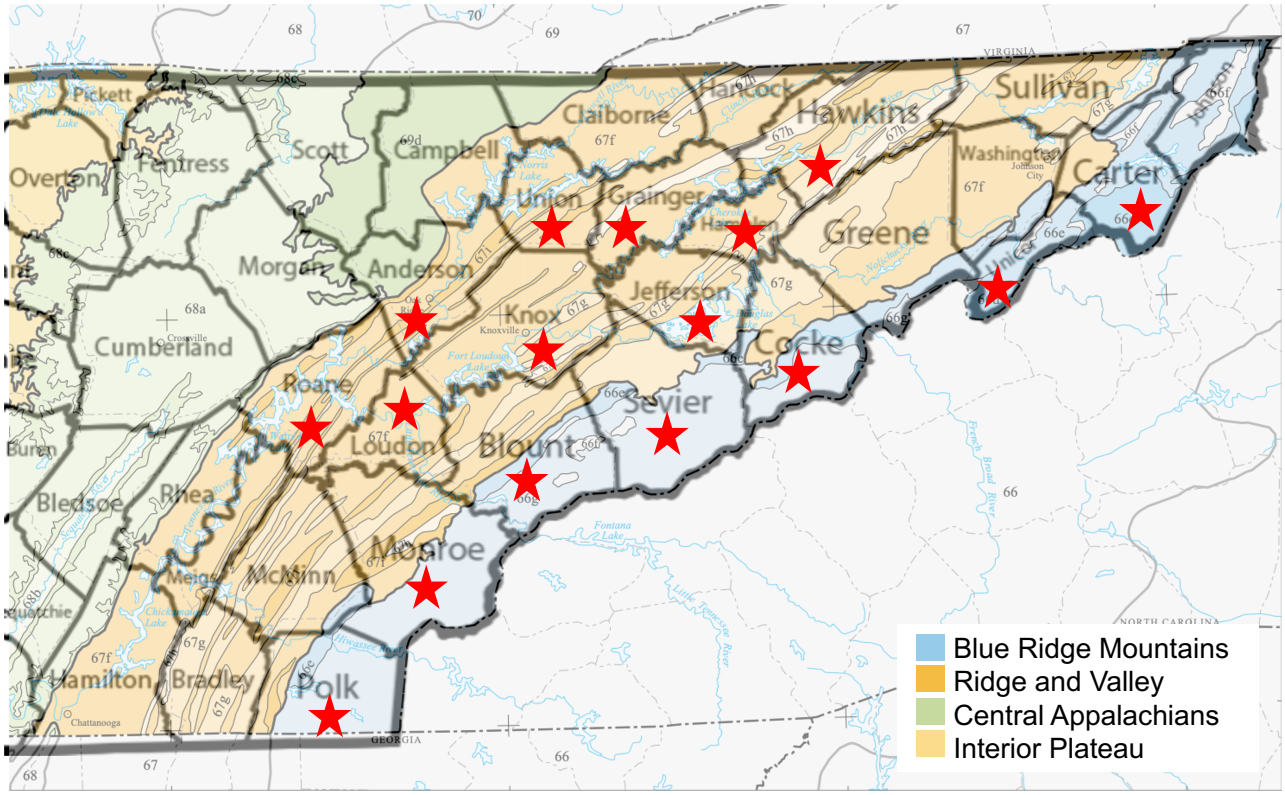

Figure 2.1 Map of ecoregions within eastern Tennessee counties. Red stars indicate counties chosen for inclusion in this study. Ecoregion map adapted from USGS Level IV TN Ecoregions Map (https://store.usgs.gov/assets/MOD/StoreFiles/Ecoregion/21632_tn_front.pdf).

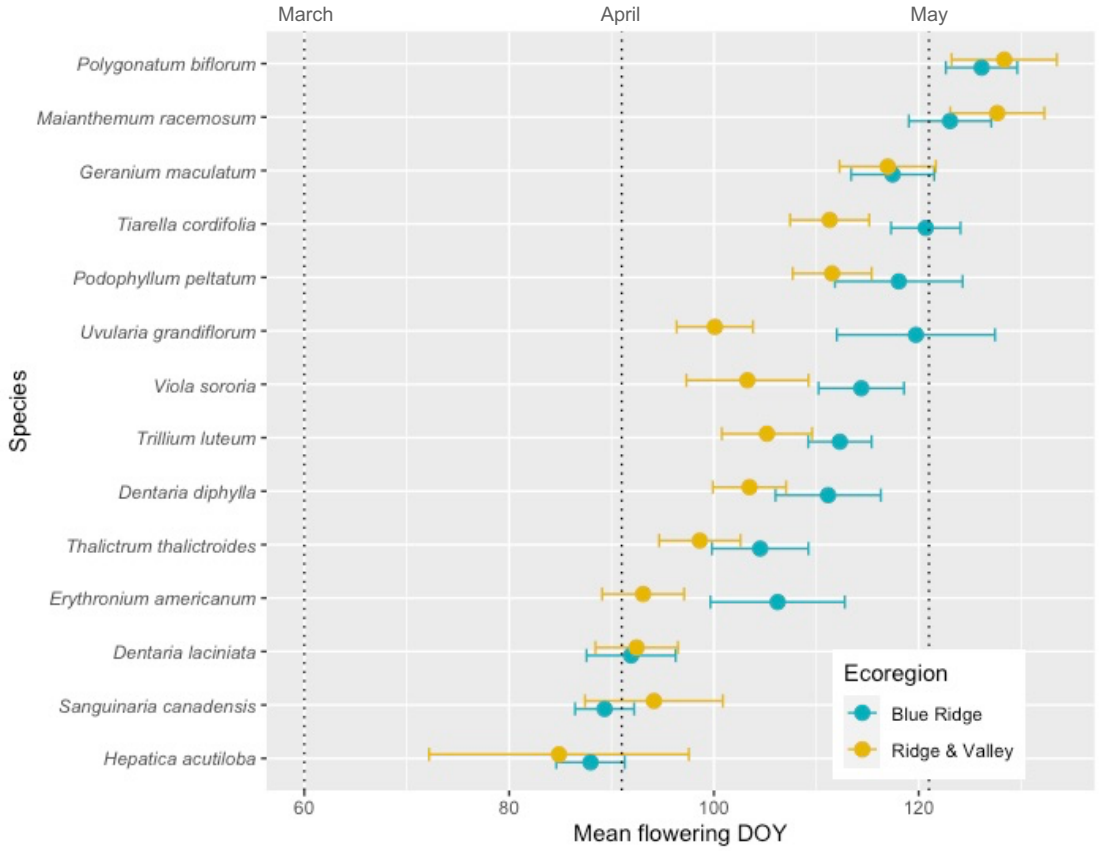

Figure 2.2 Mean flowering dates for focal species in the Blue Ridge and Ridge & Valley ecoregions. Flowering dates were calculated across all years of observation for each species. Points represent means and 95% confidence intervals.

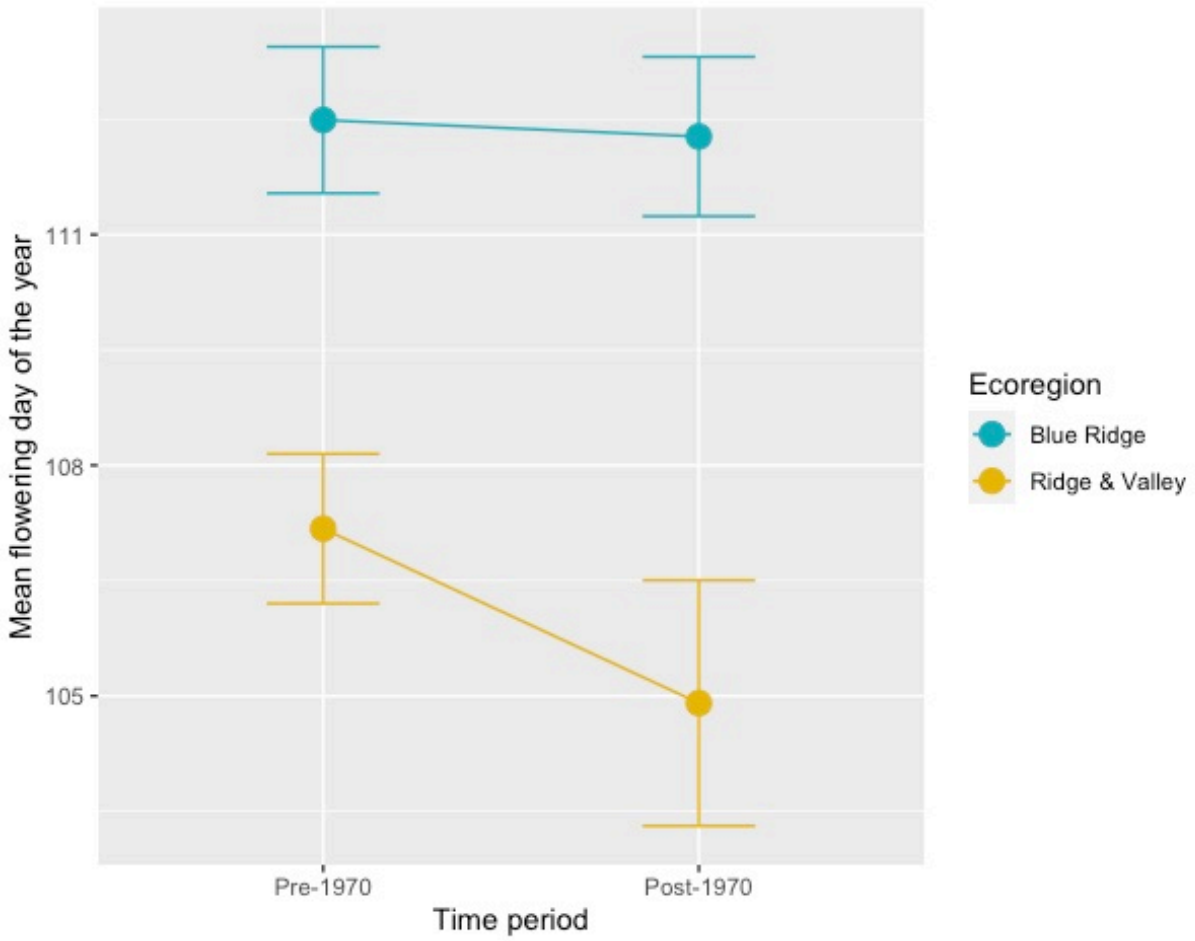

Figure 2.3 Changes in mean flowering times between historical and recent time periods in the Blue Ridge and Ridge & Valley ecoregions. Each point represents the mean and 95% confidence interval for flowering time in a given region and time period.

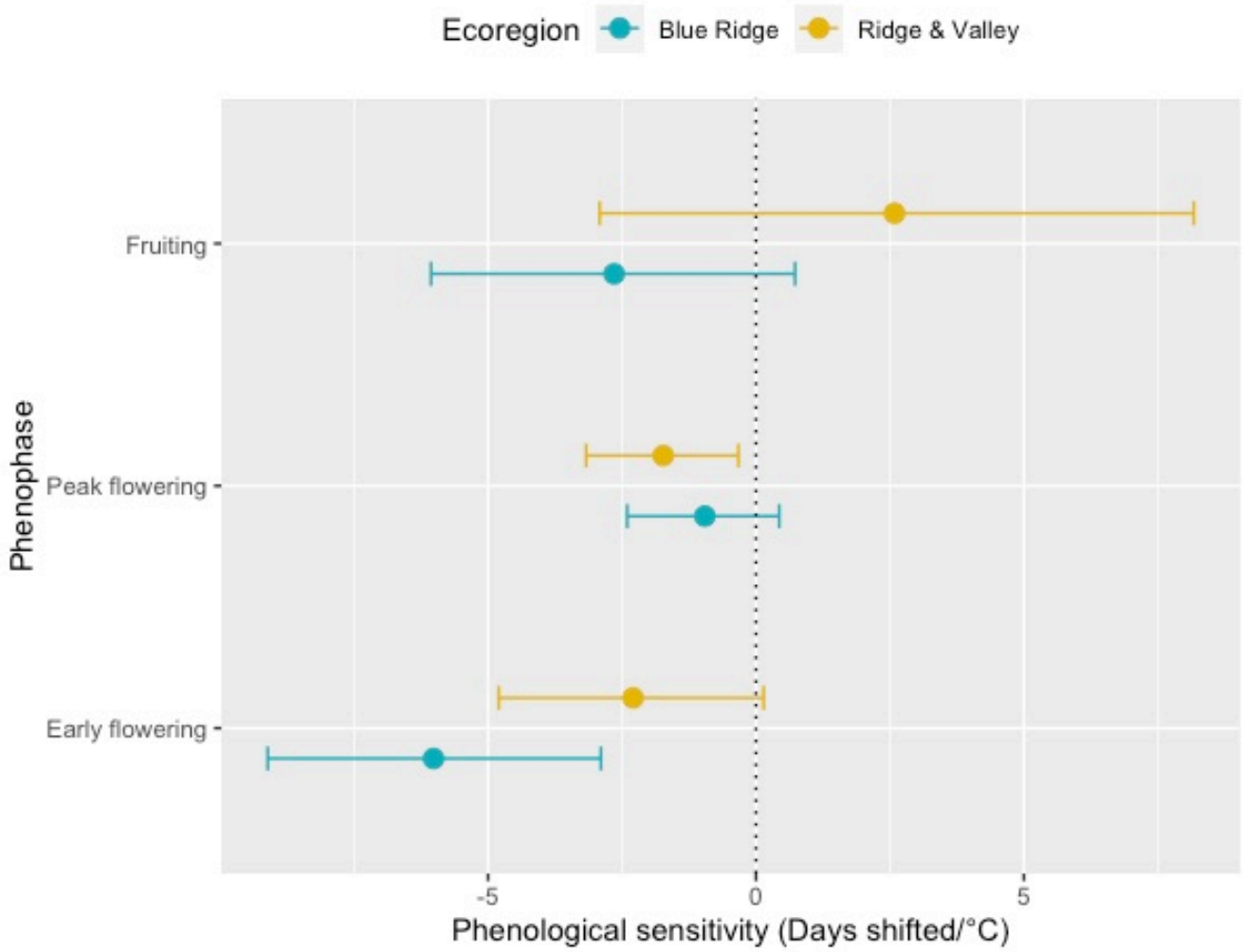

Figure 2.4 Variation in sensitivity of different phenophases to spring temperature in the Blue Ridge and Ridge & Valley ecoregions. Points represent means and 95% confidence intervals.

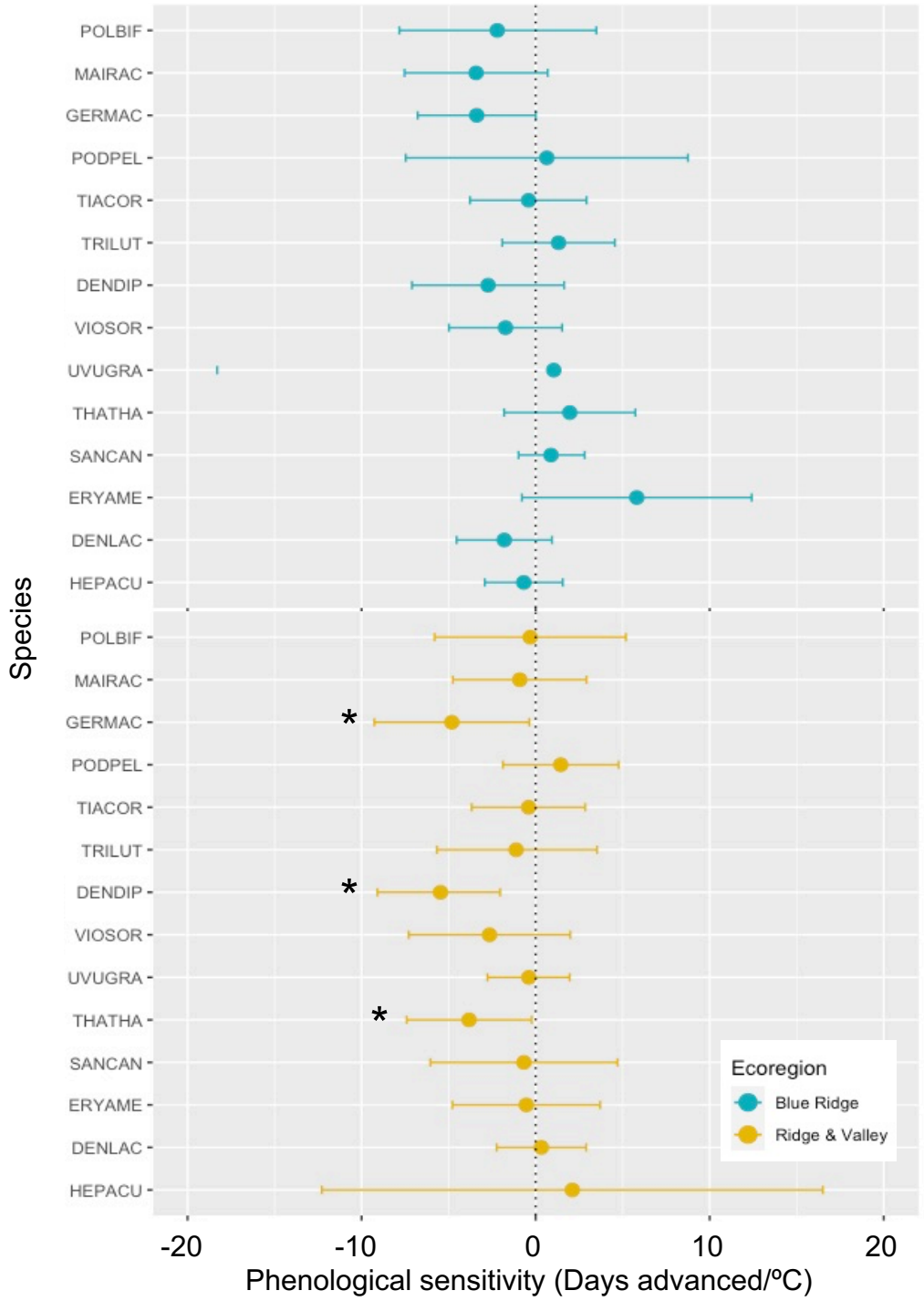

Figure 2.5 Variation in sensitivity of flowering to spring temperature (days advanced/ºC) within species among the Blue Ridge and Ridge & Valley ecoregions. Points represent means and 95% confidence intervals. Asterisks indicate species whose confidence intervals for their estimate of phenological sensitivity do not overlap zero. See Table 2.1 for species codes.

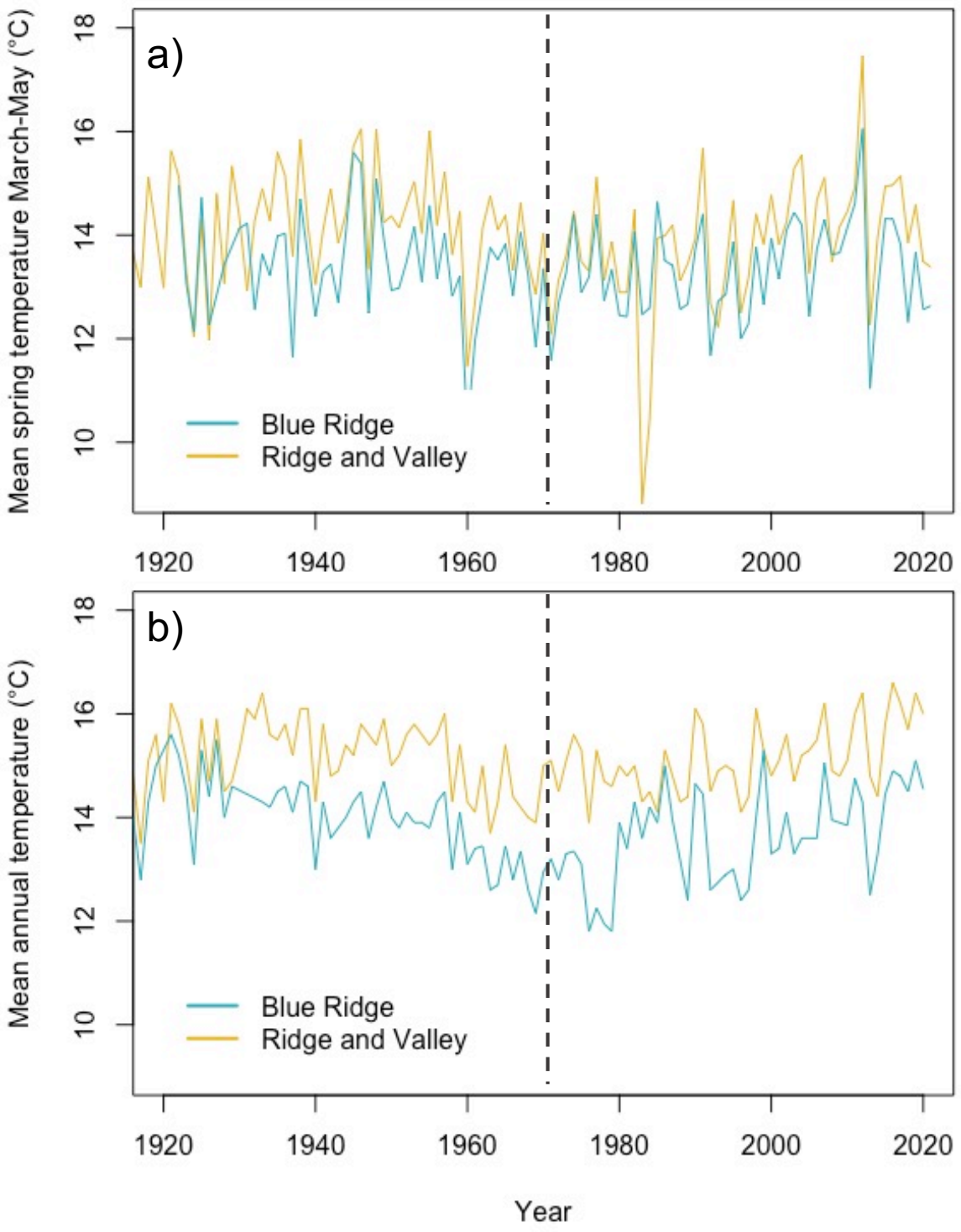

Figure 2.6 Changes in a) mean spring temperature and b) mean annual temperature in the Blue Ridge and Ridge & Valley Ecoregions over the past century. Dotted lines indicate the division between historical and recent time periods (year 1970) used in analyses of phenological shifts over the past century.

VITA

Originally from Cleveland, Ohio, Alex attended Case Western Reserve University where she received a Bachelor of Science degree in Biology. During her time at Case Western, Alex studied the impacts of invasive *Lumbricus terrestris* earthworms and invasive plant *Alliaria petiolata* (Garlic Mustard) on the native spring-flowering species *Podophyllum peltatum* (Mayapple). In her third year at Case Western, Alex was awarded a Research Experience for Undergraduates (REU) grant from the National Science Foundation to conduct research on the evolutionary dynamics of an *Ipomopsis* spp. hybrid zone at the Rocky Mountain Biological Laboratory in Gothic, CO. After developing her research interests in plant evolutionary ecology during her time as an REU student and during her time working with plant populations in Northeast Ohio, Alex chose to pursue graduate school. She attended the University of Tennessee, Knoxville to pursue a Master of Science degree in Ecology and Evolutionary Biology. Her research interests include understanding the impacts of anthropogenic change on plant reproduction, fitness, and mating system evolution. After graduation, she will return to Northeast Ohio to pursue a career in science education. She is incredibly grateful for all the support from her partner, friends, and family as she begins the next chapter in her career.