INTRA- AND INTER-SPECIFIC VARIATION IN PLANT RESPONSES TO CLIMATE

CHANGE

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ABSTRACT

Plants are sensitive to changing climates and are vulnerable to environmental conditions. In many regions, climate change is shifting temperatures and precipitation patterns, both of which affect various traits in living organisms, including those linked to survival and reproduction. Understanding how plants respond to climate change is increasingly important for conservation efforts. We addressed sources of trait variation and the responses utilized to cope with changing conditions within and among plant species in three different studies. We found evidence of local adaptation in temperature tolerance presumably due to divergent selection across geographic distance in *Solanum carolinense*. In the same species, we found phenotypic plasticity in reproductive traits when exposed to heat. Lastly, we determined that flowering phenology is driven by temperature in tallgrass prairie herbs rather than winter precipitation. These studies provide examples of how plant species are vulnerable to changing temperatures but have the capacity to adapt or acclimate.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

CMS ....Cell Membrane Stability

HCMS ....Hot Cell Membrane Stability

CCMS ....Cold Cell Membrane Stability

CHPL ....Chlorophyll Content Ratio

HCHPL ....Hot Chlorophyll Content Ratio

CCHPL..Cold Chlorophyll Content Ratio

PS ....Photosynthetic Temperature Tolerance

HPS ....Hot Photosynthetic Temperature Tolerance

CPS ....Cold Photosynthetic Temperature Tolerance

PTGR ....Pollen Tube Growth Rate

Tmax ....Temperature Maximum for Variable of Interest

Topt..Temperature Optimum for Variable of Interest

Tmin...Temperature Minimum for Variable of Interest

PCA..Principal Component Analysis

Germ ....Pollen Germination

ROS..Reactive Oxygen Species

SI...Self Incompatibility System

Herm ....Hermaphroditic Flower

Stam ....Staminate Flower

SEM ....Structural Equation Modeling

ST...Spring Temperature

TSNOW ....Total Snow Fall

DOBG ....Date of First Bare Ground

FFD ....First Flowering Date

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INTRODUCTION

Global warming is changing local conditions at alarming rates. Temperature and precipitation regimes are shifting and leading to biotic changes in communities. Plant species are especially vulnerable due to their sessile nature. Plant populations are expected to respond to environmental change either through adaptation, with phenotypic plasticity in traits allowing acclimation, or shifting ranges to locations with more favorable conditions.

Generally, for a plant species to adapt, natural selection must act on traits with heritable variation in a population and favor phenotypes that increase fitness (Kawecki & Ebert, 2004). Selective pressures can be abiotic, such as temperature, water availability, and nutrient availability or biotic, such as competition between individuals for space, pollinators, and seed dispersers. While selection is often acting on vegetative traits in the diploid phase of the life cycle, selection can also operate during the haploid phase of the life cycle including during competition among pollen grains and among ovules for fertilization during sexual reproduction. Both selection in the diploid phase (sporophyte) and the haploid phase (gametophyte) can facilitate local adaptation (Beaudry et al., 2020). Genotypes that have the highest chance of survival and reproduction will pass on their alleles to the subsequent generations. The shifting of allele frequencies within a population represents adaptation.

While adaptation allows a population to persist by avoiding local extinction as conditions change, plasticity in the phenotype allows acclimation at the individual level. If a trait is plastic, then environmental conditions can shape the phenotype and the genome is not necessarily shifting over multiple generations. The degree of phenotypic plasticity, however; is genetically controlled and can evolve (Schlichting, 1986). Hypotheses have been proposed to describe patterns in the degree of phenotypic plasticity geographically (Janzen, 1967; Molina-Montenegro

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& Naya, 2012; Schlichting, 1986) and throughout a species range (Zettlemoyer & Peterson, 2021).

A third way a plant species can cope with climate change is to shift ranges to areas with tolerable conditions. Since conditions of a novel ecosystem typically do not exactly mirror the state of a species' native range, adaptation and phenotypic plasticity can facilitate range expansion. Phenotypic plasticity can provide the variability in phenotype for a species to become established in a novel ecosystem and local adaptation can tailor the species' phenotype to persist (Molina-Montenegro & Naya, 2012).

While adaptation, phenotypic plasticity, and range shifts are integrated with one another there are nuances of each that are worth exploring in the context of climate change. The objective of this thesis was to examine how plant populations could or are responding to shifts in temperature and precipitation regimes through local adaptation and phenotypic plasticity. In the first chapter, we compare temperature tolerance in *Solanum carolinense* from populations in Minnesota and Texas to determine if there is evidence of divergent selection and thus, local adaptation. Since selection occurs independently in the gametophytic and sporophytic life stages, we measured temperature tolerance in both to determine if there is a correlation between the two and if there are implications for rates of evolution.

The second chapter addresses the effect of long-term moderate heat on traits in sexual reproduction of *Solanum carolinense*. We identified reproductive traits that are plastic, characterized by phenotypes that differed between the control and long-term moderate heat treatments. We also explored the potential for gene x environment interactions by comparing the responses of plants from Texas and Minnesota to elevated heat.

In the third chapter, we used a historical data set and climate data to identify how changes in temperature and winter precipitation have influenced flowering phenology in 24 tallgrass prairie forbs since the 1940's. We used structural equation modeling with the variables of spring temperatures, snowfall, snowpack, first date when the ground is bare, and first date when a species was observed flowering. We found interspecific variation in responses to changes in climate conditions for flowering across the species included in this study. For most species, flowering date was strongly related to temperature.

From these three studies, we can conclude that temperature has a profound influence on plant populations. We provide evidence of local adaptation in temperature tolerance traits and phenotypic plasticity in reproductive traits including flowering phenology due to changes in thermal conditions. As the climate continues to change, temperatures are bound to influence plant populations. Based on the results we attained, we predict that populations will not adapt to temperature extremes, but rather adopt life history strategies of avoidance, by favoring individuals capable of timing growth and reproduction with acceptable environmental conditions. We also expect that phenotypic plasticity in reproductive traits will allow individuals to acclimate to changing conditions to an extent, but this may not be enough to maintain survival and recruitment levels necessary to persist. Thus, gene x environment interactions may be integral in broadening the potential responses to climate change by increasing plasticity of traits through selection favoring individuals with greater plasticity. Plasticity in flower timing using temperature cues may provide another method for plant species to avoid extreme temperatures.

CHAPTER 1: EVIDENCE OF LOCAL ADAPTATION IN TEMPERATURE TOLERANCE TRAITS OF THE GAMETOPHYTIC AND SPOROPHYTIC STAGES IN *SOLANUM CAROLINENSE* (HORSENETTLE)

Abstract

Climate change is rapidly altering local temperature regimes and in different ways across the landscape. To cope with these rapid changes, plants species must have the capacity to respond to changes in temperature stress or risk extinction. We compared temperature tolerance traits in *Solanum carolinense* populations from Texas and Minnesota to understand how a species adapts or acclimates to extreme temperature stress. We included traits in both the gametophytic and sporophytic stages to distinguish between these distinct phases of selection. We found that mechanisms in temperature tolerance differ between populations of the south that face extreme heat regularly in Texas and northern populations that do not, in both the sporophyte and gametophyte. Our results are consistent with local adaptation and divergence of thermotolerance traits between northern and southern populations. These findings suggest that populations have the potential to adapt to rising temperatures due to climate change in the future.

Introduction

Climate change is rapidly altering environmental conditions at the local level and in particular, temperature and precipitation regimes and the severity of weather events. How will plants, a mostly sessile taxonomic group, cope with these rapid changes? Given the rapid change in local conditions, there are three ways plants can respond while avoiding extinction; quickly adapt, tolerate changing conditions through plasticity in phenotype that allows acclimation to the new conditions, or shift ranges (Janzen 1967; Molina-Montenegro and Naya 2012; Schlichting

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1986). We conducted a study that focuses on the variation within populations and addresses the potential for the first two of these options in a widespread, weedy species.

The conditions across a species range are almost always heterogeneous and can have a variety of selective pressures that act on the populations differently. Divergent selection in two different locations can result in differing trait optima in separate populations, leading to local adaptation (Kawecki and Ebert 2004). How a species adapts or acclimates to separate locations provides a clue to how a species in one location might respond as global warming changes local conditions. Temperature is a variable that can determine species distributions and can vary greatly in both severity and consistency with geographic region. There have been many adaptations in different species that improve survival in extreme temperatures, but how do populations of the same species persist in different temperature regimes? To understand local adaptation to diverging temperature regimes, we must understand the biology of plants and how they are vulnerable to extreme temperatures.

Temperature can impact plant physiology and cell structure in a few ways. Temperature stress changes the fluidity of phospholipid bilayers. Heat increases fluidity and dissociation of membrane components from one another (Zhu et al. 2018), while cold decreases lipid adhesion and increases rigidity (Valitova et al. 2019). Both heat and cold stress results in cytoplasm leaking from the cell membrane. Plants that are more tolerant of temperature stress would have the capacity to maintain optimal cell membrane fluidity and reduce cytoplasm leakage. The incorporation of sterols in membranes can maintain fluidity and expand temperature range for plants (Dufourc 2008a, 2008b; Valitova et al. 2019). On the other hand, saturated fatty acids can be incorporated in the cell membrane to reduce fluidity and are often associated heat tolerance (Knight and Ackerly 2001; Zhu et al. 2018).

High temperatures also affect photosynthesis via the decreased affinity of Rubisco (enzyme responsible for carbon fixation in photosynthesis) to CO_2 and increase in its affinity to O_2 (Bauwe et al. 2010; Zhu et al. 2018). The fixation of O_2 produces compounds that are needless and requires photorespiration to recycle components necessary for photosynthesis, in the process, reducing the efficiency of photosynthesis (Bauwe et al. 2010). In extreme temperatures, hot and cold, proteins and enzymes can be damaged or rendered inactive. This can have an immense effect on photosynthesis because the protein complexes in photosystem II and the electron transport chain can unfold (Zhu et al. 2018). The degradation of integral proteins and enzymes can also lead to the production of reactive oxygen species (ROS) through the excess absorption of light energy and prolonged excitation of chlorophyll molecules (Mishra et al. 2019; Wahid 2007; Wahid et al. 2007). Chlorophyll excitation isn't exclusively in the thylakoid membrane, where the light reaction typically takes place. Temperature stress can damage thylakoid membranes resulting in a release of chlorophyll. Chlorophyll will continue to absorb light energy even when free from the membrane. Without a source to receive the light energy (normally photosystems in the thylakoid membrane), excess energy forms free radicals that are typically donated to oxygen molecules forming ROS, which are highly reactive and damaging to cellular components. Plants typically degrade free chlorophyll or transform chlorophyll into alternative configurations quickly, and as a result chlorophyll fluorescence decreases (Kariola et al. 2005). Plants that are capable of tolerating temperature stress have less chlorophyll degradation in the context of relatively high temperature. Oxidative stress due to ROS hinders physiological mechanisms such as photosynthesis, metabolism, and cellular structure directly or indirectly by reacting with metabolites or damaging macromolecules. Some of these cellular processes are not unique to diploid cells of the plants (sporophyte), but also occur in the haploid

cells such as pollen and ovules (gametophyte). Extreme temperatures can limit pollen production, tube growth rate, and viability (Gajanayake et al. 2011; Kakani et al. 2002; Singh et al. 2008).

There is variation in the sensitivity to temperature stress and thus adaptations do lead to populations that are less sensitive. For example, cell membrane stability can be maintained in high or low temperature stress with the incorporation of fatty acids (Zhu et al. 2018) or sterols (Dufourc 2008a, 2008b; Valitova et al. 2019). The production of heat shock proteins, a chaperone protein, also reduces temperature stress by preserving the shape of other proteins and enzymes required for normal function (Frank et al. 2009; Goswami et al. 2010; Knight and Ackerly 2001; Lin et al. 2018; Liu et al. 2016; Nurminsky et al. 2018; Rhoads et al. 2005). For these adaptations to occur, temperature must be a selective pressure that influences the survival or reproduction of the species. In angiosperms, selection can act independently in the two life stages, the sporophyte (diploid; full plants, vegetation) and the gametophyte (haploid; ovules, pollen). It has been shown that there is a substantial overlap in genes and gene expression between the two stages (Beaudry et al. 2020; Pedersen et al. 1987; Tanksley et al. 1981; Willing and Mascarenhas 1984). There is also evidence of a correlation between the gametophytic and sporophytic stages in temperature tolerance traits (Hedhly et al. 2005; Poudyal et al. 2019).

In this study, we compared plants from Minnesota and Texas for temperature tolerance to extreme hot and cold conditions. Since temperature-based selection in the two life stages has the potential for inter-generational adaptations (thermotolerant pollen yields progeny with thermotolerant leaves), we incorporated variables from both the sporophyte and gametophyte. Sporophytic tolerance was measured using leaf measurements such as net photosynthesis, chlorophyll content, and cell membrane stability. The gametophytic variables were pollen

germination (viability) and pollen tube growth rate. The first objective was to determine if local

thermal conditions have divergently selected for temperature tolerance traits to fit regional

climate regimes. We hypothesized that if the temperature regimes in the north and south have

resulted in divergent selection and local adaptation of temperature tolerance, then the plants in

the north would be more tolerant of cold stress and plants from the south would be more tolerant

of heat stress. The second objective was to determine if there is a correlation between

temperature tolerance in the gametophyte and sporophyte. If temperature stress is similar in both

stages and gene expression patterns in the gametophyte and sporophyte overlap, then there would

be a positive correlation for temperature tolerance in the two life stages.

Methods

Species Description

Solanum carolinense L. (Solanaceae), commonly known as horsenettle, is a weedy,

herbaceous perennial that originated in southeastern North America. *Solanum carolinense* is in

the Carolinense clade of the subgroup Leptostemonum characterized by abundant prickles and

spines on the calyx of the flowers (Wahlert et al. 2014). Since all other species in this clade are

neotropical, this species likely arose through dispersal to North America and independent

diversification. Recently, this species has been reported in states across the United States, along

both coasts, as far south as Texas and Florida and as far north as Minnesota and Idaho (Figure

1.1). *Solanum carolinense* reproduces both sexually and asexually. Asexually, this species

utilizes clonal recruitment by growth from rhizomes. Sexual reproduction in *Solanum*

carolinense is complex. First, *Solanum carolinense* is indeterminate and andromonoecious,

producing mostly hermaphroditic flowers with some staminate flowers (Connolly and Anderson

2003). Second, *Solanum carolinense* has a self-incompatibility system that reduces the

occurrence of self-fertilization through multiple alleles at the S-locus (Mena-Ali and Stephenson 2007; Mena-Ali et al. 2009). S-RNases are produced within the style of a flower in accordance with the S-allele and degrade RNA of pollen tubes with like S-alleles. However, the self-incompatibility system is plastic and degrades with flower age (Travers et al. 2004). This species is buzz-pollinated, meaning that a certain frequency of vibration must be applied to the anthers for pollen to release. The primary pollinators for this species are bumble bees. Once ovules are fertilized, a small, round, green to yellow tomato-like fruit develops on a truss and is dispersed by small mammals, such as skunks, and birds (Cipollini and Levey 1997).

Plant Collection

Solanum carolinense plants from three populations in Texas and two populations in Minnesota were collected between October 2019 and August 2020 (Figure 1.1). The three southern populations were from Collin County, Texas near McKinney (Oil Patch: 33.173465 N, -96.615402 W; Reserve: 33.159962 N, -96.619011 W; and Cemetery: 33.173672 N, -96.615096 W). At the time of collection, each population consisted of between 10 and 50 mature plants. The Reserve population was located approximately 1.5 km from the Oil Patch and Cemetery populations which were adjacent to each other (Figure 1.2). The two populations from the north were from Houston County, Minnesota and from here on will be referred to as plants from the northern region or Prairie Island (44.07959 N, -91.684545 W) and Frontenac (44.523056 N, -92.338611 W). These populations are separated by approximately 80 Km (Figure 1.3). In Colin County TX, the average monthly low temperature is 18°C (65°F) and the average monthly high is 43°C (111°F). In Houston County, MN, the average monthly low temperature is -14°C (7°F) and the average monthly high is 29°C (85°F).

Since *Solanum carolinense* reproduces asexually by growing individual plants from rhizome material, plants in close proximity may be genetically identical or ramets of the same genet. To avoid sampling two plants of the same genotype, plants with a minimum inter-plant distance of 1 meter were collected. Collections involved digging up and cutting rhizome of at least 10 cm in length and placing them in ziplock bags. Rhizomes were stored in a cooler with blue ice and shipped to Fargo, where the collections were stored in a 4°C refrigerator. The rhizomes were potted in one-gallon containers with a standard potting mix and grown throughout the summer of 2020. In October, all above ground matter was cut and the rhizomes were again stored in a 4°C refrigerator to induce a period of dormancy.
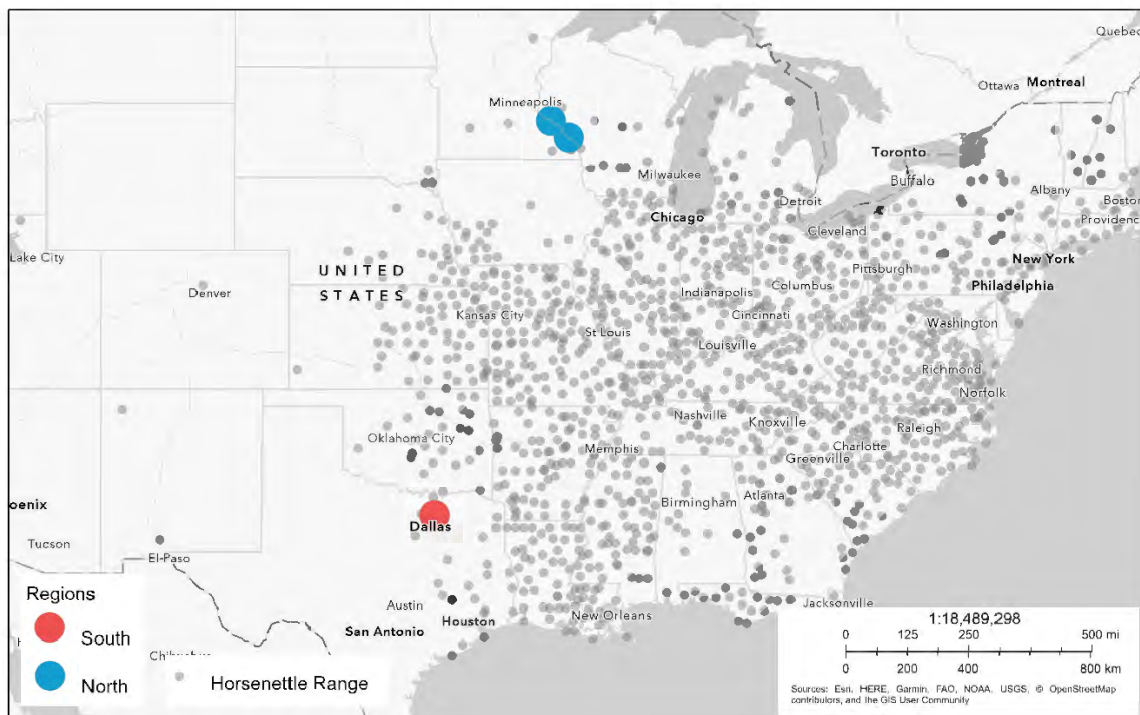
Figure 1.1. Map with collection site. Northern sites in blue and southern sites in red. Grey points indicate sites where *Solanum carolinense* was observed (EDDMapS 2022).

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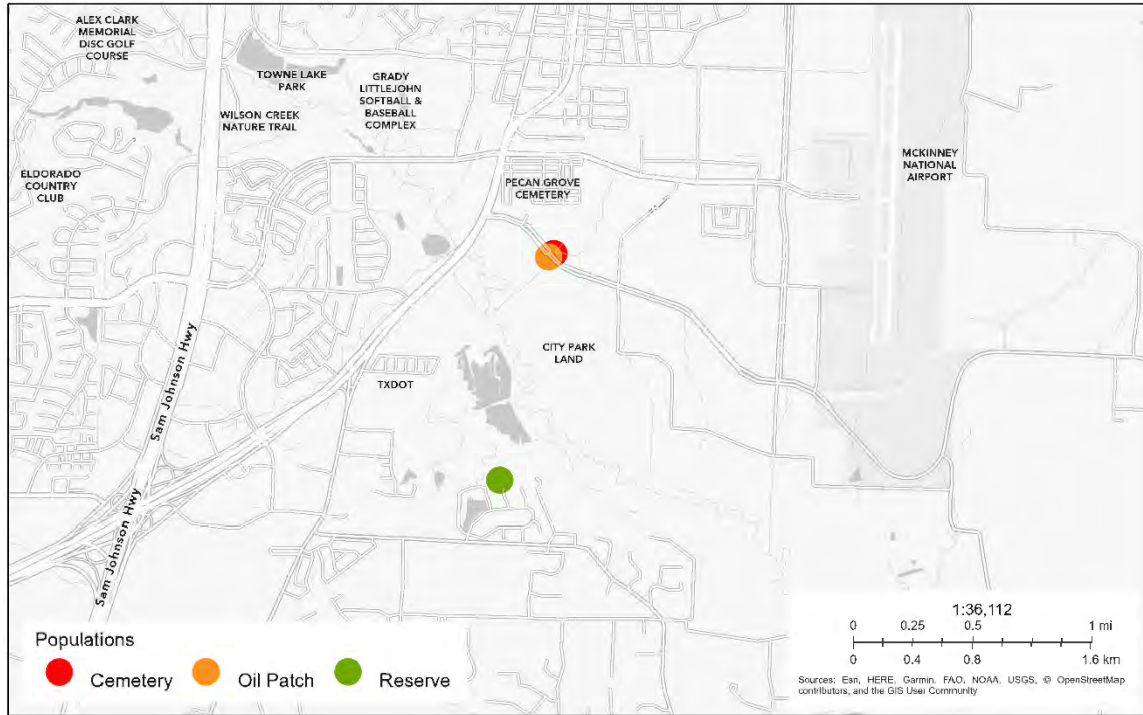
Figure 1.2. Populations in the southern region. Cemetery in red, Oil Patch in orange, and Reserve in green.

Figure 1.3. Populations in the northern region. Frontenac in blue and Prairie Island in purple.

Greenhouse Experiment

After the dormancy period (3 months), equal sections of rhizome (at least 2 cm for thick rhizomes and increased lengths for thinner rhizomes) were cut to grow ramets (genetically identical copies) in 3.8 cm diameter cone-shaped containers in the greenhouse. In total, four ramets (blocks A, B, C, and D) were grown from each genet (genetically independent), separated temporally. We started 10 or 12 ramets each week (sub-block 1-20), randomly selected from the 52 genets. Of the ramets planted each week, half were from the southern region and half were from the northern region. All ramets in block A were planted over five weeks prior to the planting of the ramets in block B and so on. The northern ramets were randomly assigned to either the left or right side of the respective southern pair within the tray that held the cone-shaped containers. The plants were fertilized regularly with 10-10-10 fertilizer and transplanted to larger, 4.5 L containers when they outgrew the small cone-shaped containers. Once the plants had leaves of a reasonable size, we began collecting sporophytic measurements from one sub-block each week. Gametophytic data were measured when plants began flowering.

Sporophytic Traits

Cell Membrane Stability

We used a handheld conductivity meter to measure cell membrane stability (CMS) of leaves after a temperature treatment following the protocol of Gajanayake et al. (2011) and Fang and To (2016). Two large, intact leaves were removed from the middle of a plant and rinsed with deionized water. One leaf was used for the high temperature treatment and the second leaf was used for the cold temperature treatment. Twenty leaf rounds were punched from each leaf with a hole puncher. Ten of the 20 leaf rounds were placed in a test tube for the temperature treatment (high or low) and 10 were placed in a test tube for a control treatment.

Prior to the high temperature treatment, 10 mL of deionized water was added to the control and temperature treatment test tubes. The high temperature treatment test tubes were placed in a water bath at 55°C for 20 minutes, while the control test tubes were left at room temperature. Both tubes were moved to room temperature for 10 minutes prior to the first conductivity measurement.

The low temperature treatment test tubes were placed at 10°C for 24 hours followed by 24 hours at 4°C to acclimate the leaf rounds to cooler temperatures. The treatment tubes were then placed at -18°C for 1 hour. The control treatment tubes remained at room temperature. After the temperature treatment, 10 mL of deionized water were added to all tubes for both the treatment and control. The tubes were placed at room temperature for 1 hour prior to the first conductivity measurement.

All tubes previously measured after treatments were subjected to a maximum damage treatment after the first conductivity measurements to control for absolute amounts of leaf material. All test tubes were placed in a water bath at 98°C for 1 hour and then left to cool at room temperature for 15 minutes before the second conductivity measurement.

The cell membrane stability value (CMS) used for data analysis was calculated as one minus the proportion of treatment final conductivity to treatment group maximum conductivity divided by one minus the proportion of control final conductivity to control group maximum conductivity. Thus, larger values correspond with higher tolerance to temperature stress.

$$CMS = \frac{1 - (Treatment_{value}/Treatment_{max})}{1 - (Control_{value}/Control_{max})}$$

Chlorophyll Content

Mishra et al. (2011) reported on the use of chlorophyll fluorescence as a measure of cold tolerance and Wahid et al. (2007) discussed the correlation between chlorophyll fluorescence and

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heat tolerance. We were interested in both cold and heat tolerance in this study. We used a chlorophyll meter (Opti-Sciences CCM-300) to measure chlorophyll content. The chlorophyll meter measures the fluorescence emitted at 735nm/700nm and uses a ratio based on experiments by Gittelson et al. (1998) to measure chlorophyll content in mg/m^2. Two intact leaves were removed from the middle of the plant. One leaf was used for the heat treatment and the other was used for the cold treatment. Each leaf was cut in half and one half was placed in the treatment temperature and the other half was placed in a control setting at room temperature. The chlorophyll content was measured for both halves before and after the temperature treatment.

The high temperature treatment was 60°C for 1 hour. The leaf halves in the cold treatment were subjected to 4°C for 1 hour followed by 1 hour in -18°C. The leaf halves were moved to room temperature for two hours prior to the second cold treatment measurement. Leaves in all treatments were kept in complete darkness.

To incorporate the control and treatment groups in one measurement, the chlorophyll content ratio (CHPL) was calculated as the compliment of the difference between the proportions of the final treatment chlorophyll content to the initial treatment chlorophyll content and final control chlorophyll content to initial control chlorophyll content. Thus, larger values correspond with higher temperature tolerance.

$$\text{CHPL} = 1 - \left(\frac{\text{Control}_{\text{final}}}{\text{Control}_{\text{initial}}} - \frac{\text{Treatment}_{\text{final}}}{\text{Treatment}_{\text{initial}}} \right)$$

Photosynthesis

We used a LI-6400 infrared gas analyzer with a red/blue light source to measure net photosynthetic rate (μmol CO_2m^{-2}s^{-1}) on leaves before and after the whole plant was exposed to the temperature treatment. The following settings were used for photosynthesis measurements:

flow rate 500 μmol s^{-1}, reference CO_2 420 μmol CO_2 mol^{-1}, reference H_2O 0 mmol H_2O mol^{-1},

ParIn_μml 400 μmol m^{-2} s^{-1}.

The high temperature treatment was 33°C and the low temperature treatment was 10°C.

Since full plants were placed in the temperature treatments, measurements were taken later in the

fall after data were collected for all other sporophytic and gametophytic traits. All four ramets, if

alive, for the 52 genets were subjected to both treatments with a rest period of one week between

the temperature treatments. Several plants died or lost leaves by the time net photosynthetic rate

was measured and thus were not included. Ramets A and C were subjected to the high

temperature treatment first and ramets B and D were subjected to the low temperature treatment

first. The proportion of the photosynthetic rate measurement after the treatment to before was

calculated as our measure of photosynthetic temperature tolerance (PS). Any value below zero

and above one was omitted prior to analysis.

$$PS = \frac{\text{Net Photosynthetic rate}_{final}}{\text{Net Photosynthetic rate}_{initial}}$$

Gametophytic Traits

We measured two pollen traits as estimates of male thermotolerance during the

gametophytic stage: 1) the propensity for pollen grains to germinate (pollen germination) and 2)

the growth rate of pollen tubes while exposed to a range of temperatures. Once a plant from the

north and from the south flowered (not necessarily the established pairings within the same sub-

block), we removed a mature flower from both plants. Since *Solanum carolinense* is buzz-

pollinated, a device crafted from a nose hair trimmer and a paper clip was used to mimic the

vibrations needed to release pollen from the anther. Pollen from each flower was thus dispersed

over five petri dishes containing 3% Bacto-Agar based growth medium (sucrose, $Ca(NO_3)_2$,

$MgSO_4$, KNO_3, H_3BO_3) following the protocol of Reddy and Kakani (2007). The dusted plates

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were each placed at one of the five temperature treatments (10°C, 20°C, 25°C, 30°C, 40°C) for 16 hours in a refrigerator (10°C), Conviron E7/2 environmental chamber (20°C), and three drying ovens (25°C, 30°C, 40°C). After the temperature treatments, each plate was covered with a thin layer of ethanol to halt further pollen tube growth and stored at 4°C until data collection could begin. Four pictures of each plate were taken using a microscope (Leica DM500 microscope, Leica ICC50 HD camera) and the LAS EZ 2.1.0 software. Pollen did not evenly cover petri dishes; therefore, pictures were taken in locations where pollen was visible. The petri dish was positioned so pollen visible to the naked eye (miniscule white spots) was under the objective. The petri dish was not repositioned once pollen grains were viewed magnified to avoid sampling bias when taking the pictures.

Pollen germination (Germ) was measured by counting the number of pollen grains that produced pollen tubes and the number of pollen grains that did not produce pollen tubes in a picture. All pollen grains in a picture were counted until at least 100 pollen grains were observed starting with the first picture taken. Pollen was considered germinated if it produced a tube that was at least half the diameter of the pollen grain. We used the percent of pollen grains with tubes out of the total number of pollen grains as our measure of pollen germination.

Pollen tube growth rate (PTGR) was determined by first measuring the 10 longest pollen tubes in each of the 4 pictures using the software ImageJ (Schneider et al. 2012). Pollen tubes were only included if they were completely visible in the picture. The actual length of each tube was calculated by calibrating each photo with a measurement of a stage micrometer. We calculated the mean of the 20 longest tubes out of the 40 measured per plate and estimated growth rate by dividing the mean length by the time allowed for growth (16 hours).

Data Analysis

All data were analyzed in R 4.1.2 (R Core Team 2020). In order to measure differences in sporophytic traits between regions and among genets, we fit linear mixed effects models using the lmer function from the *lmerTest* package (Kuznetsova et al. 2017). Region (north vs. south) was considered the fixed effect and block (A, B, C, D) and genet nested in population as random effects. We dropped the genet nested in population term for cell membrane stability and both random effects terms for hot net photosynthetic rate to avoid overfitting the model. Since the genet nested in population term was significant for some variables, we compared population and genets independently. Populations were compared using a linear mixed effects model (*lmerTest*; function lmer) with population as the fixed effect and block as the random effect. We used an analysis of variance model in the *stats* package (R Core Team 2020), to determine if there were differences between genets for each of the sporophytic variables. Since there was a significant block effect in some of the variables, we compared plants from the north and south within block using a paired t-test (*stats*; function t.test). To determine if variation within the northern and southern regions differed, we used the Bartlett's test of homogeneity of variance (*stats*; function bartlett.test).

For the gametophytic variables, we fit temperature performance curves to the multiple temperature measurements taken for each plant that flowered using the nls.multstart function in the *rTPC* package (Padfield and O'Sullivan 2021). Of the 25 temperature performance curves available in the *rTPC* package, the quadratic_2008 and the weibull_1995 models had the lowest AIC values. The weibull_1995 model was eliminated from our analyses because maximum values extracted by the weibull_1995 model were infinite for some of the northern plants. From the quadratic curves of each plant that flowered, we extracted three key values for both pollen

germination and pollen tube growth rate: the temperature minimum, temperature optimum, and temperature maximum. We then used the key values in an analysis of variance (*stats*; function aov) to determine if there were differences between region and among genets. One outlier was identified using the Grubbs' test for outliers, grubbs.test function in the *outliers* package (Komsta 2011), and subsequently dropped from the analysis.

We used correlation analysis (*stats*; function cor) using Pearson's method to determine if there were any correlations between sporophytic and gametophytic variables. We conducted correlation analysis for all plants together and then the northern and southern plants separately. The Holm-Bonferroni method (*stats*; function p.adjust) was used to adjust p-values to account for multiple correlations. To incorporate relationships between all the variables and examine amalgamated differences among regions and populations, we conducted principal component analysis (PCA) (*stats*; function prcomp). We first conducted PCA on all the sporophytic variables and all gametophytic variables separately and then all variables collectively. Photosynthetic rate was not included in the collective PCA because of limited sample size. We extracted the eigenvalues for the first three principal components for all three PCAs. The eigenvalues for each principal component were compared for the two regions using t-tests (*stats*; function t.test).

<div align="center">**Results**</div>

Sporophytic Variables

Cell Membrane Stability

Cell membrane stability (CMS) equals the ratio of a conductivity measurement after a temperature treatment to a conductivity measurement after a maximum damage treatment. An increased CMS ratio indicates higher tolerance of the temperature treatment (Gajanayake et al.

2011). When *Solanum carolinense* plants from the north were compared to the south, we found no significant difference in the hot treatment (HCMS), but there was a significant difference in the cold treatment (CCMS; Figure 1.4, Table 1.1). Southern plants had significantly higher CCMS values than northern plants. We found a significant difference among genotypes in the hot treatment, but not in the cold treatment (Figure 1.5, Table 1.1). For both the hot and cold treatments, there were significant differences between the populations. Population differences mostly followed the regional patterns in CCMS. For HCMS, one population (Oil Patch) from the southern region was less tolerant than all other populations (Appendix Figure A1, Table A1).

Because we could not grow all the experimental plants at the same time due to lack of space, we made the above comparisons among regions and genotypes in five different temporal blocks over the course of the spring and summer. To avoid confounding treatments with temporal effects, plants from different regions were paired with each other within blocks. When we tested for the presence of block effects, we found significant effects for both hot and cold CMS (Figure 1.6). Plants grown at different times in the greenhouse had different CMS ratios. We started growing the plants in the winter and early spring and outside temperatures gradually rose during that time (Appendix Figure A2). Acclimation to higher temperatures later in the year could account for the block differences observed. To remove block effects, we conducted paired t-tests of northern versus southern plants for each of the variables. When plants from the north and south were compared for HCMS, there was a significant difference between the regions (Figure 1.6) but only in the first block. In that block, northern plants had a higher HCMS than those from the south. For CCMS, there was a significant difference between regions for blocks B and C (Figure 1.6). In both cases, southern plants were more tolerant of the cold temperatures than northern plants.

Chlorophyll Content Stability

Chlorophyll content was measured before and after either a heat stress (HCHPL) or cold stress (CCHPL) and the calculated value that incorporates the two measurements was used as a proxy for temperature tolerance. As the CHPL increases, the individual sporophyte is more tolerant of the temperature treatment (Gajanayake et al. 2011). There was a significant difference between plants originating in the north and south for both the hot and cold treatments (Figure 1.4). Northern plants were more tolerant of both heat and cold than were southern plants regardless of block (Table 1.1). We found a significant difference among individual genotypes (Figure 1.5, Table 1.1) and populations (Appendix Figure A1, Table A1) for the cold treatment, but not for the hot treatment. The two regions also differed in variation for HCHPL. In the hot treatment, northern plants had significantly more variation than southern plants (Bartlett's test p-value = 1.68E-4; Figure 1.7).

Net Photosynthetic Rate

We used net photosynthetic rate after thermal stress as a physiological indicator of temperature tolerance. PS is the proportion of the net photosynthetic rate after the treatment (heat and cold) to the net photosynthetic rate before the treatment. Increased PS indicates higher temperature tolerance of either hot or cold thermal stress (Poudyal et al. 2019). For both the cold (CPS) and hot (HPS) treatments, there was no significant difference between north and south (Figure 1.4, Table 1.1). There were also no significant differences among blocks and genotypes for both the hot and cold treatments. There was a significant difference between populations for CPS (Appendix Figure A1, Table A1).

Table 1.1. Results from the mixed linear model for the difference in region (north vs south) and the one-way analysis of variance results for the difference between individual genets. Red font color highlights observed outcomes when the result was different from the expected pattern. Asterisk indicates analysis with one outlier removed determined using Grubbs' test for one outlier. Bolded values represent relationships that were statistically significant. Statistic values reported in the Appendix (Table A2), as well as results from a mixed model using only control values (Appendix Table A3).

| | Variable | | Region | | | Genet | |
		Expected	Observed	p-value	Observed	p-value
Sporophyte	Cell Membrane Stability (Heat)	S > N	-	0.06102	**Yes**	**0.013**
	Cell Membrane Stability (Cold)	N > S	**S > N**	**0.0117**	No	0.886
	Chlorophyll Content (Heat)	S > N	**N > S**	**0.0405**	No	0.38
	Chlorophyll Content (Cold)	N > S	N > S	**9.96E-11**	**Yes**	**1.05E-07**
	Photosynthetic Rate (Heat)	S > N	-	0.997	No	0.127
	Photosynthetic Rate (Cold)	N > S	-	0.77	No	0.883
Gametophyte	Pollen Germination (Tmax)	S > N	**N > S**	**0.00037**	**Yes**	**0.0251**
	Pollen Germination (Topt)	S > N	**N > S**	**0.000685**	**Yes**	**0.0351**
	Pollen Germination (Tmin)	S > N	-	0.331	**Yes**	***0.0135**
	Pollen Tube Growth Rate (Tmax)	S > N	-	0.568	No	0.418
	Pollen Tube Growth Rate (Topt)	S > N	-	0.77	No	0.608
	Pollen Tube Growth Rate (Tmin)	S > N	-	0.683	No	0.496
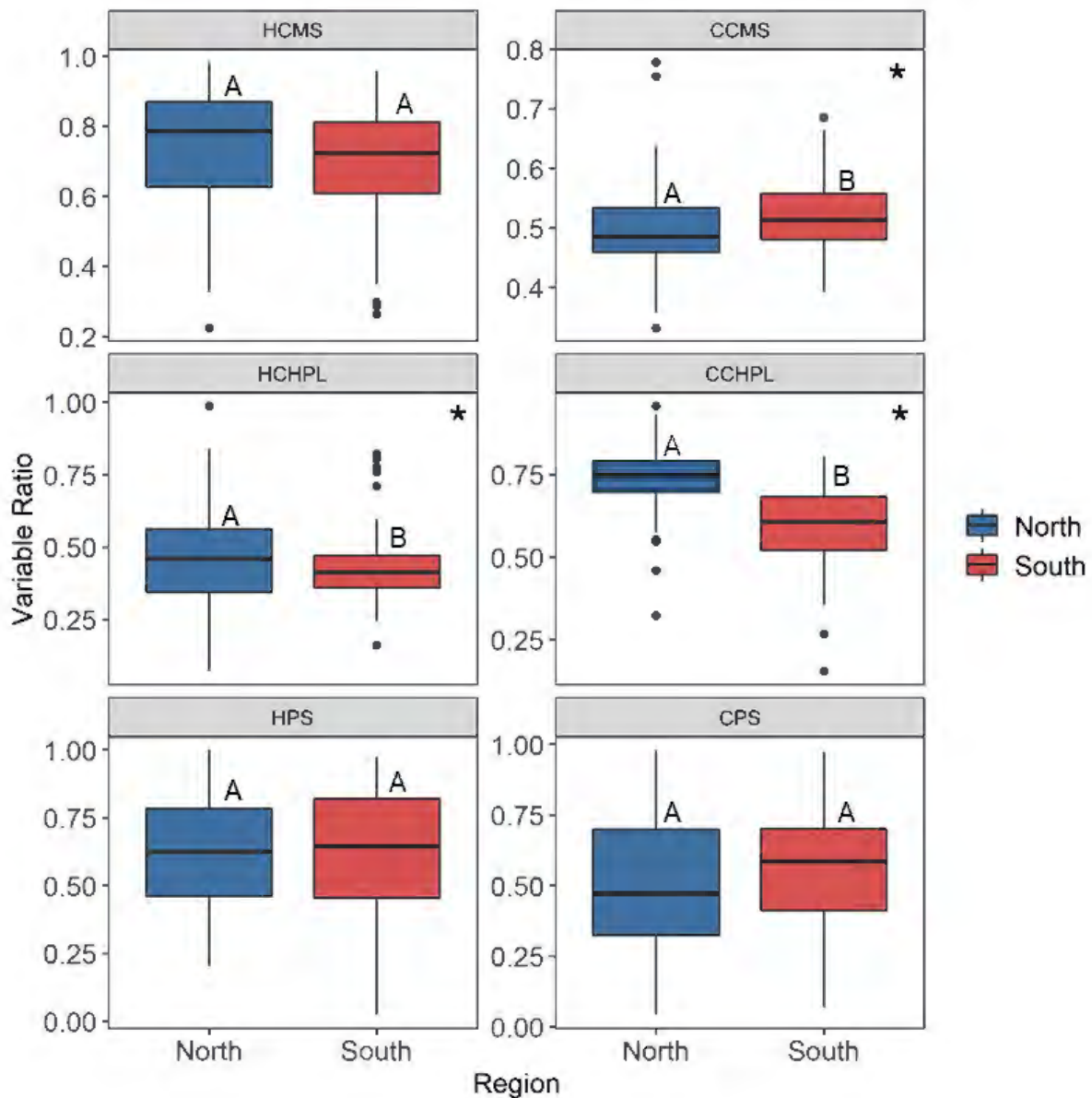
Figure 1.4. Regional differences for temperature tolerance traits including hot and cold cell membrane stability (HCMS, CCMS), hot and cold chlorophyll content stability (HCHPL, CCHPL), hot and cold net photosynthetic rate (HPS, CPS). Center line of boxplot is the median value for the region. The letters represent statistically significant differences between regions. Variables with significant differences denoted with asterisks: CCMS ($F_{1,50} = 7.792$, $p = 0.006$), HCHPL ($F_{1,51} = 4.334$, $p = 0.043$), and CCHPL ($F_{1,50} = 64.652$, $p = 1.6e-10$).
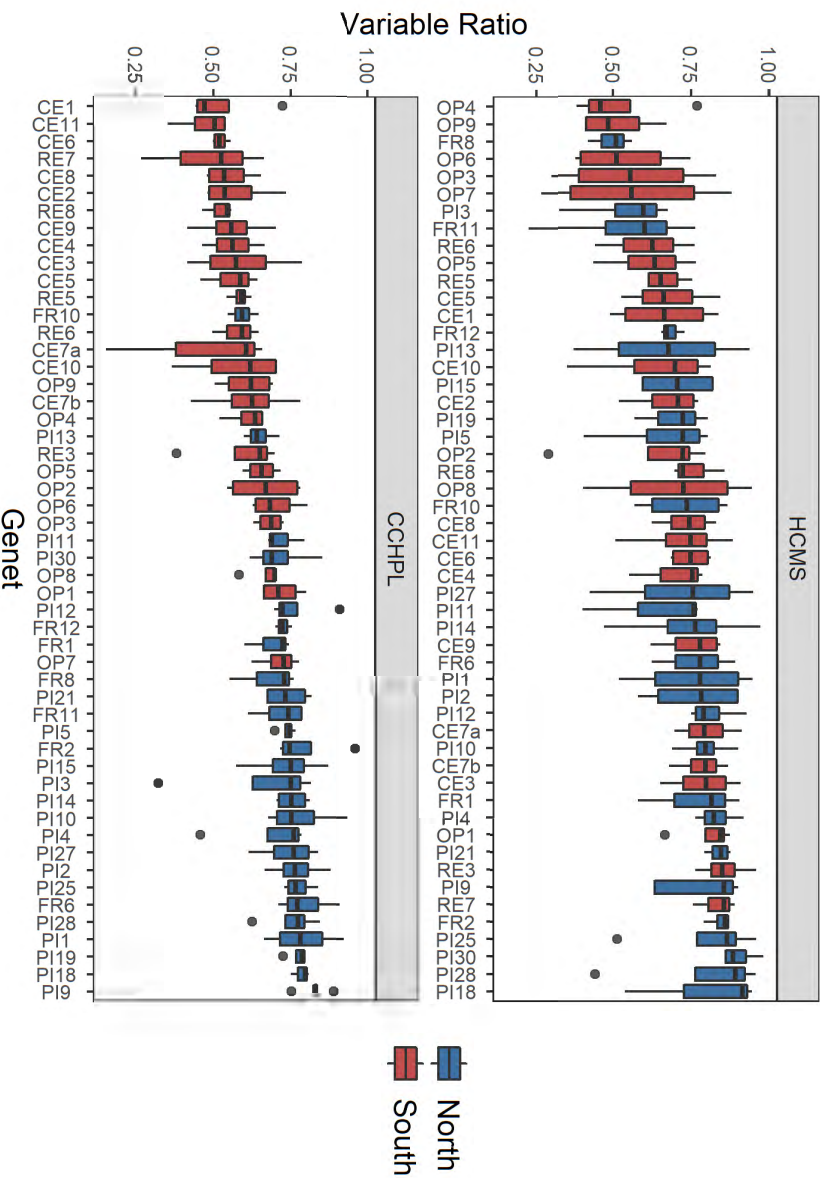
Figure 1.5. Genotype differences for temperature tolerance traits including hot cell membrane stability (HCMS) and cold chlorophyll content stability (CCHPL). Genets ordered by increasing ratio for each variable. Center line in boxplot is the median of the measurements taken for the ramets of one genet. There is a significant difference among the genets for HCMS (F = 1.5, p = 0.029) and CCHPL (F = 3.341, p = 6.1e-9). Plots of genet effect for other variables in appendix (Figure A3).
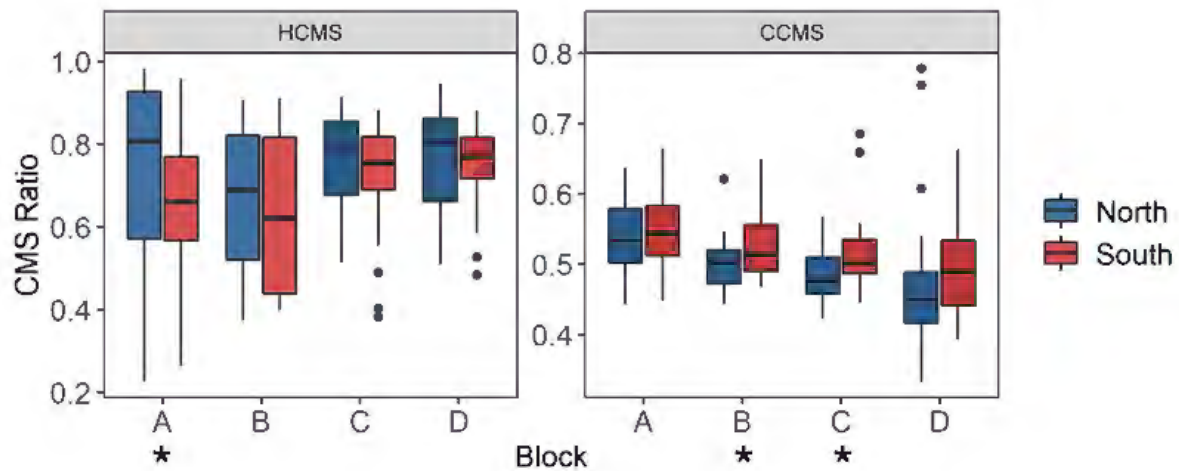
Figure 1.6. Cell membrane stability across temporally independent blocks and colored by region. The center line of the boxplot is the median of the measurements taken for each region within a ramet. There is a significant difference between blocks for hot cell membrane stability (HCMS, p = 0.0297) and cold cell membrane stability (CCMS, p = 7.30e-05). Asterisks indicate a significant difference between regions from a paired t-test of regions for each block independently. There was a significant difference between regions for HCMS block A (t = -2.910, p = 0.015), CMS block B (t = 2.190, p = 0.040), and CMS block C (t = 2.073, p = 0.049). Results from paired t-tests between blocks for each variable located in the appendix (Table A4).
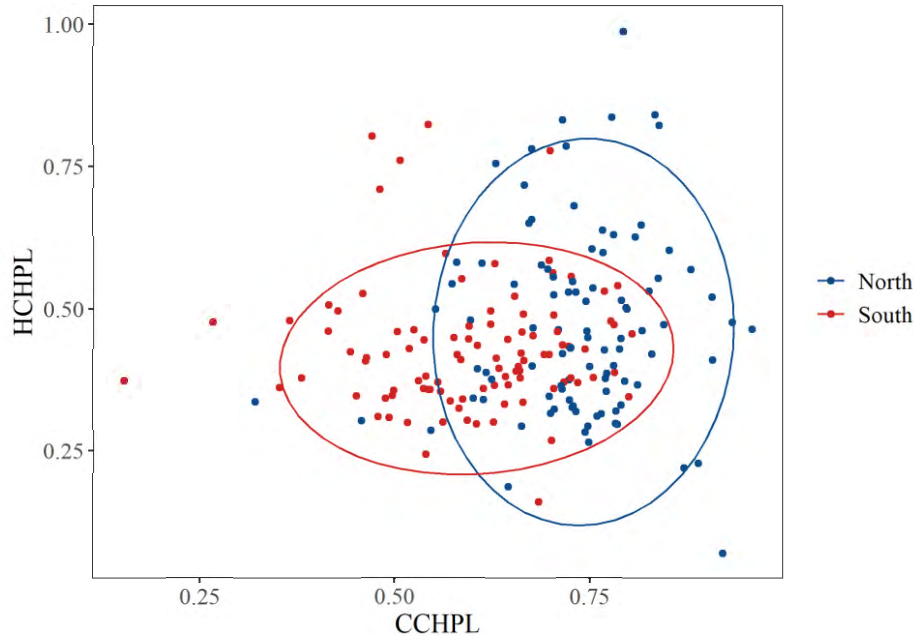
Figure 1.7. Hot chlorophyll content (HCHPL) vs cold chlorophyll content (CCHPL) for plants from the north and south. Ellipse indicating 95% confidence interval for multivariate T distribution. Results from Bartlett's test for heterogeneity of variance between regions for all variables located in the appendix (Table A5).

Gametophytic Traits

Pollen Germination

Of all genets included in this study, 20 from the north flowered and 10 from the south flowered. The number of ramets that flowered for each genet differed, so the total number of plants that flowered were 32 from the north and 29 from the south. We fit quadratic curves (Appendix Figure A4) to temperature performance profiles of each plant for pollen germination at five temperatures (Figure 1.8). From the quadratic fit, we calculated the minimum (Tmin), maximum (Tmax), and optimal (Topt) temperature of pollen germination for each individual. There was a significant difference between regions for Tmax and Topt (Figure 1.8, Figure 1.9). Plants from the north germinated more readily at high temperatures and had higher thermal optima than plants from the south. There was no significant difference between the two regions

for Tmin. The genets were significantly different from one another for Tmin, Tmax, and Topt (Figure 1.10, Appendix Figure A5). One outlier was identified using the Grubbs' test for outliers and subsequently dropped from the analysis.

Pollen Tube Growth Rate

The pollen tube growth rates for each individual were also fit with a quadratic curve to estimate the Tmin, Tmax, and Topt. There were no significant differences between plants from the north and south for any of the three variables (Appendix Figure A6). There were also no significant differences among genets (Appendix Figure A7, Figure A8).
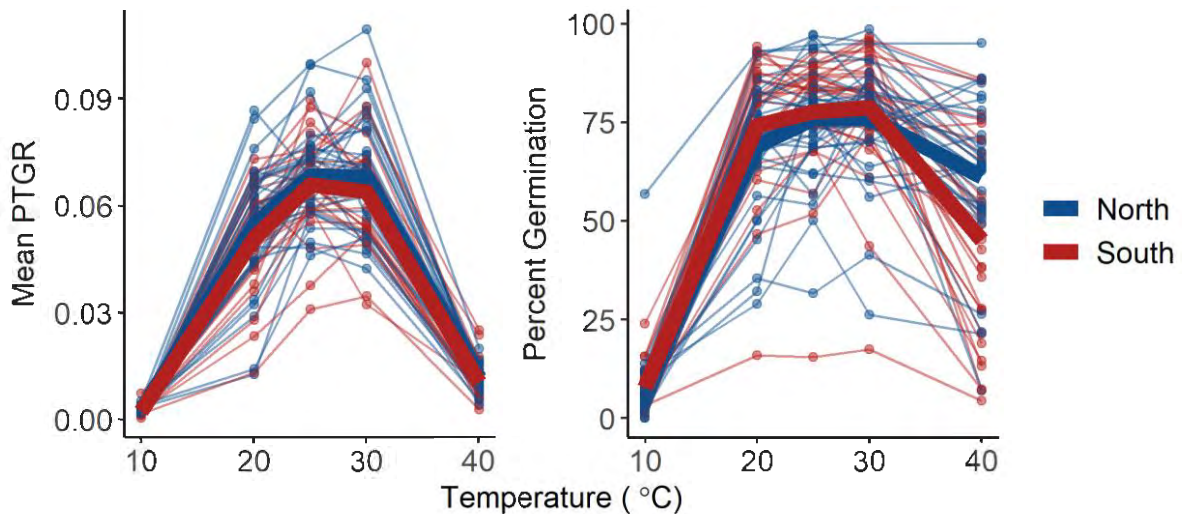
Figure 1.8. Percent germination and mean pollen tube growth rate (PTGR) for *Solanum carolinense* pollen grains from the north (blue) and south (red) across a temperature gradient (10°C, 20°C, 25°C, 30°C, 40°C). Thin lines and points represent each individual plant that flowered. Thick lines indicate the mean value for the region at each temperature.
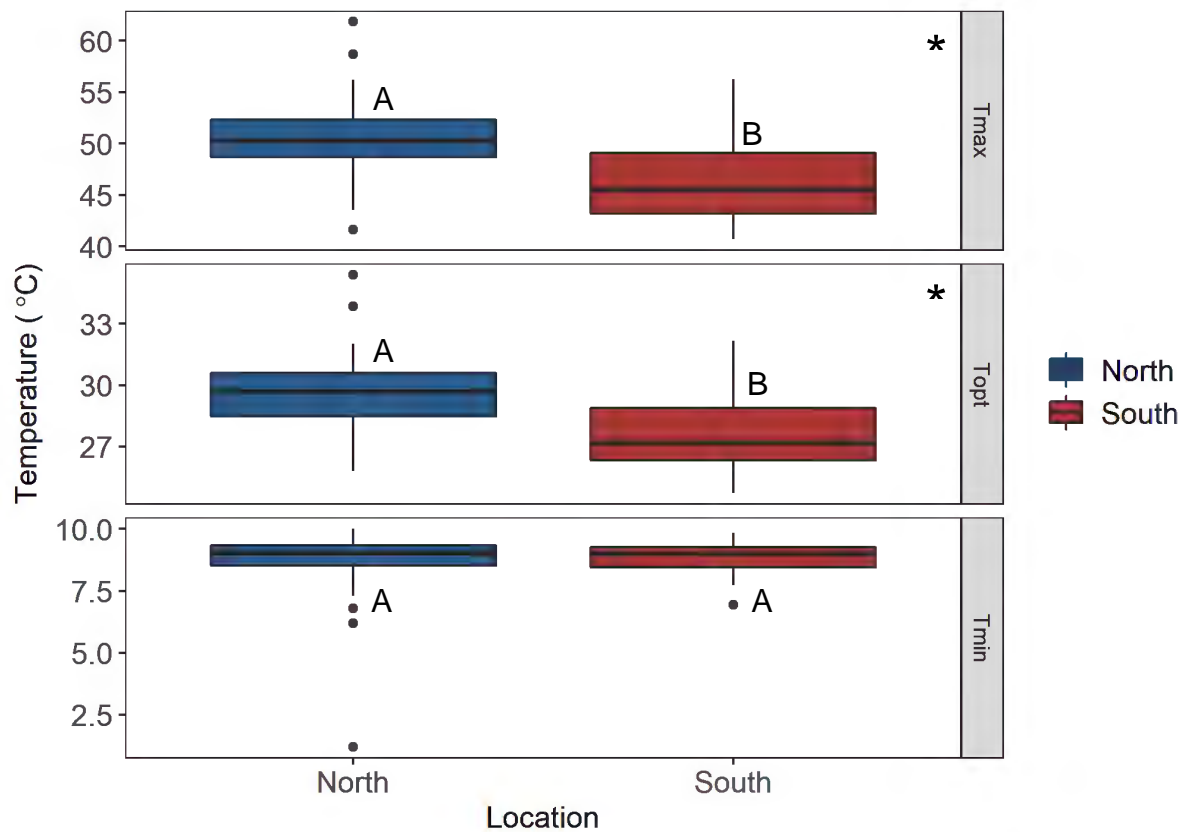
Figure 1.9. Estimates for the maximum (Tmax), optimal (Topt), and minimum (Tmin) germination temperature extracted from quadratic fits of the germination data for each individual. Asterisks and different letters indicate significant differences. There was a significant difference between regions for Tmax (F = 14.28, p = 3.7E-4) and Topt (F = 12.85, p = 6.85E-4).
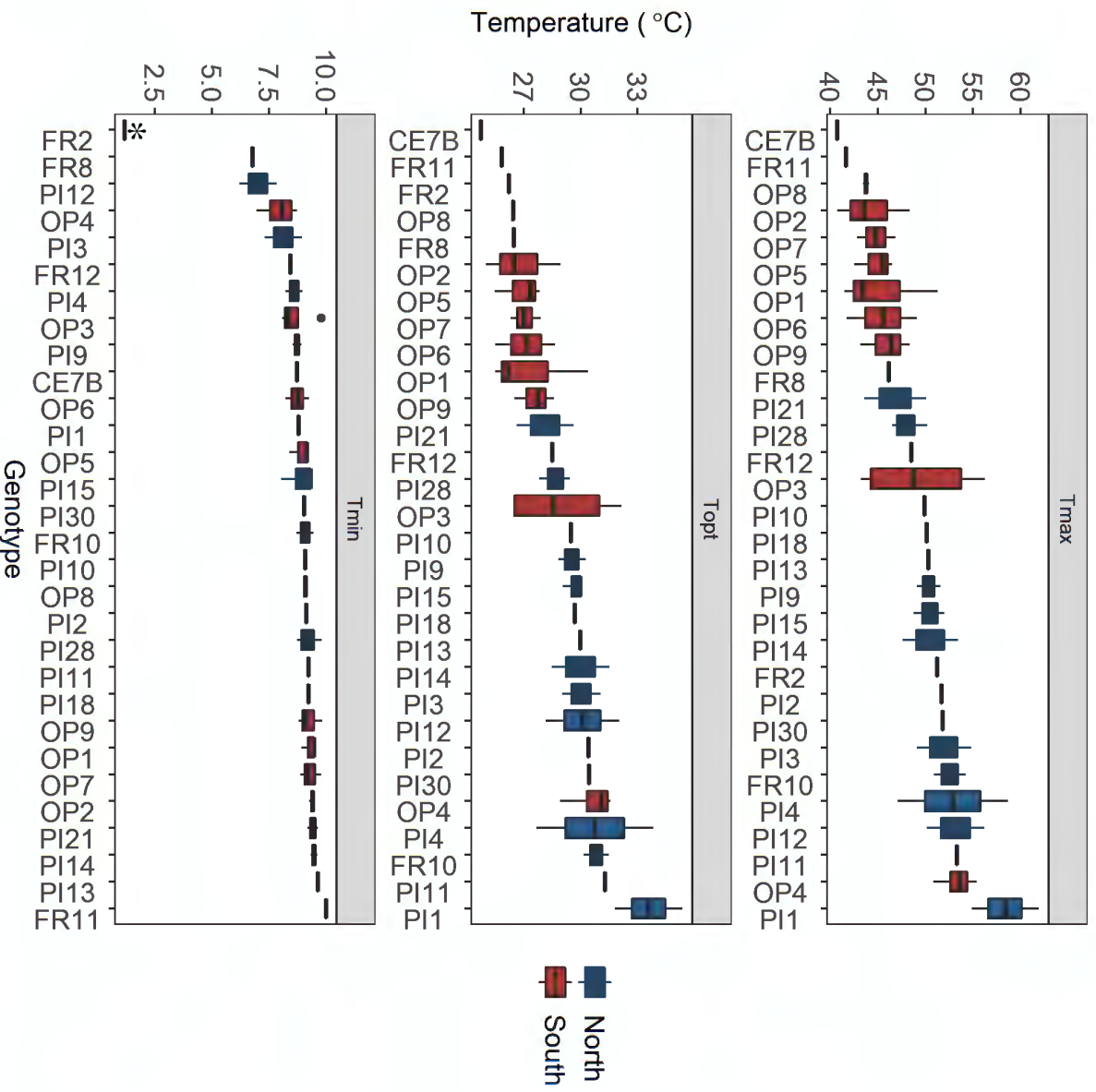
Figure 1.10. Boxplots of the maximum (Tmax), optimal (Topt), and minimum (Tmin) pollen germination temperatures by genet. There was a significant difference between the genets for Tmax (F = 2.064, p = 0.025), Topt (F = 1.952, p = 0.035), and Tmin (F = 2.284, p = 0.0135). Asterisk indicates the outlier removed for analysis.

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Correlations

We used correlation analysis to identify relationships between hot and cold tolerance for the sporophytic and gametophytic variables. Pearson's correlations were determined for all pairings of variables. When all plants were included, there were no significant correlations between the gametophytic and sporophytic variables (Figure 1.11, Appendix Table A6). However, there were two significant correlation coefficients between gametophytic variables. Maximum and minimum pollen tube growth rate were positively correlated (r = 0.46). Maximum pollen tube growth rate and maximum pollen germination were positively correlated (r = 0.3).
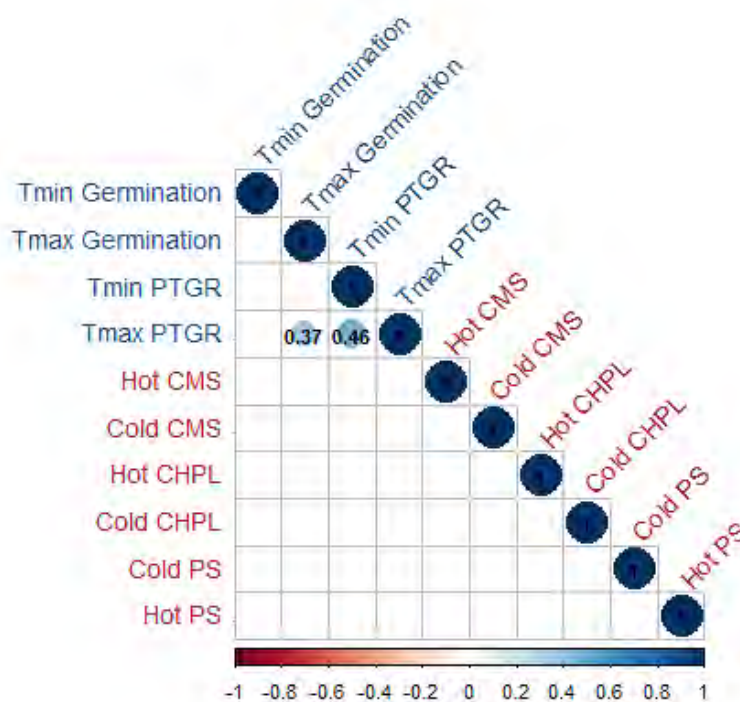
Figure 1.11. Correlation matrix of all plants. Gametophytic (labels blue font) and sporophytic variables (labels red font) with significant Pearson's correlations for all study plants.

When the correlation analysis was performed on all variables for the regions separately, there were different results. For the northern plants, there were no significant correlations. The southern plant had one significant correlation between Tmin germination and Tmax germination (r = -0.63; Appendix Figure A9).

Principal Component Analysis

We conducted principal component analysis to further explore relationships among all variables and the sporophytic and gametophytic variables separately. For the full PCA, we included all gametophytic and sporophytic variables, except HPS and CPS due to inadequate sample size. The first three principal components accounted for 57% of the variation (full PCA plots and loadings in the appendix Figure A11, Table A7). There was little divergence between regions. When the eigenvalues of the principal components were compared between regions, PC2 was the only principal component that showed a significant difference (t_{58} = -2.69, p = 0.0092). Chlorophyll content (HCHPL and CCHPL) loads primarily on PC2 and is likely driving the divergence between northern and southern plants.

Sporophytic PCA

In the sporophytic variables PCA, the first three principal components explained 60% of the variation. The variables HCMS and HPS primarily loaded on PC1 (Table 1.2, Figure 1.13). The second and third principal components were mostly influenced by CCHPL and HCHPL respectively. There was a significant difference between the regions for the eigenvalues extracted from both PC2 (t_{78} = -5.09, p = 2.39e-06) and PC3 (t_{101} = 2.38, p = 0.019). The divergence in PC2 can be explained by the opposite responses we observed for CCMS and both chlorophyll content treatments. Northern plants have a higher chlorophyll content ratio for both treatments, while southern plants had less cell membrane damage in the cold treatment. PC1 did divide HCMS and CCMS, suggesting an antagonistic relationship between the two variables, though there was no correlation between the two that was statistically significant. Hot and cold treatment variables were also divided on PC3. HPS and HCHPL were opposite in direction to CPS and CCHPL.
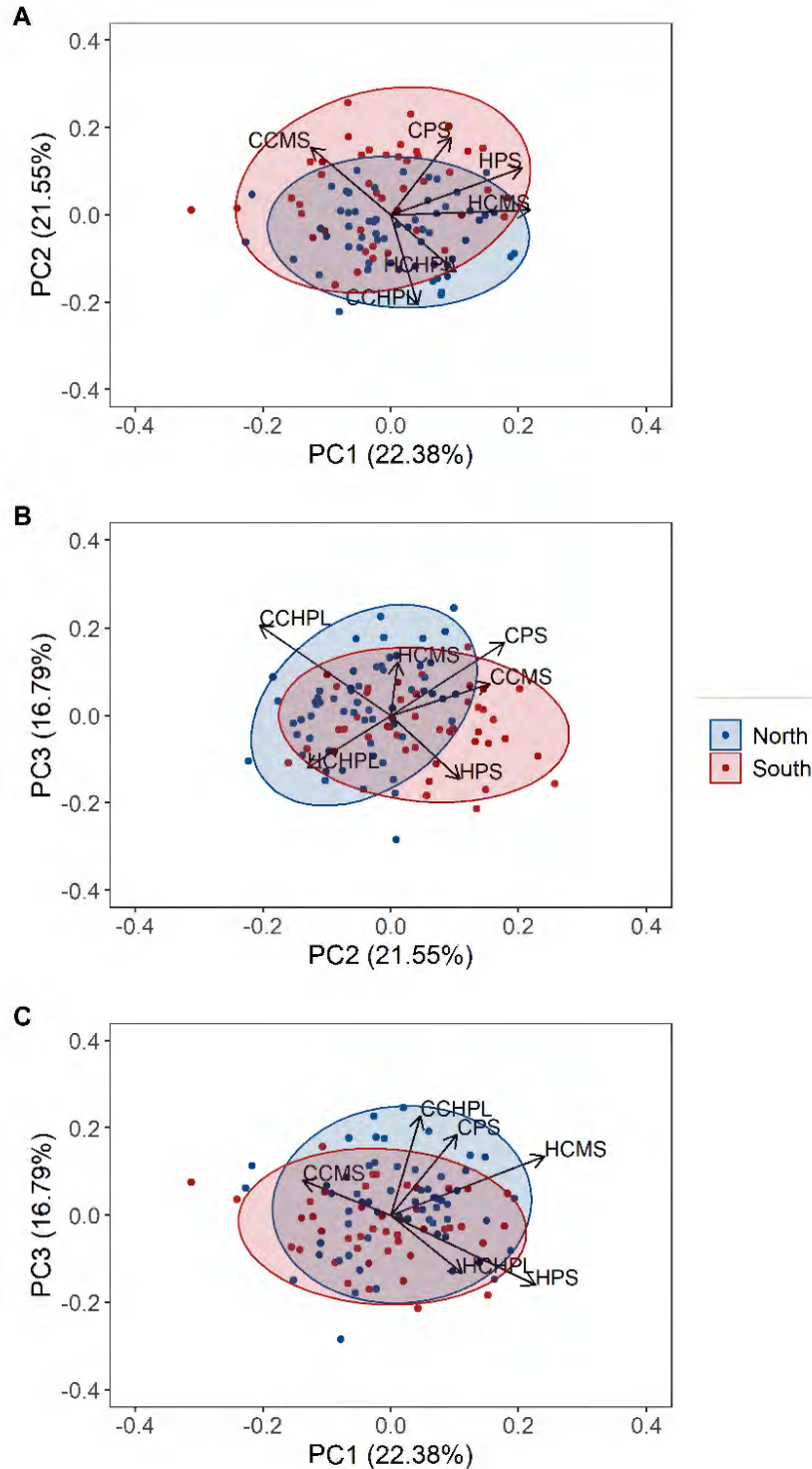
Figure 1.12. Plots of the results of principal component analysis for the sporophytic variables. A) PC1 and PC2, B) PC2 and PC3, C) PC1 and PC3. Ellipsoid indicating 95% confidence interval. PC1 explains 22.38% of the variance, PC2 explains 21.55% of the variance, and PC3 explains 16.79% of the variance. Tables with principal component importance for PC1 through PC6 in the Appendix (Table A8).

Table 1.2. Results from principal component analysis of only sporophytic variables. Loadings for each of the variables on the principal components

	PC1	PC2	PC3	PC4	PC5	PC6
HCMS	0.613999	0.02998	0.344975	0.147492	0.573797	0.390002
CCMS	-0.35207	0.435008	0.204072	-0.57534	0.520509	-0.20789
HCHPL	0.284794	-0.36797	-0.33836	-0.75803	-0.08744	0.294536
CCHPL	0.117118	-0.57752	0.579996	-0.14114	-0.03843	-0.5431
HPS	0.577968	0.302596	-0.40867	0.000171	0.044159	-0.63673
CPS	0.264909	0.499375	0.470594	-0.22955	-0.6235	0.13244

Gametophytic PCA

In the gametophytic PCA, the first three components explained 92.5% of the variance. Pollen germination variables divided the northern and southern plants (Figure 1.14). Tmax and Topt loaded evenly in the opposite direction of Tmin for both PC1 and PC2 (Table 1.3, Figure 1.14). There was a significant difference between north and south for the eigenvalues extracted from PC2 (t_{46} = -3.17, p = 0.0025). PTGR variables loaded evenly on the first two principal components, indicated by the common diagonal direction among the PTGR variables (Table 1.3, Figure 1.14).
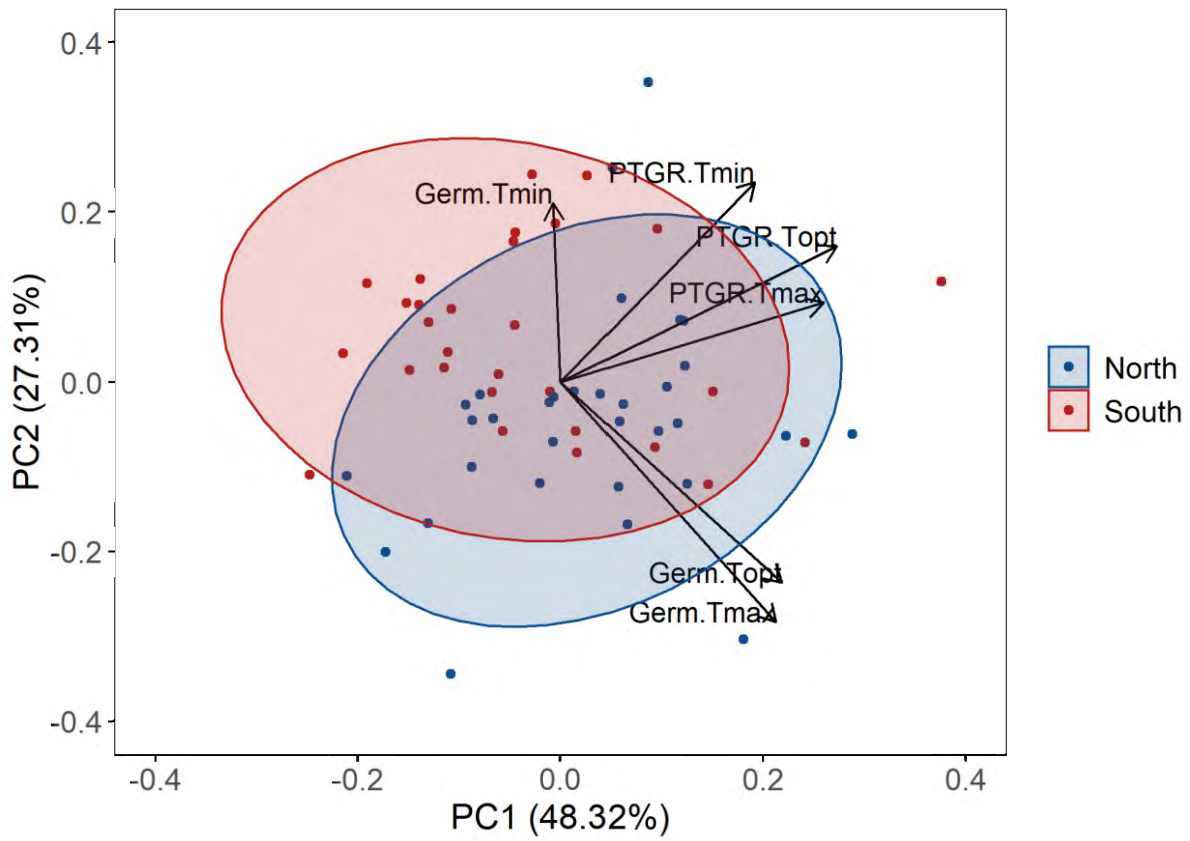
Figure 1.13. Plot of the results of principal component analysis of the gametophytic variables. PC1 describes 48% of the variation and PC2 explains 27%. A table of importance of principle components 1 through 6 is in the Appendix (Table A9).

Table 1.3. Results from principal component analysis of only gametophytic variables. Loadings for each of the variables on the principal components

	PC1	PC2	PC3	PC4	PC5	PC6
Germ.Tmin	-0.01334	0.405643	0.812217	0.376238	-0.00058	-0.18446
Germ.Topt	0.418665	-0.45436	0.390064	-0.00262	0.001723	0.682727
Germ.Tmax	0.407763	-0.5446	0.164764	-0.10069	-0.00179	-0.707
PTGR.Tmin	0.367661	0.452838	0.127813	-0.75119	-0.28131	0.00071
PTGR.Topt	0.523981	0.308129	-0.20261	0.123985	0.757676	-0.00193
PTGR.Tmax	0.498538	0.180049	-0.3219	0.518304	-0.58888	0.001496

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<div align="center">**Discussion**</div>

Regional Differences

If *Solanum carolinense* has locally adapted to the respective temperature regimes in TX and MN, we would expect that plants from the north would be more tolerant of cold temperatures and plants from the south would be more tolerant of hot temperatures. Rather than a clear-cut difference between north and south for hot and cold treatments, there were mixed results that support divergence between regions in ways we hadn't anticipated. In contrast to our expectations, northern plants were generally more tolerant of extreme heat than southern plants, but also had more variation in certain trait values. Northern plants had higher chlorophyll content (HCHPL) and baseline cell membrane stability (HCMS; Figure 1.6) under hot conditions, as well as higher maximum and optimal temperatures for pollen germination in comparison to southern plants (Table 1.1). Conversely, southern plants had increased tolerance for cell membrane stability in cold conditions (CCMS). These results suggest that adaptation to extreme temperatures is complex and may evidence the involvement of avoidance strategies rather than physiological mechanisms to withstand thermal stress.

There was no significant difference between regions for HCMS for all study plants together, but there was a significant difference for plants in block A. Temperatures in the greenhouse progressively rose throughout the spring and summer leading to a block effect in both the hot and cold treatments of CMS. In block A, northern plants had a higher HCMS, but this difference degraded in the later blocks during the times when greenhouse temperatures during plant development increased. A possible explanation for the block effect on CMS is that plants have the capacity to induce heat tolerance as they acclimate to warmer conditions (Clarke et al. 2004). Block A is the best representative measurement of baseline heat tolerance for

<div align="center">34</div>

HCMS, and later blocks likely represent induced heat tolerance, which may be more dramatic in southern plants. Conversely, plants from the south had more stable cell membranes when exposed to an extreme cold treatment. The counter gradient pattern we measured for northern and southern plants may be due to constraints of adaptation to extreme heat or cold. Adapting to match the extreme environmental conditions may not be advantageous or possible, reducing the variation in a population for tolerance in extreme conditions. Thus, populations in locations that do not experience extreme temperatures on one end of the spectrum may have more variation than those that do experience extreme temperatures, leading to the counter gradient results we attained for CMS that go against our expectations.

Plants from the north had more stable chlorophyll content in both the hot (HCHPL) and cold treatments (CCHPL; Table 1.1). More stable chlorophyll content may be explained by northern plants experiencing a larger range of temperatures. Between 2018 and 2021, temperatures during the growing season (March to September) in Houston County, MN ranged from -28°C to 34°C (62°C difference), while in Collin County, TX they ranged from -7°C to 42°C (49°C difference). Since the temperate conditions of Minnesota are more variable and rarely exceed temperatures likely to stop plant growth (Hatfield et al. 2011), populations in the north may have evolved to acclimate to temperature stress, while plants in the south do not. Furthermore, northern plants also had significantly more variation in HCHPL than southern plants. This may suggest that there is stabilizing selection occurring in the southern region for heat tolerance in chlorophyll content. Less variation in HCHPL in the south may contribute to the counter-gradient results we attained. If northern plants experience less heat stress selection and have greater variation, then there may be more potential to have individuals with high HCHPL.

Pollen from the north had a higher propensity to produce pollen tubes (Germ) at high temperatures than their southern counterparts. Pollen germination was higher in pollen grains from the northern plants than those in the south for both Tmax and Topt (Table 1.1). The distinct difference between north and south suggests that there might be sensitivity to high temperatures and an adaptive response occurring. Since southern populations experience extremely high temperatures more regularly than northern plants, there may be an avoidance strategy in southern populations and not adaptation to germinate under extreme heat in the north. Rutley et al. (2022) proposed the two-baskets model categorizing pollen, which states that there are high-ROS and low-ROS subpopulations of pollen within anthers of flowering species. The low-ROS pollen have a lower metabolic rate than high-ROS pollen due to partial dehydration during development. The two subpopulations of pollen are adaptive and beneficial under different conditions as they allow for asynchrony in pollen germination, permitting some pollen to remain dormant in a stressful environment, such as extreme heat or drought, and grow pollen tubes later in more favorable conditions. Keller and Simm (2018) compared the transcriptome and proteome in *Solanum lycopersicum* (tomato) and determined that pollen have two responses during heat stress – direct and delayed translation. Luria et al. (2019) later showed that *Solanum lycopersicum* has pollen that fall in the low-ROS and high-ROS groups, supporting the two-basket model in a species closely related to *Solanum carolinense*. We hypothesize that *Solanum carolinense* populations in the south have higher proportions of low-ROS to high-ROS pollen grains than those in the north due to stronger selection from increased exposure to extreme heat in the south. Low-ROS pollen that remains dormant would not be adaptive in northern populations, with little exposure to high temperature stress.

There was a significant negative correlation between Tmax and Tmin germination in southern plants. This correlation supports the two-basket model. The negative correlation means that plants with pollen that germinate readily at high temperatures also germinate at low temperatures, while those that have a lower Tmax have a higher Tmin. Plants with a higher proportion of high-ROS pollen would germinate in any condition (extreme heat and cold stress). Plants with a higher proportion of low-ROS pollen would not germinate as freely during stressful conditions. Since plants of the south may have evolved to have the dual pollen types, there may be more variation in pollen activity driving this correlation.

There was no significant difference between northern and southern plants for net photosynthetic rate in both the hot and cold treatments. Net photosynthesis was the only sporophytic variable where the whole plant was placed in a temperature treatment and leaves were measured on the plant. The plant may compensate for temperature stress through physiological mechanisms, such as increasing transpiration. Therefore, the temperature treatments may not have stressed the plants to the extent that temperature tolerance for the northern and southern plants was distinguishable.

The response of plants from the two regions to extreme cold were considerably more mixed. There was no significant difference between northern and southern populations for Tmin of either pollen germination or pollen tube growth rate. Of all cold traits only two sporophytic traits (CCMS and CCHPL) differed between regions and were not consistent. Pollen may have a low temperature limit on physiological processes necessary for pollen tube growth that are consistent across all populations.

Inter-Generational Relationships

Tanksley et al. (1981) first described the association between selection in the gametophyte and sporophyte when they found a correlation between allozyme genes expressed in both stages. Based on their findings and several studies that followed (Hedhly et al. 2005; Pedersen et al. 1987; Poudyal et al. 2019; Willing and Mascarenhas 1984), including studies on temperature tolerance (Hedhly et al. 2005; Poudyal et al. 2019), we hypothesized that there would be a correlation between temperature tolerance in the sporophyte and the gametophyte. Correlations between the two life stages have implications for the rate of temperature tolerance evolution. Selection in either stage for similar traits that are expressed independently would rapidly increase or decrease the allele frequency of associated genes in a population. Furthermore, in the gametophyte, there is a lack of dominance allowing selection to act on one allele (Beaudry et al. 2020). The alleles selected for in the gametophyte can then affect traits in the sporophyte.

There were no significant correlations between any of the gametophytic and sporophytic variables, suggesting that there are different mechanisms mitigating temperature stress in the two stages. This is not the first study to find inconsistencies in the selection for cold tolerance in the sporophyte and gametophyte. Dominguez et al. (2005) conducted a study to determine if pollen selection can be used to improve cold tolerance in the gametophyte by selecting pollen from cold tolerant plants (sporophyte). They found that pollen selection did not improve pollen viability and formation in cold and explained their results by describing how the genes mediating cold stress may be expressed in the sporophyte tissue surrounding the site of pollen formation, rather than the pollen grains themselves.

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Another explanation for the lack of coordinated response to temperature stress between the two life stages is that horsenettle hasn't been located in MN and TX long enough for selection to act on the populations. All populations included in this study were located toward the edge of the range for this species. Time for selective pressures to act on the populations may be insufficient for local adaptation to occur. The first record of *Solanum carolinense* in Minnesota is from 1939 and in Houston County 1975 (Bell Museum Plants, Minnesota Biodiversity Atlas; The University of Minnesota). The first record in Texas is from 1917 and the closest record of horsenettle to Collin County is from 2011 (Lundell Herbarium, Billie L. Turner Plant Resources Center; The University of Texas at Austin).

Conclusion

Our results are consistent with a process of local adaptation due to temperature acting as a selective pressure. The results of this study do not completely support our original predictions based on the assumption that northern latitudes are simply cooler than southern latitudes. The measurements of chlorophyll content did provide some evidence that populations from areas with larger thermal ranges, such as those in higher latitudes, have more variation and possibly more phenotypic plasticity, which is consistent with the climate variability hypothesis. The block effects observed in both HCMS and CCMS also suggest that there is plasticity in the phenotype when exposed to long-term changes in ambient temperature. Lastly, we found evidence of southern plants avoiding pollen germination in high temperatures by increasing the proportion of low-ROS to high-ROS pollen.

These results could inform restoration efforts by changing the way we think about seed sourcing and adaptive potential in a rapidly changing environment. Seeds from the south may have evolved stress responses to temperature that are lacking in northern populations or vice

versa. The evidence for the two-basket model in a wild species is also a novel finding that could add to our perception of the influence gametophytic traits have on species persistence in extreme environments.

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CHAPTER 2: THE EFFECT OF LONG-TERM MODERATE HEAT ON SEXUAL REPRODUCTION IN *SOLANUM CAROLINENSE* (HORSENETTLE) AND IMPLICATIONS FOR EVOLUTIONARY RESPONSES TO ENVIRONMENTAL CHANGE

Abstract

Temperatures worldwide are gradually increasing due to climate change. *Solanum carolinense* has a range that spans much of the United States, including locations where temperature increases are projected. Previous studies found that moderate heat substantially influenced reproductive processes in crop species, particularly accessions sensitive to heat suggesting that climate change has the potential to impact agriculture. Our understanding of the effects climate change has on wild species is more limited. We investigated the impact of long-term moderate heat during flower development before pollination and post-pollination on reproductive traits in *Solanum carolinense* from populations in Texas and Minnesota. We found that heat affects flower morphology, pollen size, and viable seed number suggesting that there is plasticity in the phenotype that may or may not be adaptive and could obscure evolutionary responses. We also found evidence of initial divergence among plants of the two regions, including traits that were differentially affected by long-term moderate heat, indicating the potential for gene x environment interactions. These results have implications for the persistence of wild populations in locations with gradually rising temperatures.

Introduction

The relative fitness of a species is determined by the propensity of individuals to survive and successfully reproduce relative to other individuals. Environmental conditions can directly influence the relative fitness of individuals by affecting reproductive traits and ultimately

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reproductive success. Reproductive traits have been shown to be affected by several different environmental variables. Female reproduction is broadly influenced by growth conditions such as nutrients (Burkle and Irwin 2009; Conner and Zangori 1998; Haileselassie et al. 2005), moisture (Fang et al. 2010; Galen 2000), and heat (Xu et al. 2017). Male reproductive success is also dependent on environmental conditions. Pollen viability decreases with high temperatures (Din et al. 2015; Müller et al. 2016; Poudyal et al. 2019; Sato et al. 2006; Xu et al. 2017) and drought stress (Fang et al. 2010). Because environmental conditions influence both female and male reproductive success, the number of seeds, and thus progeny, can vary as environmental conditions change, influencing the evolution of a species. Variation in reproductive traits within or among populations can be due to genetic variation or environmental variation, which can obscure selection based on genes alone. If a response to the environment is genetically mediated and increases the chances of survival or reproduction, then variation can also be due to gene x environment interactions. To fully understand the vulnerability a species has to environmental change, we must understand the variation driving evolutionary responses.

Global climate change is resulting in rapidly changing environmental conditions including higher mean daily air temperatures and minima. According to the National Climate Assessment (USGCRP 2018), temperatures in the Midwestern and Southeastern United States have been steadily rising since the 1970's. Average daily maximum temperatures in the southeastern region have made moderate increases compared to other regions in the United States, such as the Midwest, but minimum and average temperatures have been rising. The subtle increases of temperature regimes will lead to long-term temperatures that are above optimal for plant cellular processes, especially affecting reproductive success (Jiang et al. 2019; Müller et al. 2016; Sato et al. 2006; Xu et al. 2017). Thus, climate change has increased the relevance of

understanding the effects environmental temperatures have on male and female reproductive traits. If environmental temperatures do indeed affect reproductive success, then adaptation to climate change may involve not only the genetic variation within a population, but also the environmental effects and gene x environment interactions.

There is evidence that environmental temperatures affect reproductive phenotype. In crop species, development in moderately high temperatures affected floral morphology (Charles and Harris 1972; Müller et al. 2016; Sato et al. 2006), ovule viability (Xu et al. 2017), pollen viability (Din et al. 2015; Müller et al. 2016; Poudyal et al. 2019; Sato et al. 2006; Xu et al. 2017), fruit set (Charles and Harris 1972; Din et al. 2015; Sato et al. 2006), and seed set (Din et al. 2015). Sato et al. (2006) found that elevated temperatures decreased fruit set and pollen viability as well as stamen height in tomato. Poudyal et al. (2019) found that pollen viability decreased in heat, but more tolerant tomato accessions had higher pollen germination than sensitive accessions. Xu et al. (2017) found that long-term mild heat decreased pollen viability, pollen number, female fertility, and fruit set. Charles and Harris (1972) found that flower production, fruit set, fruit size, pollen germination, and distance between the stigma and antheridial cone all decreased at high temperatures in tomato. Muller et al. (2016) found that long-term mild heat resulted in floral deformations and low pollen viability in tomatoes. Thus, heat has been shown to have consistently negative effects on reproductive traits and correlates of male and female reproductive success in crop species.

While there are many studies examining how high temperatures affect sexual reproduction (Lohani et al. 2020), there are few studies that have addressed the effect of high temperatures on wild, non-crop species. Wild populations that grow in natural, heterogeneous conditions, and have endured evolution by natural selection for many generations likely have

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different levels of genetic diversity than artificially selected crop accessions. It is unclear how natural levels of genetic diversity in the context of natural conditions will ultimately determine rates of evolution and whether species will acclimate and adapt to a rapidly changing climate or not. Rising temperatures could restrict the success of sexual reproduction and thus, persistence, of wild populations in several ways. Changes in flower morphology has the potential to influence how pollinators interact with flowers and reduction in ovule and pollen viability decreases chances of fertilization, seed formation, and fruit development. Each process reduces the potential number of offspring and in that, fitness. Wild, non-crop species may be just as vulnerable to high temperatures, if not more than crops. We examined high temperature sensitivity in a wild species closely related to eggplant and tomato, *Solanum carolinense*.

We wanted to further investigate the effect of heat on sexual reproduction and identify the sources of variation driving differences among traits. We wanted to understand how environment affects reproductive phenotype and potential gene x environment interactions to comprehend and predict evolution in a warming environment. Broadly, our goals are:

1. To measure key reproductive traits in a weedy herb exposed to different temperatures during flower and fruit development as a means of quantifying phenotypic plasticity in these traits.

2. To test for local adaptation and differences in response to environmental conditions between divergent populations from warmer and cooler regions using a common garden approach.

3. To distinguish between environmental effects on traits associated with male and female reproductive success.

In this study, we investigated the effect of long-term high temperatures on reproductive traits in *Solanum carolinense*. We included both pre-pollination traits and post-pollination traits to understand how heat may influence phenotype throughout the process of sexual reproduction. If *Solanum carolinense* responds to long-term heat stress as does tomato, then we predict significant negative effects on floral morphology, male and female viability, and fruit and seed set. However, because southern populations are in warmer environments and may have adapted to growth and reproduction in a relatively warm climate, we also predict negative responses to heat will be reduced relative to northern populations. Our specific objectives to assess these patterns were:

1. To grow plants from northern (Minnesota) and southern (Texas) regions in a common garden setting to remove environmental variation between divergent genotypes

2. Experimentally test the effects of hot (32°C) temperatures versus control (25°C) temperatures during flower and fruit development on phenotypic expression of pre and post pollination reproductive traits

3. Compare the responses of plants from different regions to heat treatments in order to measure potential gene x environment effects and the potential for environmental effects to reduce the response to selection.

Methods

Species Description

Solanum carolinense L. (Solanaceae), also known as horsenettle, is an herbaceous perennial with spines that line the stem and midrib of the variably lobed leaves. This species reproduces both sexually and asexually by rhizome. *Solanum carolinense* grows indeterminately and is andromonoecious, meaning that both staminate and hermaphroditic flowers are produced.

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The flowers are "buzz-pollinated", requiring bumblebee pollinators that vibrate their abdomens at a relatively high frequency to release pollen from the anther. Fertilization is complicated by a gametophytic self-incompatibility (SI) system. The SI system reduces inbreeding by degrading pollen tubes of self and closely related pollen, prior to fertilization (Mena-Ali and Stephenson 2007; Mena-Ali et al. 2009). However, as flowers age, the SI system deteriorates and the potential for successful self-fertilization with fruit production increases (Travers et al. 2004). The fruit are small yellow to green, tomato-like berries that are dispersed by small mammals and birds.

Field Collection

Solanum carolinense plants were collected from two populations in Houston County, Minnesota and three populations in Collin County, Texas between October 2019 and August 2020 (Chapter 1, Figure 1.2). The Minnesota plants collectively will be referred to as the northern plants and include the populations Prairie Island (44.07959 N, -91.684545 W) and Frontenac (44.523056 N, -92.338611 W). Approximately 80 Km separated the two populations (Chapter 1, Figure 1.3). In Houston County, MN, the mean daily low temperature is -14°C (7°F) and the mean daily high is 29°C (85°F). The Texas plants together will be referred to as the southern plants. All three TX populations were located within a circle with a 1.5 Km radius near McKinney TX (Oil Patch: 33.173465 N, -96.615402 W; Reserve: 33.159962 N, -96.619011 W; and Cemetery: 33.173672 N, -96.615096 W). In Colin County TX, the mean daily low temperature is 18°C (65°F) and the mean daily high is 43°C (111°F).

Solanum carolinense is a perennial that reproduces asexually by the growth of ramets (genetically identical plants connected by rhizomes). Genets (individual genotypes) were sampled by collecting the below ground portion of individual plants and saving 10 cm of root

and rhizome. Sampled plants were a minimum of 1 meter apart, ensuring that unique genotypes were collected with each plant. The rhizomes were given unique ID numbers, placed in zip lock bags, and shipped to Fargo in a cooler with blue ice. The rhizomes were stored in a 4°C refrigerator until they were planted in one-gallon containers and allowed to grow under greenhouse conditions. In October, the above ground material was cut and the pots plus below ground tissues were stored again at 4°C for a three-month period of dormancy. During the spring and summer of 2021, four ramets (A, B, C, and D) were cut from the rhizome of each genet, grown in separate plots and used in a previous study (methods described in Chapter 1). In October and November, the above ground material for all ramets of each genet was cut and the plants were returned to 4°C for a dormancy period.

Growth Conditions and Experimental Design

On January 12, 2022, ramets A and B for all genets (26 from north and 26 from south) were placed in a randomized grid pattern in a Conviron PGC-FLEX growth chamber. Due to space constraints in the environmental chambers, only two per genet were grown at a time. For initial growth, all plants were placed in the same, "control" conditions. In the control growth conditions, the chamber was set at 25°C day/25°C night with fluorescent lights at setting 2 and incandescent lights at setting 1 for 14 hours per day. As plants grew to heights at which the incandescent bulbs damaged upper leaves on some plants, the incandescent setting was reduced to 0. Plants were fertilized once every two weeks with a high phosphorus fertilizer to promote flower production (Super Bloom, Scotts).

Once a plant flowered, all flowers and buds were removed, and it was moved to its heat treatment. The control treatment chamber (Conviron PGC-FLEX) was set at the same conditions used for initial growth. The heat treatment chambers (Conviron E7/2) was set at 32°C day/25°C

night with the same light settings as the control. One ramet from each genet was randomly assigned to the heat treatment. The other was assigned to the control treatment. Plants were watered daily. The date of first flowering (prior to treatment) and the date when a ramet flowered again (during the treatment) were recorded. The flower type (hermaphroditic or staminate) produced for the first flowering in the treatment was also recorded.

Pre-Pollination Dependent Variables

The first three hermaphroditic flowers that developed in the respective treatments were collected and used for flower morphology measurements, ovule counts, and pollen size measurements. The ovules were stained following a modified protocol adapted from Diaz and Macnair (1999). The flowers with petals removed were stored in Eppendorf tubes (1.5 mL) with ethanol for 24 hours and then washed with deionized water. The tubes were then filled with 1M NaOH and placed in a heat block at 70°C for 2 minutes to soften the floral structures before a final wash in deionized water. The flowers were then stained in 0.1% aniline blue with 0.1M K_3PO_4 for 24 hours in darkness. The length of the style plus the stigma and the length of one anther were measured under a dissecting scope. The ovary and anther were sectioned and mounted on a microscope slide with 50% glycerol. The number of ovules in each ovary was counted. Pollen diameter of at least 100 grains was measured with the use of a microscope (Axio Scope A.1 Carl Zeiss, Germany) at 400x total magnification and the circle diameter measurement tool on the Zen 3.1 software.

Post-Pollination Dependent Variables

Pollen germination percentage was calculated for grains on artificial media at 40°C. In the previous study (Chapter 1), there was variation among genotypes and regions in pollen germination at high temperatures. We used 40°C to determine how plants differ in germination at

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high temperatures and whether pollen development in long-term high heat affects pollen germination at high temperatures. One flower from each plant in the treatment group was collected for pollen germination. Pollen was collected from the mature flower, identified by petals in an open position perpendicular to the anthers and a fully developed stigma (if flower was hermaphroditic). Since horsenettle is naturally buzz pollinated, we used a handmade device to vibrate anthers and release pollen directly onto an agar/growth medium contained in petri dishes. We used a 3% Bacto-Agar based growth medium (sucrose, $Ca(NO_3)_2$, $MgSO_4$, KNO_3, H_3BO_3) following the protocol of Reddy and Kakani (2007). Immediately after dispersal of pollen, the plate was placed in a drying oven at 40°C for 16 hours. Three pictures of the pollen on the petri dish were then taken using a microscope mounted with a camera (Leica DM500 microscope, Leica ICC50 HD camera) and the LAS EZ 2.1.0 software. To avoid sampling bias, each petri dish was positioned so pollen visible to the naked eye was under the objective. The petri dish was not repositioned once pollen grains were viewed under magnification. Pollen germination was measured by counting the number of pollen grains that produced tubes of at least half the diameter of the pollen grain. The final pollen germination variable equaled the number of grains germinated divided by the total number of pollen grains assessed. All pollen grains in a picture were counted. The number of pictures used depended on the number required to count at least 100 pollen grains.

Female reproductive traits measured include fruit set (number of fruits produced / number of flowers pollinated) and the number of viable seeds per fruit. Once all flowers for morphological and male performance traits were collected, the subsequent three flowers on each plant were pollinated with a mix of pollen from flowers (2 to 5 flowers on average, north and south represented) in the control treatment. The goal was to isolate the effect of heat during the

development of the ovules and ovary, not during the development of the pollen. Horsenettle has a self-incompatibility system, which prevents plants with the same S allele from fertilizing one another. The self-incompatibility system is a measure to prevent inbreeding. We mixed pollen from multiple populations from the north and south to ensure that there was the opportunity for fertilization. The flowers were pollinated by applying the mixture of pollen on the stigma with a probe and labeling the flower with a jewelry tag. Once flowers were pollinated, the plant remained in the treatment for one week before we moved them into a greenhouse for the fruit to finish development (Average Daily Temperatures 25.08°C day / 21.31°C night).

Once fruits were at least one month old, they were harvested. The number of viable seeds, aborted seeds, and unfertilized ovules were counted under a dissecting scope. The variables used as measures of female performance were fruit set and seed set. Fruit set was the number of fruits produced divided by the number of flowers pollinated, which was three for all plants. Viable seed number is the number of seeds produced per fruit.

Data Analysis

All data analysis was conducted in R 4.1.2 (R Core Team 2020). Flower date was analyzed for regional differences using a linear mixed effects model in the *lmerTest* package (Kuznetsova et al. 2017) with region and population as the fixed effects and genet nested in population as the random effect. Regional differences for the number of plants that flowered in the control conditions and treatment groups were determined using a chi-squared test (*stats*; function chisq.test). Differences in flower type development between the treatments in the northern plants were analyzed using a chi-squared test in the *stats* package (R Core Team 2020). Because of low sample size in southern plants, treatment effects were only analyzed for northern plants for all variables except flower date, propensity to flower, and flower type. Anther length,

style plus stigma length, and ovule number were analyzed for regional differences in the control treatment using a linear mixed effects model (*lmerTest*; function lmer) with region and population as fixed effects and genet nested in population as the random effect. A linear mixed effects model (*lmerTest*; function lmer) with treatment and population as the fixed effects and genet nested in population as the random effect was used for treatment differences. The ratios of style plus stigma to anther length for northern plants were analyzed using a linear mixed effects model with treatment as the fixed effect and population as a random effect. To test differences in variation between the treatment groups of the ratio, we used the Bartlett test of homogeneity of variances (*stats*; function bartlett.test). We also conducted correlation analysis for mean anther and mean style plus stigma lengths (*stats*; function cor.test). Mean pollen diameter was compared between regions using a linear mixed effects model (*lmerTest*; function lmer) with region as the fixed effect and genet nested in population as the random effect. The treatment effect on mean diameter of pollen grains in the northern plants was analyzed using a linear mixed effects model (*lmerTest*; function lmer) with treatment as the fixed effect and population as the random effect.

Since there was a slightly larger sample size for southern plants in the treatment groups for pollen germination at 40°C because staminate flowers could be used, region and treatment were analyzed in a two-way analysis of variance model (*stats*; function aov). Fruit set was analyzed for only northern plants using a chi-squared test (*stats*; function chisq.test). Viable seed number, aborted seeds, and unfertilized ovules were analyzed using the same linear mixed effects models as described for ovule number.

Results

Flowering

There was no significant difference between the regions for the timing of the first flower (Appendix Figure B1). However, there was a significant difference between regions for the number of plants that initially flowered, with 48 plants from the northern region and 17 from the southern region (Figure 2.1; Table 2.1). There were 21 plants in the control group and 24 plants in the heat treatment group that flowered for the northern plants. For the southern plants, 9 in the control and 6 in the heat treatment flowered again. Since the number of plants that flowered in the two regions differed substantially, we only considered northern plants in analyses for treatment differences in style plus stigma length, anther length, ovule number, pollen diameter, fruit set, and seed number.
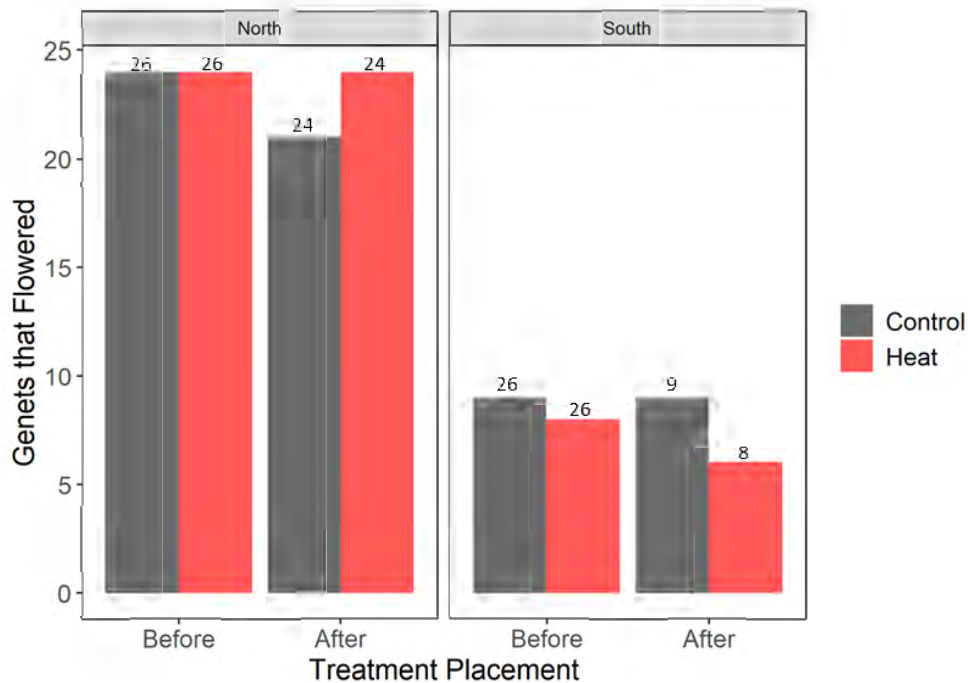
Figure 2.1. The number of genets that flowered in the control and heat treatments before and after they were placed in the treatments. Counts for the northern and southern regions are shown independently. Numbers above the bars represent the number of plants within each group that were initially placed in the environmental chambers.

Table 2.1. Results from the chi-squared tests for the number of plants that flowered the first time and the second time in the treatments and chi-squared tests for flower type and fruit set. Bolded values indicate a significant relationship.

Variable	Test	df	χ^2	p
Plants that flowered 1st time	Region	1	**36.923**	**1.23E-09**
Plants that flowered 2nd time	Region	1	**33.130**	**8.62E-09**
Plants that flowered 2nd time	Treatment	1	0.000	1.000
Flower Type	Treatment	1	0.370	0.543
Fruit Set	Treatment	3	5.547	0.136

Flower Development

The flower type for the first flower after placement in the treatment was recorded. There was no significant difference between treatment groups for flower type of northern plants (Table 2.1). Flower type did limit the data collected since staminate flowers were not used for variables such as ovule number, style plus stigma length, anther length, pollen diameter, fruit set, and seed number (Figure 2.2). Thus, treatment effects were only considered from plants from northern populations for style + stigma length, anther length, ovule number, pollen diameter, fruit set, and seed number. Southern plants had larger floral structures than northern plants. There was a significant difference between regions for style plus stigma length and anther length in the controlled (25°C) conditions (Figure 2.3, Table 2.2). We couldn't test for the effect of heat on flower morphology in southern plants, as few plants flowered and those that did flower in the heat had mostly staminate flowers.
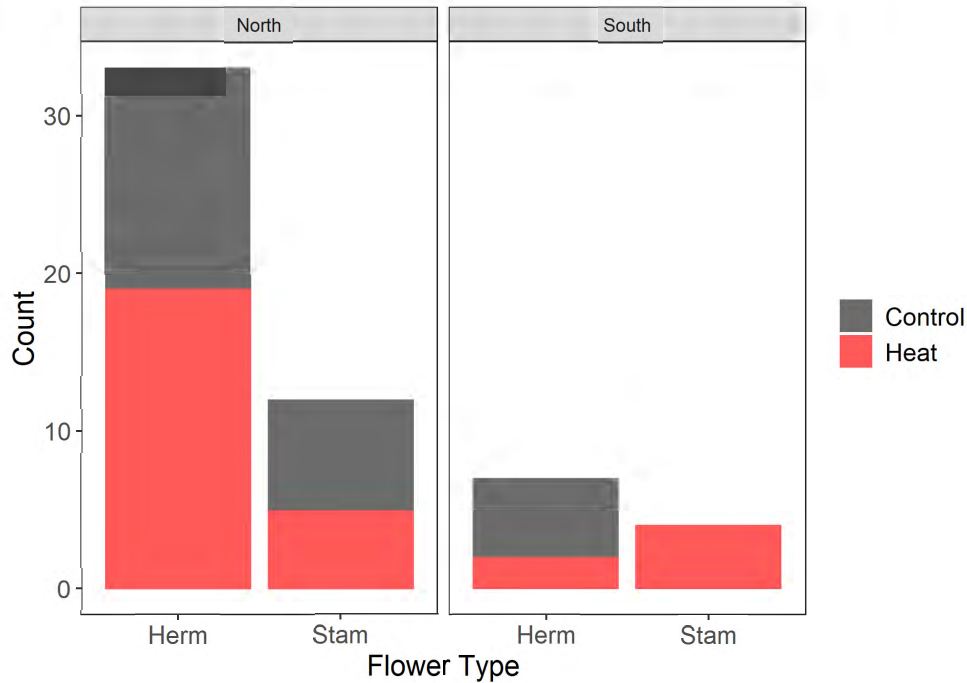
Figure 2.2. Number of plants with hermaphroditic and staminate flowers for the treatment groups. Counts for northern and southern plants displayed independently.

Table 2.2. Results from analysis of floral morphology variables using mixed effects models for regional and population differences. Analysis is just of plants in control treatment. Bolded values indicate a significant relationship.

| | Fixed Effects | | | | | | Random Effects | |
| | Region | | | Population | | | Population:Genet | Population |
Variable	F	df	p	F	df	p	p	p
First Flower Timing	1.458	38.437	0.235	0.019	40.031	0.892	0.804	-
Style + Stigma Length	**4.453**	**24.943**	**0.045**	1.200	24.854	0.284	**6.24E-11**	-
Stamen Length	**12.071**	**25.000**	**0.002**	**13.916**	**25.000**	**0.001**	**9.09E-06**	-
Ovule Number	0.093	24.105	0.763	2.822	23.848	0.106	**0.017**	-
Mean Pollen Diameter	0.522	0.738	0.633	-	-	-	-	0.449
Viable Seed Number	0.189	16.507	0.669	2.032	16.602	0.173	**5.38E-06**	-
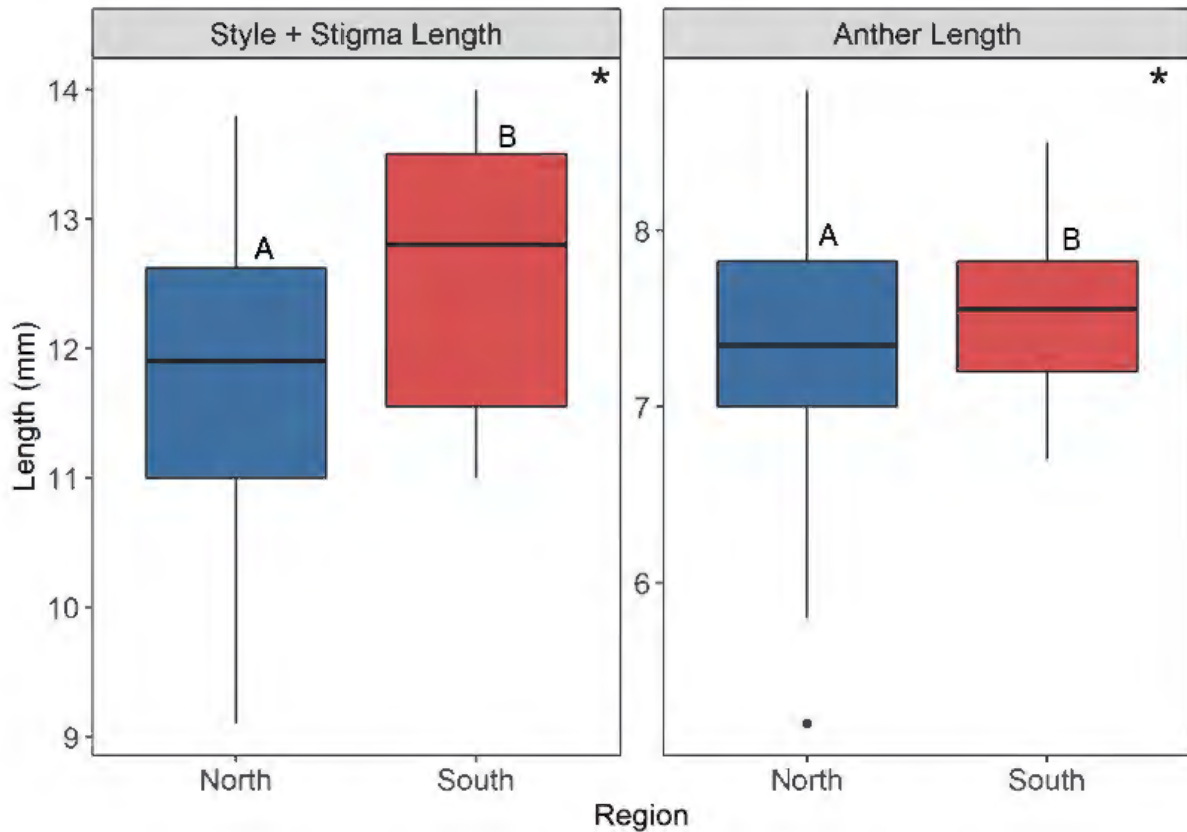
Figure 2.3. Regional differences for the length of the style plus stigma and the length of the anther from flowers that developed in the control treatment. Midline in boxplot indicates the median of the sample. Asterisks and letters indicate differences that are statistically significant. There are significant differences between regions (north (n) = 20; south (n) = 8) for style plus stigma length ($F_{25} = 4.453$, $p = 0.045$) and anther length ($F_{25} = 12.071$, $p = 0.002$).

There were significant temperature treatment effects for northern plants in both style plus stigma length and anther length (Figure 2.4, Table 2.3). In both cases, development in heat reduced the lengths of the structures. For the ratio of style plus stigma length to anther length, there was no significant difference between treatments for the means, but there was a significant difference between variances (Bartlett's K^2 = 14.51, p = 1.40e-04; Figure 2.5). There was a significant, positive correlation (Pearson's correlation = 0.761, p = 9.611e-05) between the two variables for the control treatment, but not for the heat treatment (Pearson's correlation = -0.292, p = 0.225; Figure 2.6).

There were no significant differences in ovule number between regions or treatments (Appendix Figure B2). Mean pollen diameter did not differ between the two regions (Appendix Figure B3), but there was a significant treatment difference. The diameter of pollen that developed in heat is significantly smaller than pollen that developed in the control conditions (Figure 2.7, Table 2.3).

Table 2.3. Results from mixed effects models for treatment differences in plants from northern populations. Bolded values indicate a significant relationship.

	Fixed Effects						Random Effects	
	Treatment			Population			Population:Genet	Population
Variable	F	df	p	F	df	p	p	p
Style + Stigma Length	**48.332**	**102.075**	**3.49E-10**	0.000	21.581	0.996	**5.46E-07**	-
Stamen Length	**67.849**	**108.688**	**4.33E-13**	**48.178**	**21.677**	**6.22E-07**	**0.025**	-
Ovule Number	0.730	110.609	0.395	**6.119**	**28.109**	**0.020**	0.130	-
Mean Pollen Diameter	**20.954**	**36.007**	**5.42E-05**	-	-	-	-	0.678
Viable Seed Number	**12.742**	**45.912**	**0.001**	0.163	12.620	0.693	**5.59E-05**	-
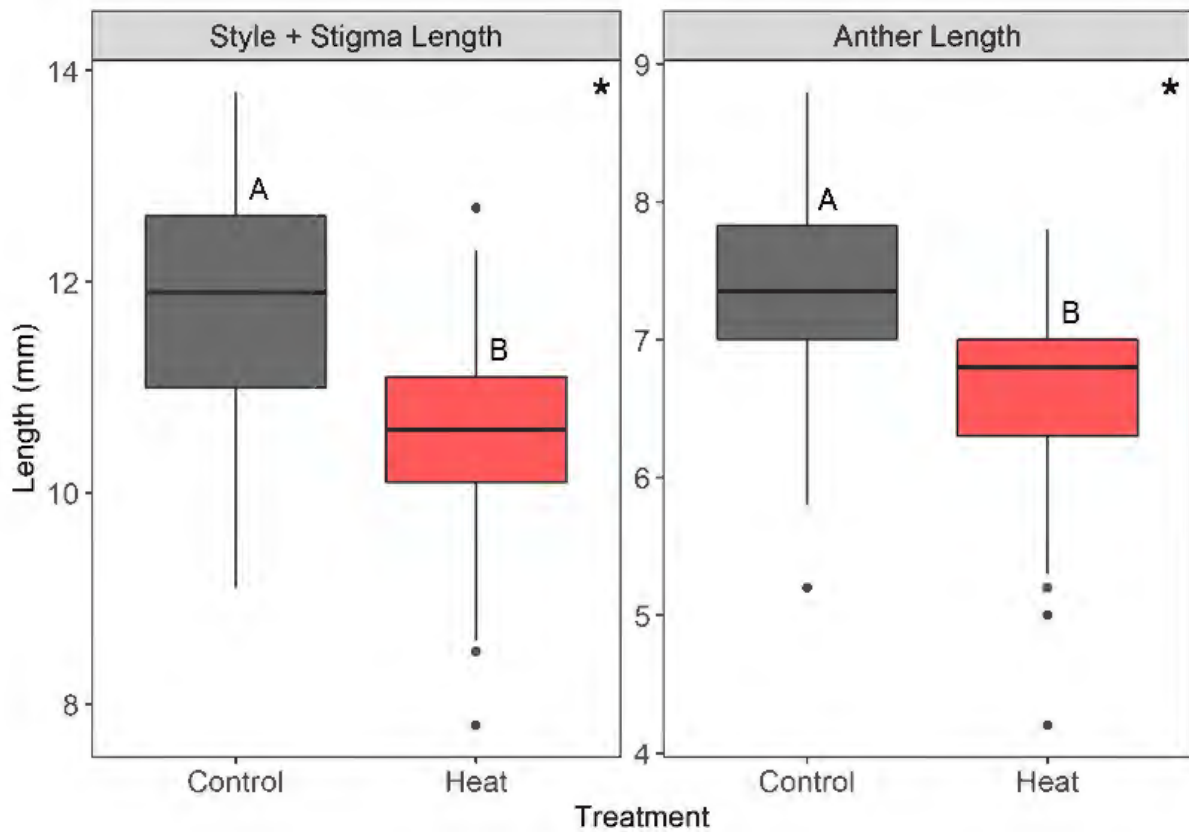
Figure 2.4. The length of the style plus stigma and length of the anther from flowers in hot and control conditions (strictly northern populations). Midline in boxplot indicates the median of the sample. Asterisks and letters indicate differences that are statistically significant. There are significant differences between regions for style plus stigma length (F_{102} = 48.33, p = 3.49-10) and anther length (F_{109} = 67.85, p = 4.33e-13).
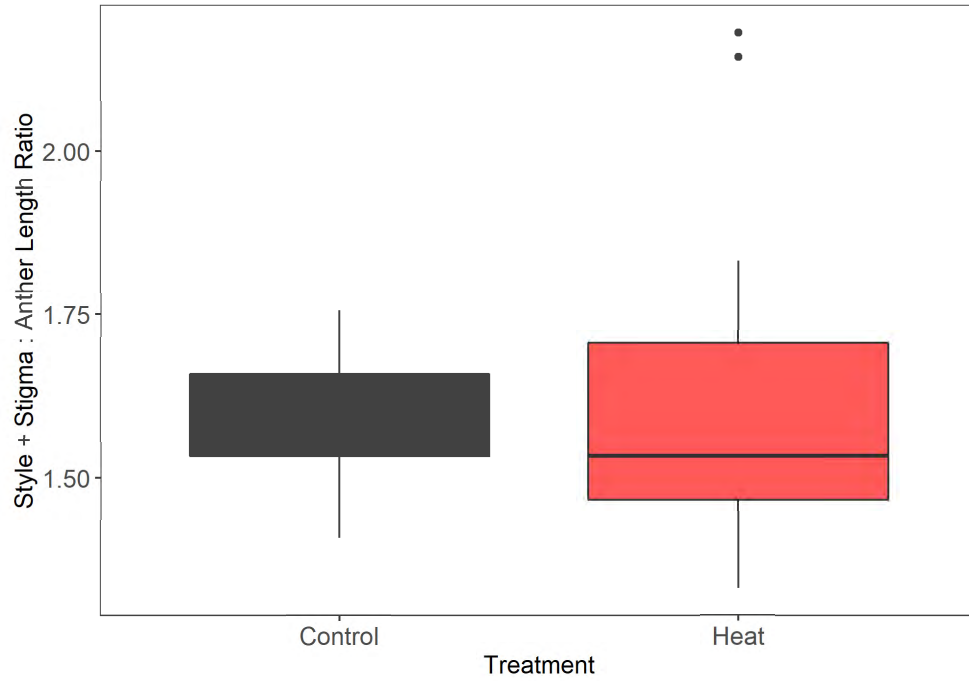
Figure 2.5. Treatment differences for the ratio of style plus stigma length to anther length. Midline in boxplot indicates the median of the sample. No significant difference between means, but there is a significant difference between variances (Bartlett's K^2 = 14.51, p = 1.40e-04).
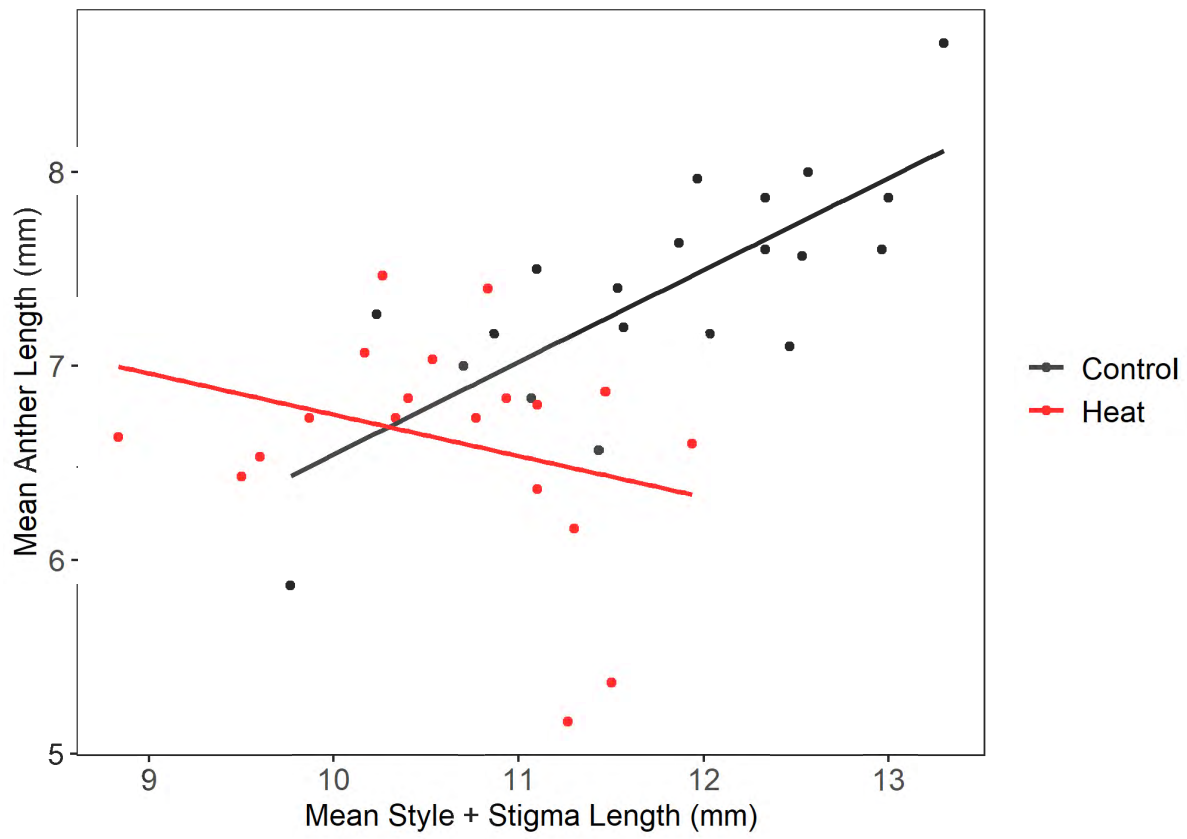
Figure 2.6. Treatment differences of correlations between the mean style plus stigma length and mean anther lengths for individual genets. The control treatment Pearson's correlation (0.761) was significant (p = 9.611e-05). The heat treatment Pearson's correlation (-0.292) was not statistically significant (p = 0.225).
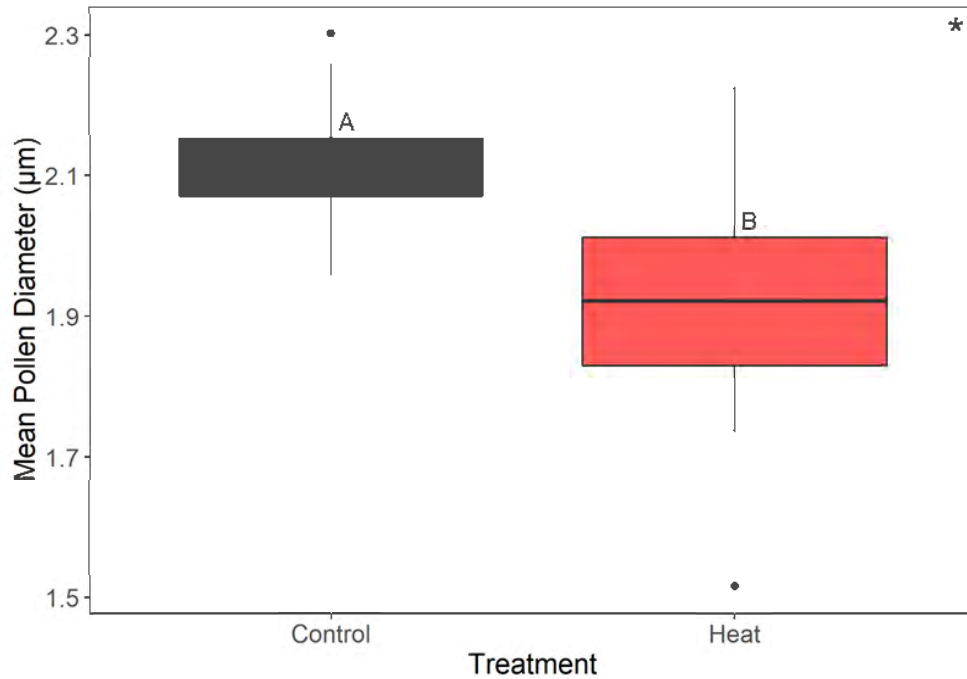
Figure 2.7. The mean pollen diameter of 100 pollen grains per flower of northern plants from flowers that developed in the respective treatment groups. Midline in boxplot indicates the median of the sample. Asterisk and letters indicate differences that are statistically significant. There was a significant difference between treatment groups (F_{34} = 25.544, p = 1.456e-05).

Post-Pollination

Pollen germination at 40°C was significantly different between regions, but not treatment groups (Figure 2.8, table 2.4). In both treatment groups, northern plants had significantly higher pollen germination than southern plants. There were no significant differences between treatment groups within northern plants for fruit set (Figure 2.9, Table 2.1). There were no significant differences between regions for viable seed count (Appendix Figure B4), but there was a significant difference between treatment groups for plants from northern populations (Figure 2.10, Table 2.2). There were fewer viable seeds produced per fruit when ovules developed in the heat treatment and underwent pollination and fertilization in the heat treatment than those in the control (25°C) treatment.

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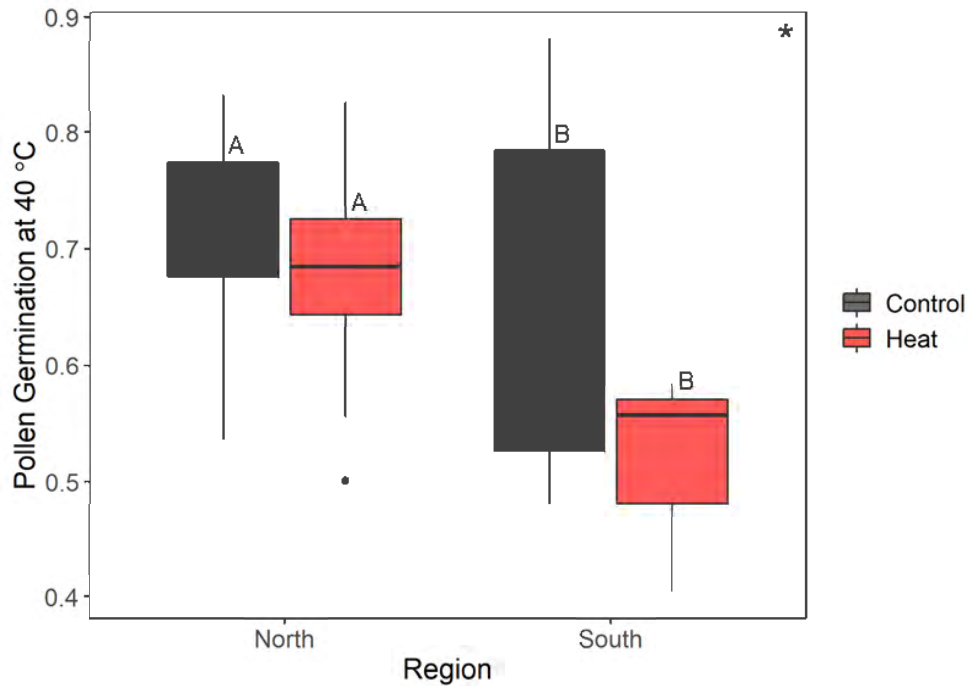
Figure 2.8. Regional differences of pollen germination at 40°C in the two treatment groups. Letters represent significant differences between groups. There was a significant difference between regions ($F_{46} = 9.180$, $p = 0.004$), but no difference between treatment groups. Sample sizes: north control (n = 20), north heat (n = 20), south control (n = 6), south heat (n = 3).

Table 2.4. Results from two-way analysis of variance for pollen germination at 40°C. Bolded values indicate a significant relationship.

		Region		Treatment	
Variable	df	F	p	F	p
Pollen Germination (40°C)	46	**9.180**	**0.004**	3.916	0.054
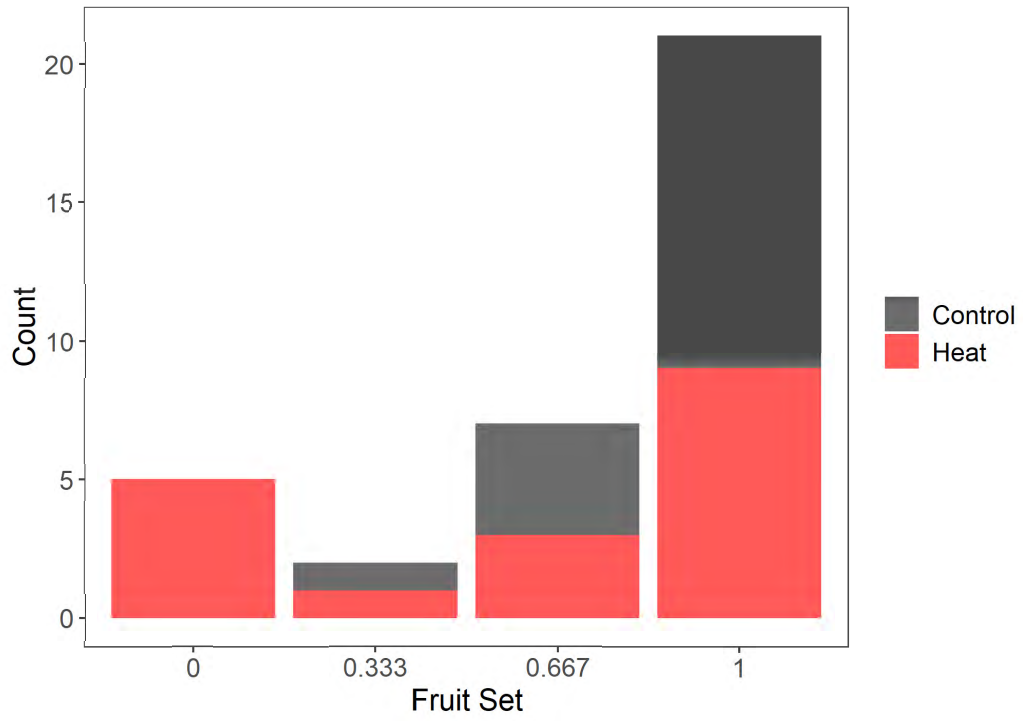
Figure 2.9. Counts of plants with the four different fruit sets based on three pollinated flowers for plants that originated in northern populations. Color indicates treatment groups.
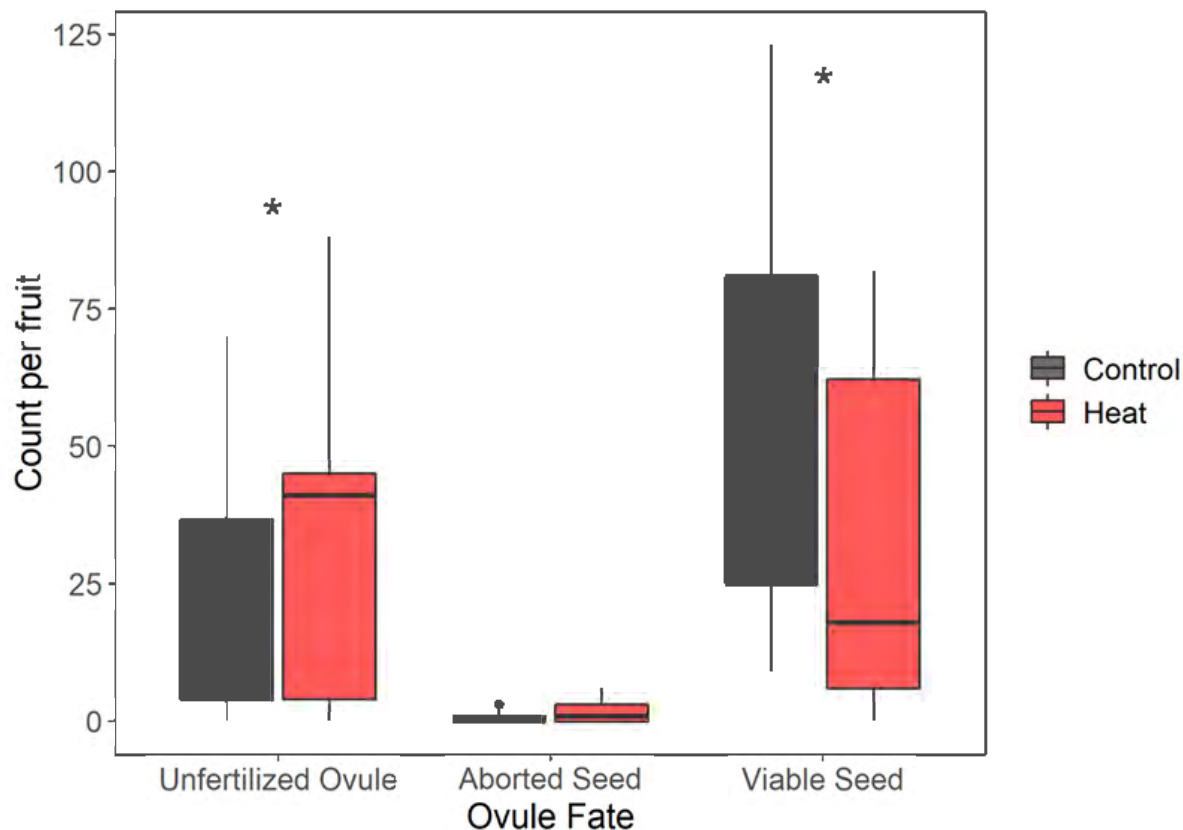
Figure 2.10. The number of aborted seeds, unfertilized ovules, and viable seeds per fruit from flowers of northern plants that developed in the respective treatment groups. Asterisks indicate differences that are statistically significant. There was a significant difference between treatment groups for unfertilized ovules ($F_{46} = 4.587$, $p = 0.038$) and viable seeds ($F_{46} = 12.742$, $p = 8.514e-04$).

Discussion

In this study we investigated how long-term heat affects sexual reproductive traits in plants from Texas and Minnesota. Based on previous studies in crop species, we predicted that heat would affect reproductive traits in *Solanum carolinense*, but more so in northern plants than southern plants. Heat did affect several of the traits including flower morphology, pollen diameter, and the number of viable seeds per fruit (Table 2.5). In all traits where we found a treatment effect, heat reduced the size or number of reproductive structures.

Table 2.5. Summary of the results for each of the dependent variables. Bolded values indicate significant relationships.

Trait	North	South	Overall
Propensity to Flower	Control = Heat	Control = Heat	**North > South**
Proportion Staminate Flowers	Control = Heat	Control = Heat	South = North
Style + Stigma Length	**Control > Heat**	NA	**South > North**
Anther Length	**Control > Heat**	NA	**South > North**
Ovule Number	Control = Heat	Control = Hot	South = North
Pollen Diameter	**Control > Heat**	NA	South = North
Pollen Germination at 40°C	Control = Heat	Control = Heat	**North > South**
Fruit Set	Control = Heat	NA	NA
Viable Seeds per Fruit	**Control > Heat**	NA	NA

In northern plants, style plus stigma length and anther length were significantly smaller in the heat treatment than the control treatment. Muller et al. (2016) found anther deformations when tomato flowers developed in mild heat (32°C/26°C). A study on blueberry found that cooler temperatures recessed anthers further in the corolla and warmer conditions increased style length (Lyrene 1994). Charles and Harris (1972) found that as temperatures increased the distance between the antheridial cone and the stigma in tomatoes decreased (longer pistil or shorter stamen). Unlike *Solanum carolinense*, the stamen of tomato flowers are fused and the style plus stigma do not extend beyond the antheridial cone. Charles and Harris found that as the stigma extended further into the antheridial cone, pollination was less likely, affecting fruit set. In horsenettle the ratio of pistil length to anther length is important because it should influence herkogamy or the distance between stigma and anther tip as well as the propensity towards self-pollination (Roldán and Ashworth 2018). We didn't specifically look at herkogamy because the ovary of the pistil and the filament of the stamen were not included in the measurements. Regardless, different sizes of the style could have implications for pollen competition (Ramesha et al. 2011) and the position of anthers relative to the stigma could affect the receipt of pollen from pollinators. We found no significant difference in the ratio between the treatments, but

flowers developed in heat did have significantly more variation than those that developed in the control. To further understand the increased variation in the heat treatment we conducted correlation analysis. We found that in the control treatment style plus stigma length was correlated with anther length, but the correlation breaks down in heat. This suggests that the fundamental proportions of floral structures are disrupted in heat. The change to position of integral reproductive structures in heat could affect rates of self-pollination and inbreeding for *Solanum carolinense.*

We found that pollen that developed in long-term low heat were significantly smaller than those in controlled (25°C) conditions. There are fitness implications for changes in pollen size as well. McCallum and Chang (2016) found evidence of pollen size influencing siring success; larger pollen grains were more competitive (sired more seeds) than smaller pollen grain size in common morning glory. Another explanation for the effect of heat on pollen diameter is that long-term heat could induce an increase in the proportion of smaller, low-ROS (reactive oxygen species) pollen. There have been multiple studies with evidence suggesting that pollen grains within a species fall into one of two categories. Rutley et al. (2022) described this phenomenon as the "two-basket" model, where the baskets are low-ROS and high-ROS pollen and are related to the dual nature of pollen found in other studies (Jegadeesan et al. 2018; Luria et al. 2019). High-ROS pollen have higher metabolic rates, are typically larger in size, and readily germinate once mature. On the other hand, low-ROS pollen grains are partially dehydrated with low metabolic rates, are smaller in size, and remain dormant when environmental conditions are not favorable for germination. While low-ROS pollen may be adaptive in locations with unfavorable conditions, the smaller pollen grain size and reluctancy to germinate is maladaptive under favorable conditions establishing a trade-off that influences

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fitness. Either through pollen dormancy or reduction in pollen performance, size affects siring success and therefore, fitness. If *Solanum carolinense* produces a higher number of low-ROS pollen when flowers develop in heat, then pollen may be more likely to fertilize ovules by avoiding times in the day when temperatures are too high. However, the smaller pollen grain size may also imply that genets with high proportions of low-ROS pollen are less competitive than pollen of other genets.

We found that heat during the development of maternal tissues and fertilization reduced the number of viable seeds per fruit (Figure 2.10, Table 2.3). Previous studies have found mixed responses to heat in tomato. Xu et al. (2017) found that heat had little influence on seed number compared to other reproductive traits. Din et al. (2015) found that seed set was reduced in heat, especially in more temperature sensitive accessions and attributed this difference to heat reducing pollen viability, or pollen tube growth in the style. Since ovule number was not affected by heat in our study, the decrease in viable seed number and increase in unfertilized ovules we attained, might also be a product of low pollen viability at 32°C compared to 25°C. Viable seeds and unfertilized ovules dominated the counts, with few aborted seeds (Figure 2.3). This suggests that male viability and pollen tube growth at 32°C after pollen developed at 25°C may be the limiting factor, and not female viability. Jiang (2019) also found disparity between ovule and pollen viability of peas when exposed to heat, ovules maintained viability in heat stress, while pollen viability decreased.

These differences in phenotype strictly due to environmental change suggests that phenotypic plasticity accounts for some of the variation in reproductive traits within this species. Since these traits are tied to fitness, environment could obscure evolutionary responses tied to natural selection by effectively decreasing the additive genetic variance in reproductive traits.

Phenotypic plasticity can partially dissociate genotype from phenotype through molecular mechanisms such as histone modification or regulation of transcription factors (Nicotra et al. 2010). However, phenotypic plasticity itself can be an adaptive trait and in our study may be the result of gene x environment interactions (Schlichting 1986). Molina-Montenegro and Naya (2012) found that phenotypic plasticity of several traits, including photosynthesis, water use efficiency, number of flowers, seed output, dry biomass, and foliar angles, increased in populations as latitude of origin increased. The increase in plasticity with latitude was justified by the authors using the climate variability hypothesis, which states that organisms have higher levels of phenotypic plasticity in locations with more variable conditions (Janzen 1967; Schlichting 1986). Since environmental conditions are rapidly changing, increased phenotypic plasticity may be advantageous and thus adaptive just as in locations with variable conditions, such as at higher latitudes. We were therefore, interested in gene x environment interactions in *Solanum carolinense* plants and whether location of origin influenced how these plants respond to heat.

There were differences between plants from the two regions for the propensity of a plant to flower under control conditions, the length of male and female floral structures, and pollen germination at 40°C (Table 4). In both heat and the control treatments, almost all the northern plants flowered. On the other hand, only one population from the southern region had plants that consistently flowered. Since temperatures in Texas are generally high and sexual reproduction seems to be disrupted by heat in this species, populations in Texas may have evolved to allocate more resources to vegetative growth and asexual reproduction through clonal recruitment than sexual reproduction. Another explanation for the dominance of asexual reproduction in some populations may be due to the location of these populations relative to the range margin for

Solanum carolinense. Eckert (2001) reviewed the variation in modes of reproduction within a species, including how the modes of reproduction vary across a species range. Ecological pressures at the range margin may decrease sexual reproductive success, favoring clonal reproduction. Barrett (2015) also reviewed clonality and sexual reproduction and mentioned that mechanisms of clonality are labile and there are few evolutionary constraints for the resources allocated to flowering or vegetative growth. Therefore, even populations within a species can differ greatly between the modes of clonality.

For the plants that did flower in both regions, there was no significant difference for the propensity to flower following heat (32°C) vs control (25°C) temperatures (Figure 2.1, Table 2.1). These results suggest that the propensity to flower phenotype is determined by local adaptation through selection acting on genetic variation rather than environmental effects.
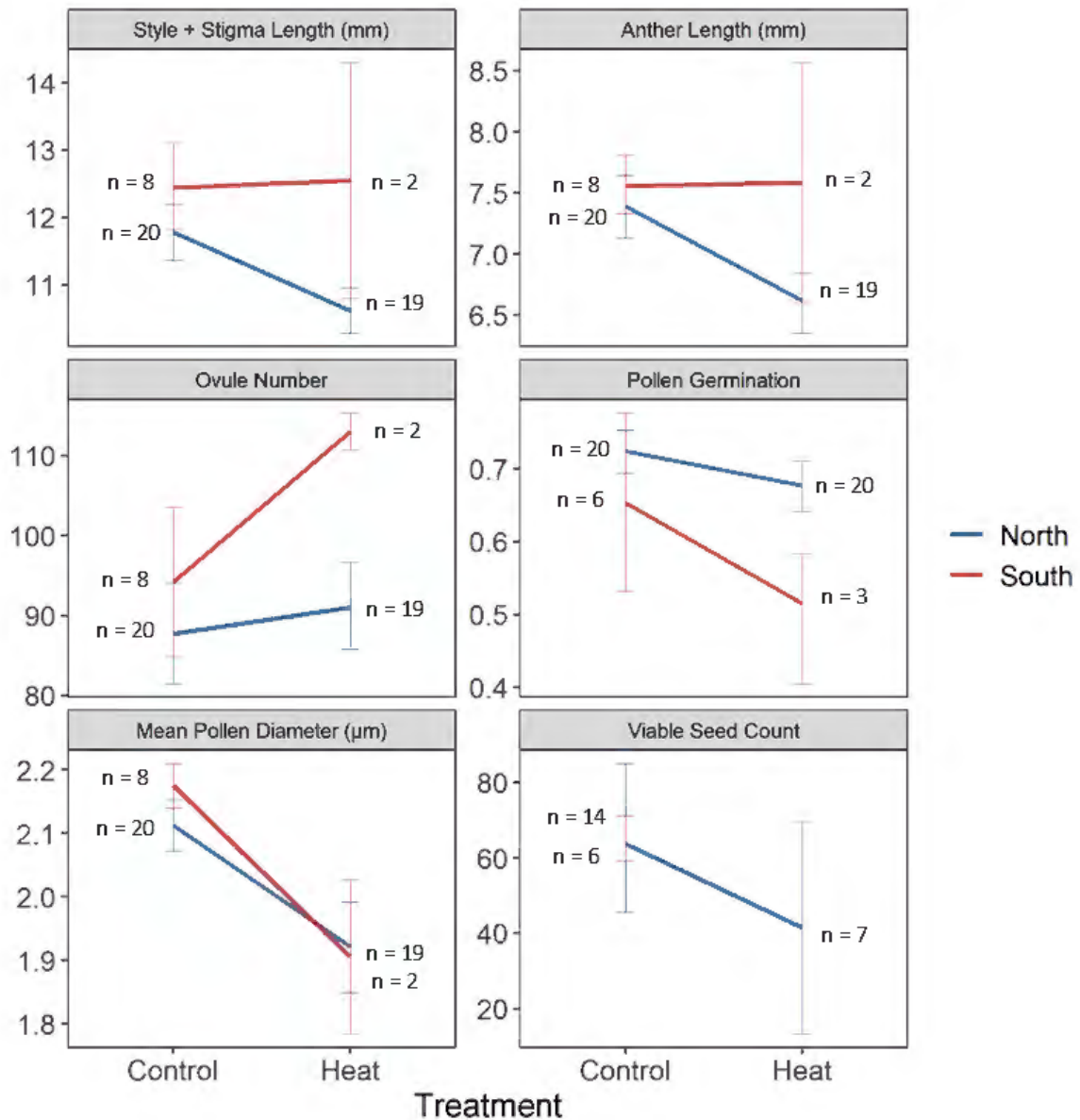
Figure 2.11. Reaction norms for variables in control (25°C day / 25°C night) and moderate heat (32°C day / 25°C night) environmental conditions. Colors indicate region of origin. Solid lines connect mean for the variable across treatments. Error bars indicate the mean standard error of a nonparametric bootstrap for the confidence interval.

The male and female floral structures were larger in plants from the south than those from the north. Based on qualitative observations in the field (Figure 2.12), the fruit size seems to also differ between the two regions. Larger floral structures and fruit may provide more protection to ovules and seeds in conditions with higher temperatures. Style plus stigma length

and anther length also differed between the two treatment groups for northern plants. Because of low sample size, we did not analyze the responses of plants from the two regions to heat treatment. However, based on the reaction norms, there may be evidence of variable responses to heat among populations in the two regions (Figure 2.11). For both style plus stigma length and anther length, northern plant structures decreased when grown in heat, while structures in southern plants maintained the same average, but varied more between genets. This suggests that there may be a gene x environment interaction involved.

Figure 2.12. Comparison of fruit sizes collected in the field from plants in Minnesota and Texas.

The reaction norm (Figure 2.11) for ovule number appears to also follow the gene x environment interaction pattern, with the elevated mean number of ovules in heat from the few southern plants included relative to the mean of the northern plants. Northern and southern plants appear to have similar responses to heat in pollen diameter. The reaction norms for pollen germination at 40°C were almost parallel with differences in trait means between regions in both treatments, suggesting that the response in southern and northern plants may be comparable.

Pollen germination at 40°C did differ significantly between the two regions. These results match those in the first study (Chapter 1) where Tmax for northern plants was higher than for southern plants. One explanation for this result is that southern plants have adapted to higher temperatures by producing a higher proportion of low-ROS pollen than plants from the north to selectively germinate and avoid high temperature stress by only germinating in favorable conditions. Our study confirmed that the temperature at which pollen develops doesn't affect germination; pollen either does or does not germinate at 40°C regardless of how warm it was during development. Muller et al. (2016) found that long-term mild heat during development did reduce pollen germination in tomato. However, we presume they tested germination after incubation at room temperature and not at high temperatures, which may be one reason our results differed from this study and others that also found that development in heat reduced pollen viability (Jiang et al. 2019; Poudyal et al. 2019; Sato et al. 2006; Xu et al. 2017).

Conclusions

Overall, our results indicate that environmental conditions and conditions associated with climate change affect reproductive traits and processes in *Solanum carolinense* and ultimately fitness. Long-term heat during flower development reduced the size of floral structures and pollen diameter, and after pollination, reduced seed production. Our findings imply that as temperatures rise, male and female success of sexual reproduction may decline in this species and potentially others. As environment directly influences fitness, adaptation of plants to a warmer world may not be a simple matter of certain environments favoring alleles advantageous for thermal tolerance. We did find evidence of local adaptation between the two regions for the propensity to flower, pollen germination at 40°C, and in the size of floral structures. Since both the region of origin and treatment group affected flower morphology, there is some evidence

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suggesting a gene x environment interaction. Understanding the sources of variation driving responses to environmental change is important in predicting how and if species will persist in this rapidly changing world.

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CHAPTER 3: THE TIMING OF SNOWMELT AND AMOUNT OF WINTER PRECIPITATION HAVE LIMITED INFLUENCE ON FLOWERING PHENOLOGY IN A TALLGRASS PRAIRIE[1]

Abstract

Prior studies showed that the timing of flowering in temperate angiosperms has shifted due to climate change. Sensitivity in flowering phenology to changing temperatures has been well-documented in temperate communities, but not the effect of changing precipitation patterns. The exception is the relationship between snowpack and flowering in alpine environments. The timing of flowering had strong associations with winter precipitation and when snowmelt occurred. Based on these results, we hypothesized that populations in northern latitudes, characterized by strong seasonality and winter snowfall would demonstrate associations between winter precipitation and flowering phenology. We combined a historical data set of first flowering dates with climatic data from the same time period to construct a structural equation model, testing hypotheses on the relationships between winter precipitation, temperature, and flowering phenology. While temperature had a strong effect on flowering phenology, winter precipitation had a significant relationship with few species. These results suggest future changes in precipitation will have differing consequences depending on region.

Introduction

One of the best documented biotic effects of climate change is changing flowering phenology, or flower timing (Cleland et al. 2007; Miller-Rushing and Primack 2008; Parmesan

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[1] The material in this chapter was co-authored by Emma Chandler and Steven Travers. Emma Chandler had primary responsibility for model construction using a previously published data set, analyzing data, developing conclusions, and drafting and revising all versions of this chapter. Steven Travers served as proofreader and checked the methods used by Emma Chandler in constructing and analyzing the models. This article has been published as Chandler, E.K., and Travers, S.E. 2022. The timing of snowmelt and amount of winter precipitation have limited influence on flowering phenology in a tallgrass prairie. Botany **100**(3): 301-311. doi:10.1139/cjb-2021-0102.

2006; Schwartz et al. 2006; Wolkovich et al. 2012). Flowering phenology is important for plant-pollinator interactions, as asynchrony between flower timing and pollinator emergence can be detrimental for plant reproduction and pollinator health (Cleland et al. 2007; Kharouba and Wolkovich 2020; Kharouba et al. 2018; Visser and Gienapp 2019). Asynchrony is problematic for plant and pollinator populations, the communities they inhabit, and the ecosystem services they provide. Plant reproductive success has also been shown to be dependent on flowering phenology. Schemske et al. (1977) found that *Claytonia* sp. had peak seed set per ovary at the end of April with seed set per ovary decreasing in organisms with early or later maturation (Schemske 1977). Thus, it is important to understand climate change effects on flowering phenology, in part because there is the potential for changes in evolutionary and conservation dynamics of natural populations.

Flowering can be triggered by several environmental cues such as photoperiod, amount and timing of precipitation or soil moisture, and temperature (Rathcke and Lacey 1985). Climate change may alter these environmental cues resulting in the changing flowering phenology. A majority of studies on flowering phenology and global climate change have focused on the effects of temperature change (Wolkovich et al. 2012).

In prairies, flowering phenology has been strongly linked with temperature (Henebry 2003; Dunnell & Travers 2011; Henebry 2013; Reed et al. 2019). Reed et al. (2019) found advancement of phenological events due to experimental warming in prairies of the Pacific Northwest. Dunnell and Travers (2011) also found prairie species shifting both earlier and later in response to temperature changes in the Midwest (Dunnell and Travers 2011). However, temperature is not the only climate or environmental variable affected by the accumulation of greenhouse gases. Changes in precipitation patterns have also been predicted as a result of a

warming globe. For example, overall precipitation is expected to increase in the Midwest (Pachauri and Meyer 2014). In the Northern Great Plains, where winters can be relatively long and harsh, changes in precipitation have the potential to influence plants as snow. Snow could affect flowering phenology in several ways. During bud emergence, snow cover decreases the amount of sunlight plants receive but also insulates buds from frost events. When snow melts and sunlight can reach the bare ground (no snow cover), soil temperature should increase quickly promoting plant growth. Earlier seed germination and plant growth in turn could lead to earlier starts to other subsequent life history stages including flowering (but see Park 2016). Additionally, substantial amounts of moisture are released into the soil and supply plants well into the summer. Warmer winter temperatures could lead to a shift from snow to rain, leading to changes in the overall timing of when snow melts and sunlight first reaches the soil in spring.

Snowpack has been found to alter flowering phenology in montane and tundra species. Inouye et al. (2002) found a significant correlation between date of first bare ground (a snowpack of zero) and date of first flowering for *Delphinium barbeyi*, a subalpine species. Similarly, Sherwood et al. (2017) found advanced emergence, bud break, and flowering in a montane forb when snowpack was reduced. However, the snow removal treatment also resulted in increased frost damage among buds due to the lack of insulation from snow and freezing night temperatures (Sherwood et al. 2017). Species in the tundra had similar responses. Bjorkman et al. (2015) found that snowmelt was strongly related to flowering time for four arctic tundra species, while temperature was not a consistent driver of flowering phenology.

Though there have been several studies on the effects of snowpack on flowering phenology for montane and tundra species, from our understanding, no studies have been conducted on the effects of snowpack on the flowering of plant species growing in lowland

grasslands. The boundaries of northern tallgrass prairies where the study described here is based are dependent on precipitation patterns and exist at an intermediate point along an increasing precipitation gradient from the rain shadow of the Rocky Mountains in the west to the wetter forests of central and eastern Minnesota (Tester and Keirstead 1995). The tallgrass prairie of Clay County, MN experienced between 78 and 300 cm of precipitation a year (average 108 cm snowfall annually) and a dramatically seasonal pattern of cold winter temperatures and warming spring temperatures leading to plant growth in the months of March and April.

Seasonal precipitation and temperature patterns in the northern Great Plains suggest that the amount of snow accumulating in the winter and the timing of snowmelt could play an important role in the timing of plant life history phases like germination and flowering. In western Minnesota where this study was conducted snowfall peaks in December and begins to decline in March when spring temperatures begin to increase (Figure 3.1). The timing of complete snowmelt where the ground is bare then varies through March and into May depending on the year. This study examines the effect that snow accumulation and the timing of zero snowpack have on flowering phenology for 24 perennial herbs that are typical of northern tallgrass prairies or woodland species that were observed in the Bluestem Prairie Reserve. The goals of this study are:

1. Simultaneously assess direct and indirect effects of temperature and winter precipitation variables on flowering phenology using path analysis.

2. Determine the importance of bare ground as an intermediate step between winter precipitation and flowering phenology.

3. Compare phenological responses to precipitation in the form of snow and temperature, whether advanced or delayed, across several species using a long-term data set.

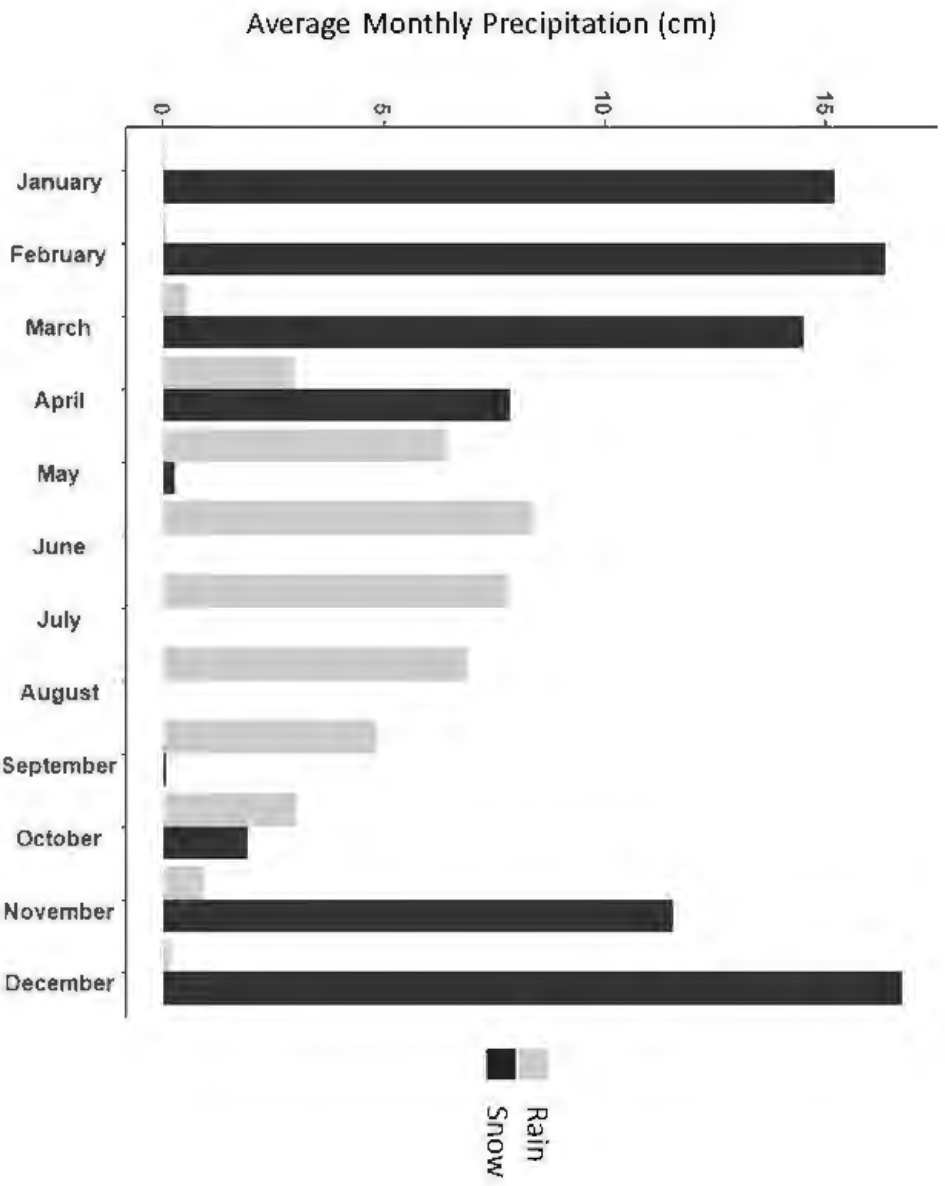Figure 3.1. Average monthly snowfall and rainfall in Fargo, North Dakota for the years of this study (1942-1961; 2012-2020).

Methods

Data Collection

We used historical data collected by O.A. Stevens and S. Travers to initially create a dataset of first flowering days (FFD) for 24 flowering plant species. The observations were made at Bluestem Prairie (https://www.dnr.state.mn.us/snas/detail.html?id=sna00996), a tallgrass

prairie site in Clay County Minnesota that has been a Nature Conservancy preserve since 1975. Individual data points represent the day of the year on which a given plant species was observed flowering at the site, although all species were not observed in all years. The Stevens dataset represents continuous data from 1910 to 1961 (Dunnell and Travers 2011); subsequent observations are from 2012 through 2020. Thus, there is a 52-year gap in data at the end of the past century. The plant species analyzed in this study were limited to those that met a series of minimum data requirements. The focal species had a minimum of five years of observations and at least one observation prior to 1962 and one after.

In order to quantify different environmental variables related to annual climate patterns, we used daily climate data collected in Fargo, North Dakota, USA, as part of the National Atmospheric and Oceanic Administration (NOAA) National Climatic Data Center (NCDC) observing network (http://www.ncdc.noaa.gov/oa/ncdc.html). The climate data collection site (46 ° 56' N, 96 ° 49' W) is located at the Fargo International Airport, 32 km west of the flowering observation site. The climate dataset includes daily estimates of maximum and minimum temperature, snowpack (0 was considered bare ground or ground with no snow cover), and snowfall beginning in 1942. However, snowpack data is unavailable for 1997 through 2004. As a result, we were able to analyze data for a total of 29 years (1942-1961 and 2012-2020).

Climate Variables

We used the raw climate data to calculate four variables regarding annual patterns of temperature or winter snowfall. The first climate variable we calculated for each year was intended to quantify the relative warmth of the late winter/early spring season, when the earliest flowering on the prairie is initiated. Spring Temperature (ST) was the average temperature over the course of February, March, and April. Three different winter precipitation variables were

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calculated. The winter snowfall amount for a given year (TSNOW) was calculated as the sum of daily snowfall (cm) over the first 90 days. A second variable associated with winter snowfall was the Date of Bare Ground (DOBG) or the day of the year when snowpack first reached zero. Eight records indicated a short period, one to two days, of snowpack late in the season which were excluded. The third variable associated with winter snowfall was Snowpack (cm) on Day X (SPDX), a variable designed to estimate the extent of snowpack just prior to the growing season. To calculate SPDX for each species we used linear regression and model selection to identify the day in March with snowpacks that best predicted the first flowering day (FFD) for that species. We ran separate linear regressions where FFD was the dependent variable and snowpack on day X was the independent variable for each day in March. Akaike Information Criterion (AIC) values were determined for each regression and the model associated with the lowest AIC value was chosen and used to assign the day in March consistently used for SPDX in that species. Thus, SPDX values increase with increasing snowpack and decrease with decreasing snowpack on the selected day of March. The most predictive day was determined separately for each plant species. We used this variable to maximize the explanatory power of snowpack on flowering in a month when temperatures and snowpack are rapidly changing. Each of the four climate variables were not independent of each other.

Because climate change has been predicted to lead to increases in both spring temperature and precipitation, we calculated mean values of three environmental variables separately for the earlier (1942-1961) and later (2012-2020) sampling periods: mean spring temperature (February, March and April), mean snowpack on March 15, and the sum of snowfall for the first 90 days of the year (TSNOW). We also calculated mean first flowering day for the two sampling periods for each species.

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Model Development

Our goal was to use Structural Equation Modelling (Grace 2006) to simultaneously assess the relationships between each of the four climate variables and the first flowering day (FFD) of the focal plant species, given the covarying nature of the climate variables (Figure 3.2). We used the *lavaan* package (Rosseel 2012) in R (R Core Team 2020) to incorporate path analysis and examine the relationships among the climate variables and the dependence of FFD on each of the climate variables individually. In our initial, full model, we included ST and TSNOW as exogenous variables and DOBG, SPDX, and FFD as endogenous variables (Figure 3.3). The assumption was that FFD could have direct and indirect effects from both temperature (ST) and winter snowfall (TSNOW), through their indirect effects on snowpack in March (SPDX) and the date at which the snow melted (DOBG). The model included regressions for each endogenous variable, variances within all variables, and residual covariances between the exogenous variables. We considered both direct and indirect regressions. To best compensate for missing data points over the course of the 29 years analyzed, we applied full information maximum likelihood (FIML) estimation to determine path coefficients and model statistics. We used regression estimates for indirect and direct effects to interpret the relationships between latent variables in each of the species.

In order to identify the best overall structural equation model for analyzing relationships among climate and flowering variables we used a model selection approach and compared the fit of the full model (above) to three other reduced models that omitted either DOBG, SPDX, or ST. After using the *lavaan* program to conduct path analyses of the three reduced models, we used AIC to select the model of the four that best represented the data, based on the lowest AIC value.
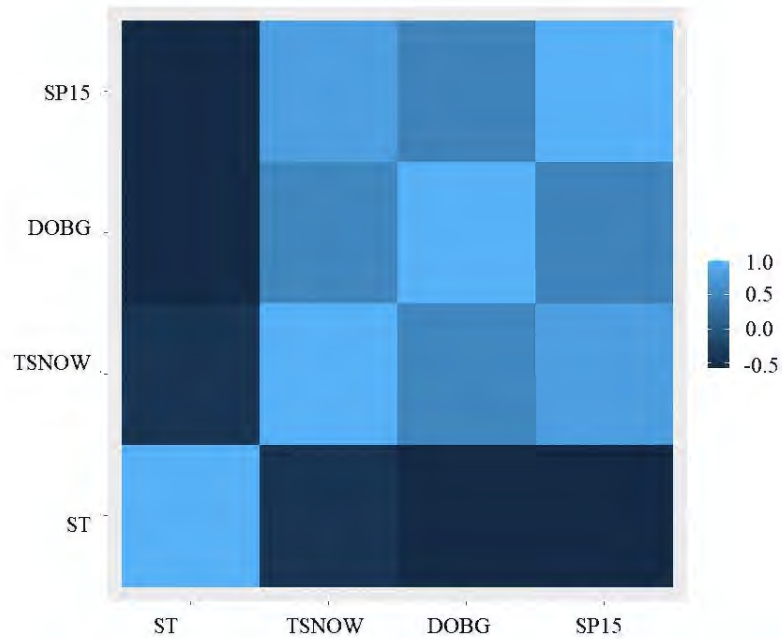
Figure 3.2. Correlation matrix showing strength of relationships among environmental variables: ST (spring temperature), TSNOW (total snowfall), SP15 (snowpack on March 15), DOBG (date of first bare ground). SP15 was used in place of SPDX to avoid species specificity. The magnitude of the correlation coefficient is indicated by the hue of color in each square.
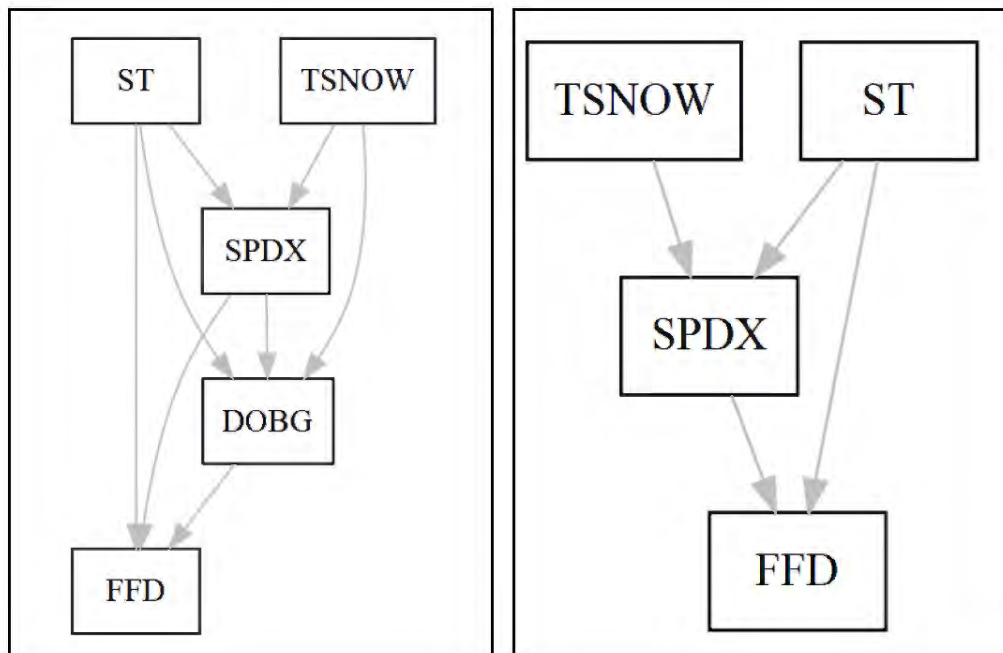
Figure 3.3. Full path diagram with ST (spring temperature), TSNOW (total snowfall), SPDX (snowpack on day X), DOBG (date of first bare ground), and FFD (first flowering day) (a) and reduced path diagram excluding DOBG (b). The reduced path diagram was used for the structural equation modeling.

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Results

Variation in First Flowering Day (FFD)

We identified 24 flowering plant species in the Stevens Data set that met the criteria for analysis described in the methods. None of the species were observed in every year of the survey; sample size by species ranged from 5 to 13. The first flowering day (FFD) varied extensively both among years within a species and among species. Median FFD varied across the species from a low of 123 (May 2) to a high of 206 (July 24) and included early, mid, and late spring flowering species (Figure 3.4).
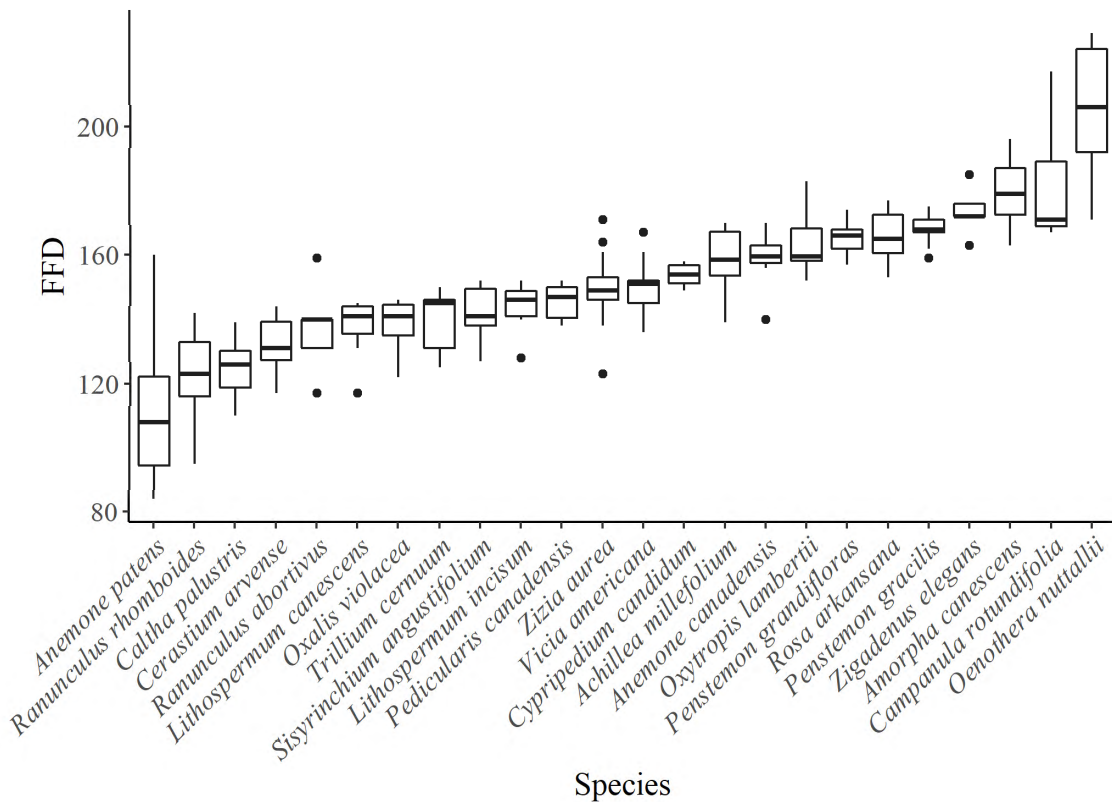
Figure 3.4. Box plots of the first flowering day (FFD) of 19 plant species from the Bluestem Prairie reserve in Clay County, MN. Observations were made between 1942-1961 and 2012-2020. Box plots indicate distribution quartiles and standard error bars.

Estimates of mean FFD in the first sampling season (1942-1961) and the second sampling season (2012-2020) indicated shifts in the flowering time of species with over time

(Table 3.1). Eleven of 17 species shifted earlier in their flowering with a maximum of over two

weeks for *Ranunculus rhomboides*. The remaining six species either did not shift mean flowering

time or shifted to flowering later (e.g., two weeks for *Oxytropis lambertii*). Concurrently, both

temperature and precipitation changed as predicted (Table 3.1). The mean spring temperature

increased 41% between the two time periods. Winter precipitation also increased as indicated by

a 51% increase in mid-March snowpack and a 43% increase in total snowfall.

Table 3.1. Comparisons of variables between early sampling periods (1942-1961) and later
sampling periods (2012-2020). Variables include mean estimates of first-flowering day (FFD) by
species and three environmental variables: Spring temperature (Feb, Mar, April), Snowpack in
mid-march and Total snow.

Species	Mean FFD (1942-1961)	Mean FFD (2012-2020)	Shift (# days)
Ranunculus rhomboides	134.7	117.2	-17.5
Achillea millefolium	170.0	155.6	-14.4
Zizia aurea	152.6	145.2	- 7.4
Penstemon gracilis	170.5	165.6	- 4.9
Cypripedium candidum	157.0	152.3	- 4.8
Ranunculus abortivus	140.0	136.8	- 3.3
Anemone canadensis	161.0	158.2	- 2.8
Sisyrinchium angustifolium	144.0	141.5	- 2.5
Pedicularis canadensis	147.0	144.8	- 2.2
Oxalis violacea	140.0	137.8	- 2.2
Penstemon grandifloras	165.3	164.6	- 0.7
Rosa arkansana	165.7	166.0	0.3
Vicia americana	150.0	151.5	1.5
Zigadenus elegans	173.3	175.0	1.7
Lithospermum incisum	140.0	144.2	4.2
Cerastium arvense	128.0	133.6	5.6
Oxytropis lambertii	152.0	166.2	14.2
Environmental Variable	Mean+SE (1942-1961)	Mean+SE (2012-2020)	Shift (%)
Mean Spring Temperature (°C)	-4.1 (0.5)	-2.4 (1.2)	41
Snowpack-3/15 (cm)	9.0 (2.5)	13.6 (6.7)	51
Total snow (cm)	41.6 (4.6)	59.4 (9.8)	43

Model Selection

Model selection comparisons of AIC values among the three reduced models and the full model indicated that the best explanatory model was the reduced model which excluded DOBG. This indicates that the influence of temperature and snowfall on flowering date was relatively negligible through an indirect effect on when the ground first became bare of snow each spring.

Based on the chi-squared statistic estimating goodness of fit (Grace 2006) (*lavaan*), the reduced model was a good representation of the relationships among the exogenous and endogenous variables (χ^2 p-value > 0.05) for 15 of the 24 (Table 3.3). For another fit index (CFI), the model was a good fit (> 0.95) for 14 of the 24 species (Table 3.3). All species were included in our analysis.

The results of path analysis are presented in Figure 3.5, showing direct effects, and Table 3.2, showing indirect effects, for each species arranged by order of seasonal flowering sequence. The direct relationship between ST and FFD was significant in 12 out of 24 species analyzed suggesting an important role of temperature in determining flowering time for a majority of species. All twelve species with significant ST effects had negative regression coefficients, indicating that warmer temperatures earlier in the year led to earlier flowering. Most species with a significant relationship between ST and FFD were early flowering species. For the direct effect of ST on SPDX, 12 of 24 species were significant and most regression coefficients were weakly negative (Figure 3.5). This means that the higher the spring temperature, the lower the snowpack on day X. Only one species (*Ranunculus abortivus*) had an indirect effect of spring temperature on flowering time (FFD) through intermediary effects on the snowpack in March (SPDX).
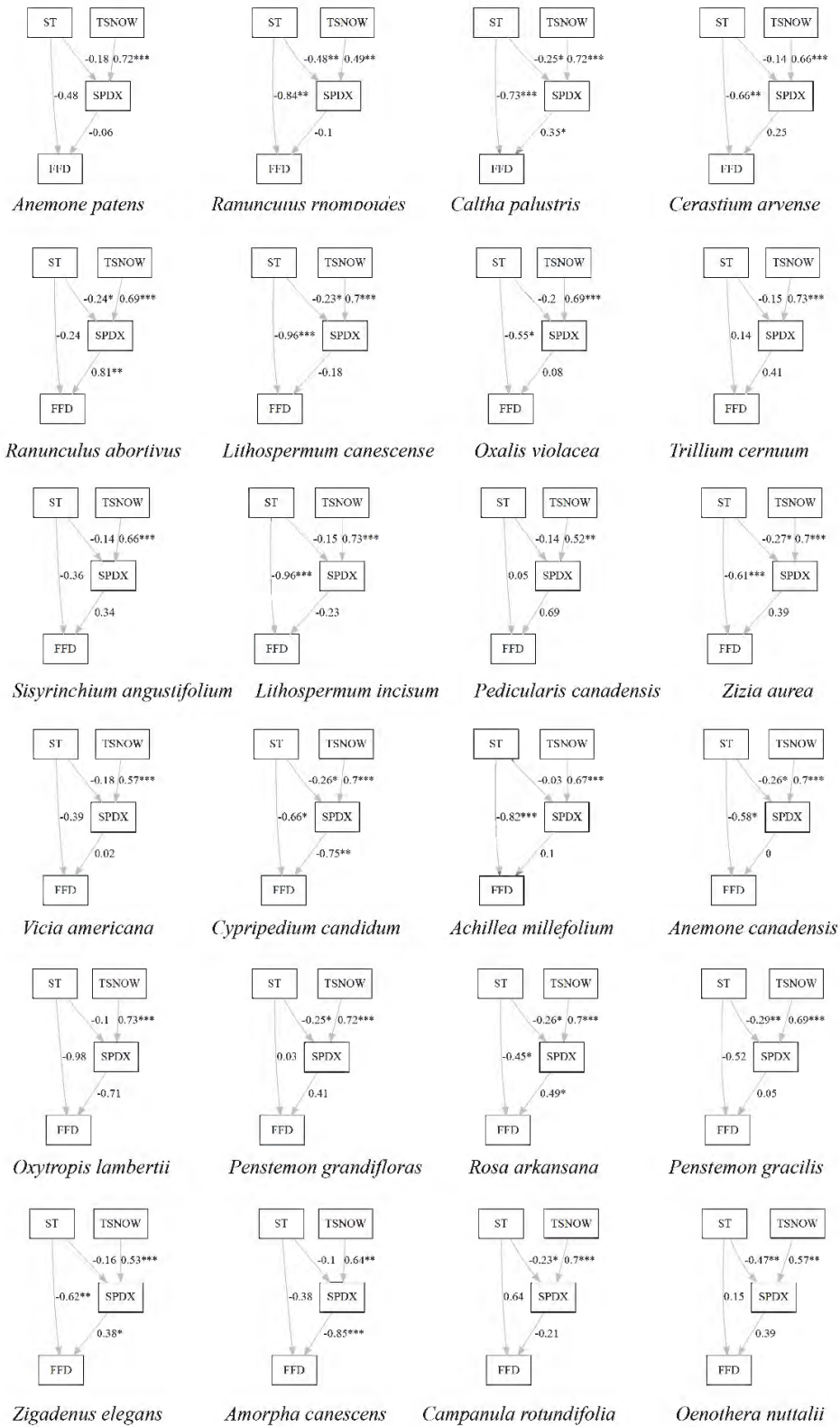
Figure 3.5. Path diagrams with standardized regression coefficient estimates labeled. Number of asterisks indicate level of significance for p-value: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Table 3.2. Statistical summary of standardized regression coefficients for indirect effects for reduced model. Number of asterisks indicate level of significance for p-value: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Species	Indirect effects of TSNOW on FFD mediated by SPDX	Indirect effects of ST on FFD mediated by SPDX
Anemone patens	-0.040	0.010
Ranunculus rhomboides	-0.049	0.048
Caltha palustris	0.248	-0.085
Cerastium arvense	0.168	-0.035
Ranunculus abortivus	0.553***	-0.197*
Lithospermum canescens	-0.107	0.068
Oxalis violacea	0.059	-0.016
Trillium cernuum	0.297	-0.061
Sisyrinchium angustifolium	0.222	-0.046
Lithospermum incisum	-0.166	0.034
Pedicularis canadensis	0.359*	-0.097
Zizia aurea	0.277*	-0.105
Vicia americana	0.012	-0.008
Cypripedium candidum	-0.524**	0.193
Achillea millefolium	0.065	-0.003
Anemone canadensis	0.002	-0.001
Oxytropis lambertii	-0.514***	0.067
Penstemon grandifloras	0.148	-0.096
Rosa arkansana	0.342*	-0.126
Penstemon gracilis	0.033	-0.014
Zigadenus elegans	0.201	-0.06
Amorpha canescens	-0.548**	0.088
Campanula rotundifolia	-0.146	0.049
Oenothera nuttallii	0.224	-0.182

The relationship between snowfall in the winter months (TSNOW) and snowpack in March (SPDX) was a predictably strong one (Figure 3. 2). The path coefficient between the two variables was positive and significant for all species. TSNOW was expected to be related to SPDX because both describe winter snowfall. However, only 6 out of the 24 species had a significant relationship between SPDX and FFD. In two species (*Cypripedium candidum* and *Amorpha canescens*) the path coefficient was negative indicating that relatively large amounts of winter snowfall led to earlier flowering compared to years when there was less winter snowfall

(Figure 3.5; Figure 3.6). However, in the other four species (*Caltha palustris*, *Ranunculus abortivus*, *Zigadenus elegans* and *Rosa arkansana*) the regression coefficients were positive indicating that greater snowpack delayed flowering (Figure 3.5; Figure 3.6). There were significant indirect effects of TSNOW on FFD through the intermediary effects of SPDX seven of the 24 species (Table 3.2). Four of the species with significant indirect effects had significant direct effects for SPDX on FFD.
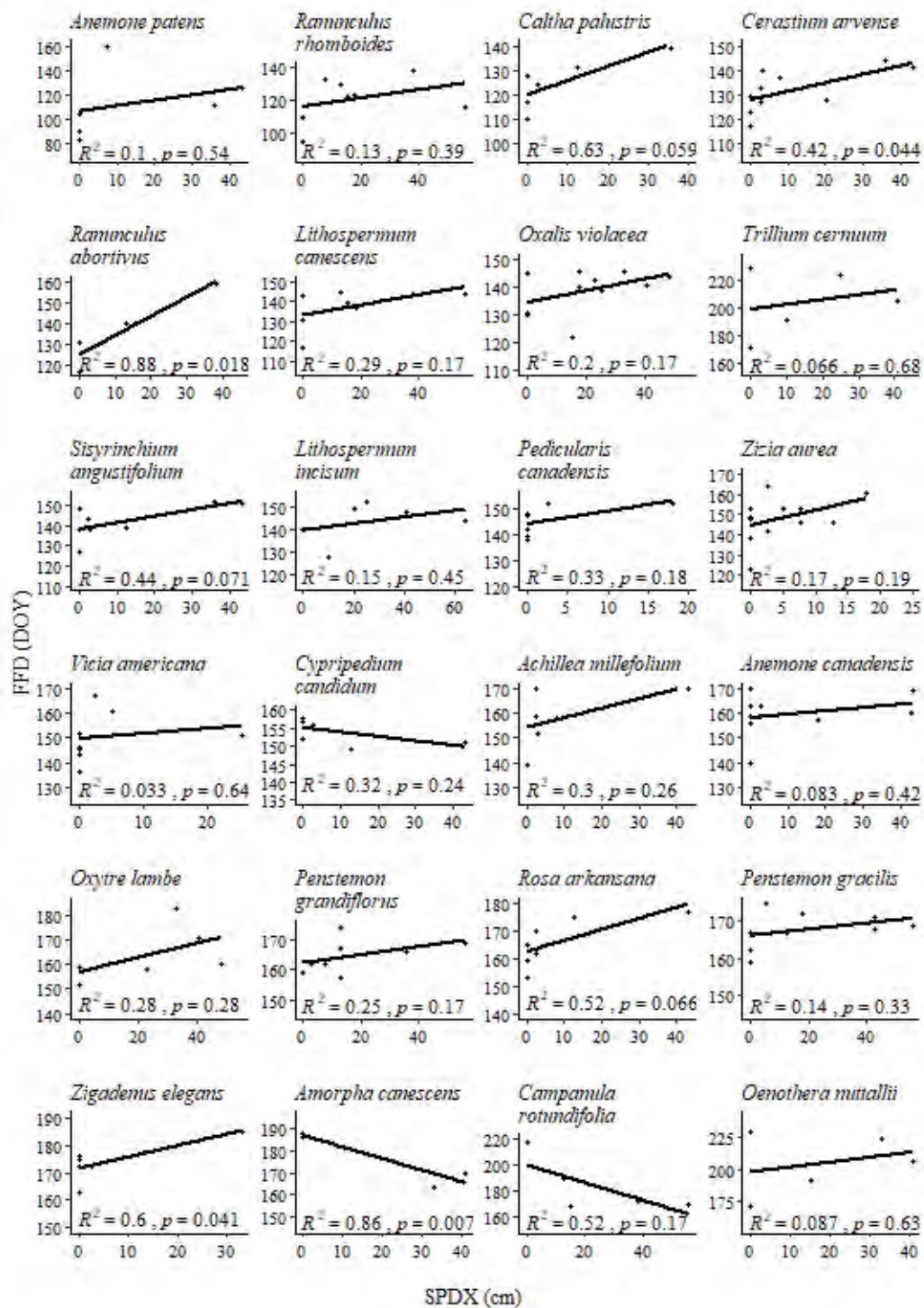
Figure 3.6. Simple linear regressions of first flowering day (day of year) as a function of snowpack on day X (cm) for each species with best-fit lines based on least-square estimates. Results for species are organized in order of flowering sequence over the season from early spring to late summer.

Table 3.3. Goodness of fit values for each model. An asterisk indicates a good fit (X^2 p-value > 0.05 and CFI > 0.95).

Species	p-value for X^2 test	CFI
Anemone patens	0.001	0.777
Ranunculus rhomboides	0.234*	0.981*
Caltha palustris	0.014	0.913
Cerastium arvense	0.653*	1*
Ranunculus abortivus	0	0.679
Lithospermum canescens	0.012	0.899
Oxalis violacea	0.069*	0.904
Trillium cernuum	0.528*	1*
Sisyrinchium angustifolium	0.01	0.836
Lithospermum incisum	0.302*	0.998*
Pedicularis canadensis	0.656*	1*
Zizia aurea	0.264*	0.996*
Vicia americana	0.361*	1*
Cypripedium candidum	0.736*	1*
Achillea millefolium	0.008	0.828
Anemone canadensis	0.348*	1*
Oxytropis lambertii	0.744*	1*
Penstemon grandifloras	0.546*	1*
Rosa arkansana	0.013	0.897
Penstemon gracilis	0.308*	0.999*
Zigadenus elegans	0.156*	0.962*
Amorpha canescens	0.004	0.662
Campanula rotundifolia	0.042	0.927
Oenothera nuttallii	0.684*	1*

Discussion

Our results indicate that as climate change over the past 50 years has led to increases in temperature and winter precipitation (Table 3.1) there has been a corresponding shift in first flowering date of many plant species observed in Bluestem Prairie Reserve as seen in other studies (Dunnell and Travers 2011, Wolkovich et al. 2012). The direction and magnitude of flowering time shifts depended on the species, but over 30% of them shifted by at least 5 days on average. Likewise, spring-time temperatures and both snowfall and snowpack increased over time suggested direct and indirect relationships between environmental variables and flowering

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time. However, in contrast to studies of alpine communities we found evidence of a stronger effect of temperature on FFD than of the timing of snowmelt or total snowfall.

In this study we built a model to analyze relationships between temperature (ST), snowfall (TSNOW), snowpack (SPDX), date of first bare ground (DOBG), and first flowering day (FFD) for 24 species. The model for all species improved when we excluded DOBG. We expected that the date of first bare ground would influence first flowering day as was reported by Inouye (2002) for montane species in Colorado. However, only a few of the species had a significant relationship between DOBG and FFD suggesting that when the winter snow melt occurs is not important for determining when plants begin flowering. An explanation for the lack of relationship between DOBG and FFD is that early DOBG could lead to increased frost damage in sensitive buds while later DOBG extends the date at which buds could emerge (Sherwood et al. 2017). Plants themselves may also compensate for a late start by shortening other growth stages, resulting in the same flower timing regardless of DOBG (Semenchuk et al. 2016). In contrast, temperature seems to be a consistent determinant of flower timing.

ST and FFD had a negative and significant relationship in most of the species. This suggests that higher temperatures in late winter and early spring are important for growth and development. This was especially the case for earlier flowering species. These results mirror other studies of plants in upper Midwestern prairies (Dunnell and Travers 2011), Pacific Northwestern prairies (Reed et al. 2019), and other temperate communities (Cook et al. 2012). Interestingly, these results differ from previous research for areas that receive substantial amounts of snow such as alpine and tundra environments (e.g., average 1054 cm per year in Gothic Colorado versus 108 cm per year in northwestern Minnesota). Moreover, Sherwood et al. (2017) found that temperature manipulations, specifically heating, had no effect on flowering

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phenology in montane species. Bjorkman et al. (2015) found that temperature was not strongly related to flowering phenology in tundra species. Temperature was a significant predictor for only one of four species observed (Bjorkman et al. 2015). Temperature may be more important than date of first bare ground for flowering phenology in tallgrass prairies compared to alpine settings because of the short, intense growing season in alpine communities. In a short growing season, there is a higher premium for every day of growth.

Snowpack in March was relatively unimportant compared to temperature, with only 6 of the 24 species expressing a relationship between SPDX and FFD. Four species (*Caltha palustris, Ranunculus abortivus, Rosa arkansana* and *Zigadenus elegans*) had positive regression coefficients meaning the deeper the snow on day X in March, the later the species flowered. This outcome would be expected if snow cover impaired earlier flowering. Since three of these species flower later in the summer, developmental processes earlier in the spring could be directly affected by snowpack, shifting flowering phenology, regardless of flower timing. Two species (*Amorpha canescens* and *Cypripedium candidum*) had a negative regression coefficient meaning that increased snowpack led to earlier flowering. *Cypripedium candidum* is an obligative wetland species along with *Caltha palustris*, which may explain the strong relationship between flowering and winter precipitation in both species. Since soil moisture from snowpack can take months to dissipate, snowmelt and early evapotranspiration may affect the soil moisture available for species that flower later in the season (Wang et al. 2018). *Amorpha canescens*, a later flowering species, also had a negative regression coefficient, which might mean the increased soil moisture levels later in the summer advanced flowering. Whether due to impaired early development or quantities of soil moisture, these six species are compensating for the conditions that resulted from snowpack by shifting flowering phenology.

Conclusions

Overall, our results suggest that snowpack does not have a strong relationship with flower timing in northern Midwestern tallgrass prairies. Snowpack may not inhibit flowering because, even with a late start, growing plants may be able to catch up by shortening earlier developmental phases (Semenchuk et al. 2016). We expected snowpack to influence early flowering species and not later flowering species, but our results indicate that growth and flowering begin regardless of snow cover. In comparison with snowpack, air temperature had a much stronger effect on when plants flower. Species interspersed throughout the growing season had significant regression coefficients for the effect of early temperatures on flower timing. We can therefore draw the conclusion that temperature is more strongly associated with flowering phenology than snowpack for species in Midwestern tallgrass prairies.

Further research is needed to better understand the relationships between changing climatic conditions and flowering phenology. We only considered snow cover and melt, but other forms of precipitation might be more tightly related to triggering flowering. Patricola and Cook (2013) found that precipitation is expected to increase for April and May with climate change and decrease for July and August. These changes could have implications for flowering phenology throughout the growing season.

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APPENDIX A. CHAPTER 1 SUPPLEMENTAL TABLES AND FIGURES

Table A1. Mixed effects model results for the difference between population for all sporophytic variables. One-way ANOVA results for the gametophytic variables for the difference between population. Block overfit the model when included for the gametophyte and was therefore excluded from the gametophytic variable models. Bolded values indicate significant relationships.

	Variable	Population			Block	
		F-value	df	p-value	LRT	p-value
Sporophyte	Cell Membrane Stability (Heat)	**5.563**	**188.06**	**2.97E-04**	**4.210**	**0.040**
	Cell Membrane Stability (Cold)	**2.824**	**188.06**	**0.026**	**15.342**	**8.97E-05**
	Chlorophyll Content (Heat)	1.732	188.25	0.145	0.188	0.665
	Chlorophyll Content (Cold)	**32.341**	**188.22**	**2.20E-16**	1.477	0.224
	Photosynthetic Rate (Heat)	1.473	126.00	0.214	-2.84E-04	0.214
	Photosynthetic Rate (Cold)	**3.717**	**138.74**	**6.62E-03**	0.032	0.858
Gametophyte	Pollen Germination (Tmax)	**6.069**	**3**	**1.17E-03**	-	-
	Pollen Germination (Topt)	**6.861**	**3**	**5.02E-04**	-	-
	Pollen Germination (Tmin)	2.656	3	0.057	-	-
	Pollen Tube Growth Rate (Tmax)	0.400	3	0.753	-	-
	Pollen Tube Growth Rate (Topt)	0.197	3	0.898	-	-
	Pollen Tube Growth Rate (Tmin)	0.459	3	0.712	-	-

Table A2. Mixed effects model results for each variable. Full model included region as a fixed effect with block and genet nested in population as random effects. Random effect terms were dropped when the model overfit the data. Bolded values indicate significant relationships.

	Variable	Region			Block		Population: Genet	
		F-value	df	p-value	LRT	p-value	LRT	p-value
Sporophyte	Cell Membrane Stability (Heat)	3.673	49.948	0.061	**4.728**	**0.030**	3.320	0.068
	Cell Membrane Stability (Cold)	**6.482**	**191.020**	**0.012**	**15.731**	**7.30E-05**	-	-
	Chlorophyll Content (Heat)	**4.418**	**51.122**	**0.041**	0.222	0.637	0.018	0.892
	Chlorophyll Content (Cold)	**66.369**	**49.933**	**9.97E-11**	1.018	0.313	2.082	0.149
	Photosynthetic Rate (Heat)	1.24E-05	1	0.997	-	-	-	-
	Photosynthetic Rate (Cold)	3.269	46.752	0.07702	0.148	0.701	1.674	0.196
Gametophyte	Pollen Germination (Tmax)	**14.280**	**1**	**3.70E-04**	-	-	-	-
	Pollen Germination (Topt)	**12.850**	**1**	**6.85E-04**	-	-	-	-
	Pollen Germination (Tmin)	0.960	1	0.331	-	-	-	-
	Pollen Tube Growth Rate (Tmax)	0.330	1	0.568	-	-	-	-
	Pollen Tube Growth Rate (Topt)	0.087	1	0.770	-	-	-	-
	Pollen Tube Growth Rate (Tmin)	0.683	1	0.683	-	-	-	-

Table A3. Mixed effects model of control values used in calculation for variable proportions to determine baseline differences between regions without the temperature treatments.

Variable	Region		Genet	
	Difference	p-value	Difference	p-value
Conductivity of cell membrane max damage	No	0.445	No	0.097
Chlorophyll content initial value	No	0.795	No	0.869
Net photosynthetic rate initial value	No	0.303	No	0.380

Table A4. T-test results for differences between region within block. Paired t-tests were used as a northern and southern plant were paired with one another and experienced the same green house conditions. An unpaired t-test was used for photosynthesis because there were missing data points for some genets. Bolded values indicate significant relationships.

	Variable	Method	Block A		Block B		Block C		Block D	
			t-stat	p-value	t-stat	p-value	t-stat	p-value	t-stat	p-value
Sporophyte	Cell Membrane Stability (Heat)	Paired	**-2.910**	**0.015**	-0.853	0.403	-1.640	0.113	-0.539	0.595
	Cell Membrane Stability (Cold)	Paired	0.758	0.456	**2.190**	**0.040**	**2.073**	**0.049**	0.939	0.358
	Chlorophyll Content (Heat)	Paired	-0.374	0.712	-1.650	0.113	-1.933	0.065	-0.728	0.474
	Chlorophyll Content (Cold)	Paired	**-5.889**	**3.82E-06**	**-4.746**	**9.77E-05**	**-5.982**	**3.50E-06**	**-4.106**	**4.33E-04**
	Photosynthetic Rate (Heat)	Unpaired	0.541	0.594	1.144	0.261	-1.367	0.187	0.021	0.984
	Photosynthetic Rate (Cold)	Unpaired	-0.664	0.511	1.542	0.137	1.219	0.231	1.782	0.083

Table A5. Difference in variation between the two regions for all variables using Bartlett's test of homogeneity of variances. Bolded text indicates differences that are statistically significant. Asterisk denotes variable with one data point removed after an outlier test.

	Variable	Difference	More Variation	p-value
Sporophyte	Cell Membrane Stability (Heat)	No	-	0.896
	Cell Membrane Stability (Cold)	No	-	0.131
	Chlorophyll Content (Heat)	**Yes**	**North**	**2.48E-04**
	Chlorophyll Content (Cold)	No	-	0.057
	Photosynthetic Rate (Heat)	No	-	0.444
	Photosynthetic Rate (Cold)	No	-	0.602
Gametophyte	Pollen Germination (Tmax)	No	-	0.515
	Pollen Germination (Topt)	No	-	0.972
	Pollen Germination (Tmin)	No	-	0.1557*
	Pollen Tube Growth Rate (Tmax)	No	-	0.107
	Pollen Tube Growth Rate (Topt)	No	-	0.532
	Pollen Tube Growth Rate (Tmin)	No	-	0.487

Table A6. Correlation matrix with correlation coefficient and p-value for each combination of variables. Bolded text indicates correlations that are statistically significant with p-values adjusted using the Holm's-Bonferroni method for multiple correlations.

		Tmin Germ	Tmax Germ	Tmin PTGR	Tmax PTGR	Hot CMS	Cold CMS	Hot CHPL	Cold CHPL	Cold PS	Hot PS
Tmin Germination	Corr	-									
	p-value	-									
Tmax Germination	Corr	-0.264	-								
	p-value	0.040	-								
Tmin PTGR	Corr	0.266	0.061	-							
	p-value	0.038	0.639	-							
Tmax PTGR	Corr	-0.073	**0.371**	**0.456**	-						
	p-value	0.576	**0.003**	**2.18E-04**	-						
Hot CMS	Corr	0.015	0.112	-0.004	0.030	-					
	p-value	0.911	0.392	0.977	0.818	-					
Cold CMS	Corr	-0.130	0.167	0.042	0.106	-0.131	-				
	p-value	0.317	0.198	0.745	0.416	0.067	-				
Hot CHPL	Corr	0.112	-0.078	-0.069	-0.103	0.060	-0.131	-			
	p-value	0.392	0.552	0.596	0.428	0.401	0.066	-			
Cold CHPL	Corr	0.101	0.065	-0.127	-0.144	0.145	-0.093	0.102	-		
	p-value	0.440	0.621	0.331	0.267	0.043	0.191	0.154	-		
Cold PS	Corr	0.240	0.062	0.164	0.133	0.205	-0.084	0.092	-0.151	-	
	p-value	0.228	0.758	0.412	0.508	0.038	0.402	0.358	0.129	-	
Hot PS	Corr	-0.052	0.054	0.066	0.137	0.194	0.076	-0.069	-0.030	0.131	-
	p-value	0.730	0.719	0.662	0.358	0.019	0.362	0.408	0.718	0.186	-

Table A7. Results from principal component analysis with gametophytic and sporophytic variables. Loadings for each variable on all principal components.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Tmin Germination	-0.189	0.377	-0.586	0.224	-0.069	0.422	0.385	0.313
Tmax Germination	0.511	-0.286	0.088	-0.299	-0.103	0.625	0.324	-0.232
Tmin PTGR	0.417	0.456	-0.360	0.123	-0.009	-0.206	-0.112	-0.646
Tmax PTGR	0.628	0.195	-0.072	-0.150	-0.025	-0.069	-0.354	0.639
HCMS	0.035	-0.352	-0.516	-0.468	0.215	-0.466	0.351	0.058
CCMS	0.271	-0.325	0.021	0.575	-0.522	-0.319	0.326	0.095
HCHPL	-0.235	0.126	-0.090	-0.476	-0.815	-0.037	-0.160	-0.053
CCHPL	-0.064	-0.534	-0.489	0.215	-0.025	0.253	-0.595	-0.082

Table A8. Results from principal component analysis with sporophytic variables. Proportion of variance explained by each of the components.

	PC1	PC2	PC3	PC4	PC5	PC6
Standard deviation	1.159	1.1372	1.0038	0.968	0.8775	0.8058
Proportion of Variance	0.224	0.2155	0.1679	0.1562	0.1283	0.1082
Cumulative Proportion	0.224	0.4394	0.6073	0.7634	0.8918	1

Table A9. Results from principal component analysis with gametophytic variables. Proportion of variance explained by each of the components.

	PC1	PC2	PC3	PC4	PC5	PC6
Standard deviation	1.703	1.28	1.0046	0.673	0.0008	0.0001
Proportion of Variance	0.483	0.2731	0.1682	0.0755	0	0
Cumulative Proportion	0.483	0.7563	0.9245	1	1	1
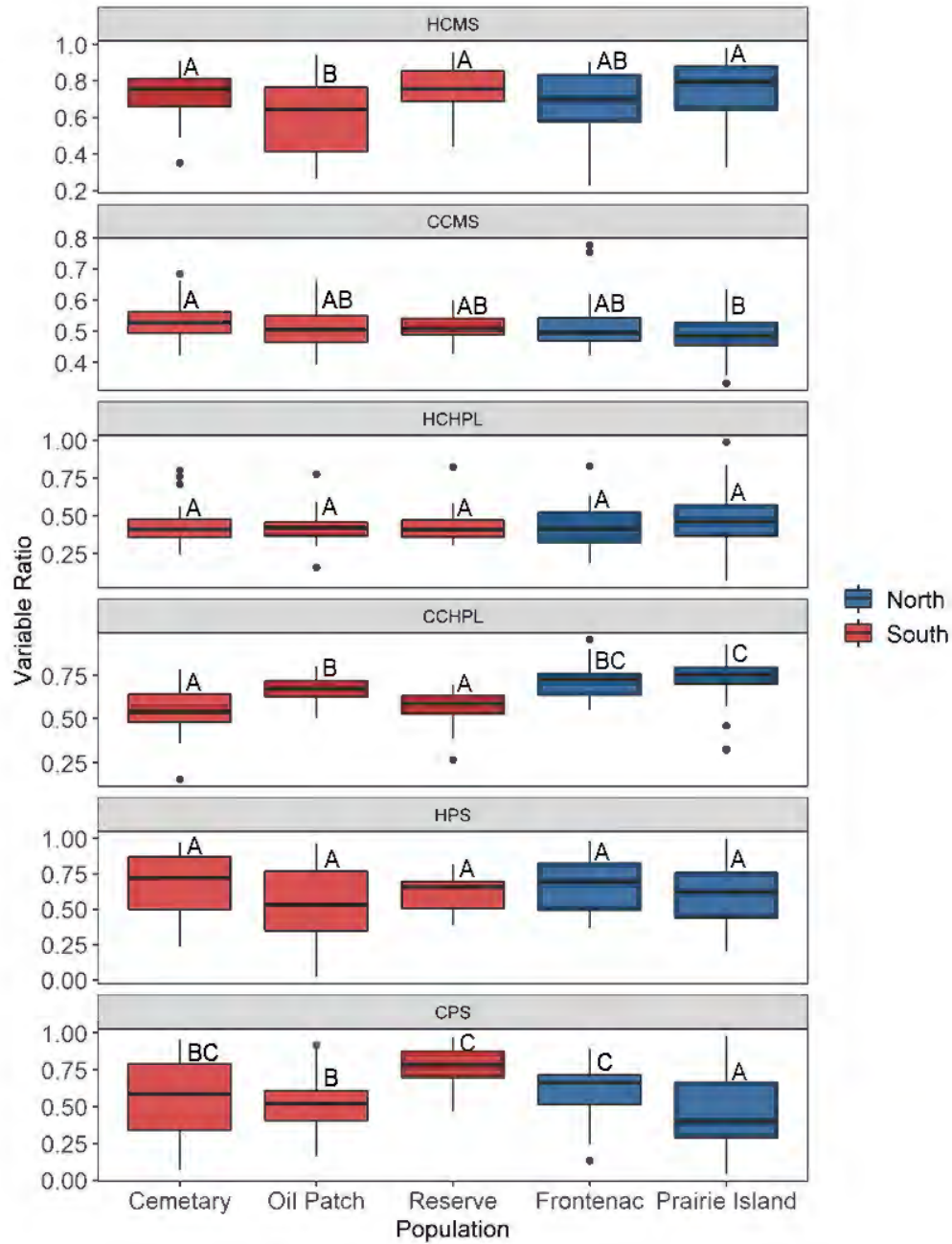
Figure A1. Differences between the populations for all sporophytic variables. Letters denote significant differences between populations from a linear mixed effects model with population as the fixed effect and block as the random effect.

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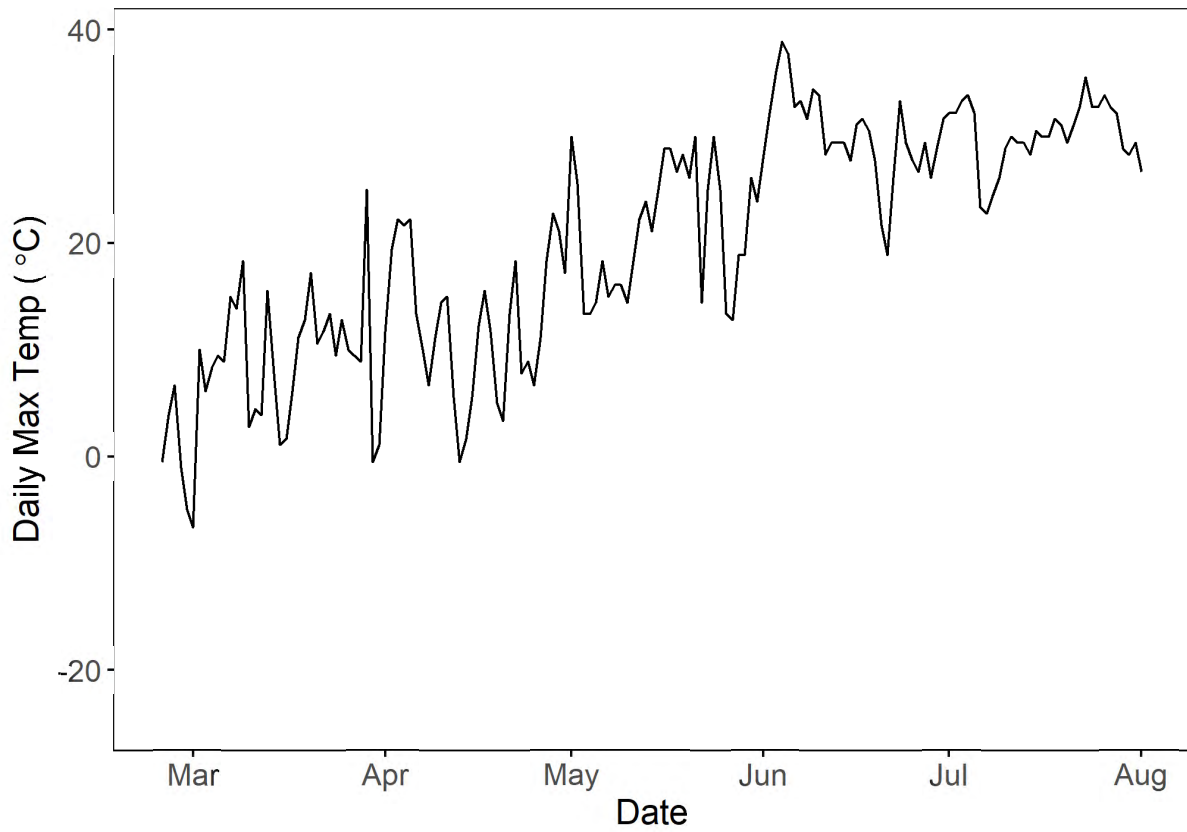
Figure A2. Daily max temperature for spring and summer of 2021 from the NOAA station at the Hector International Airport, Fargo, ND.
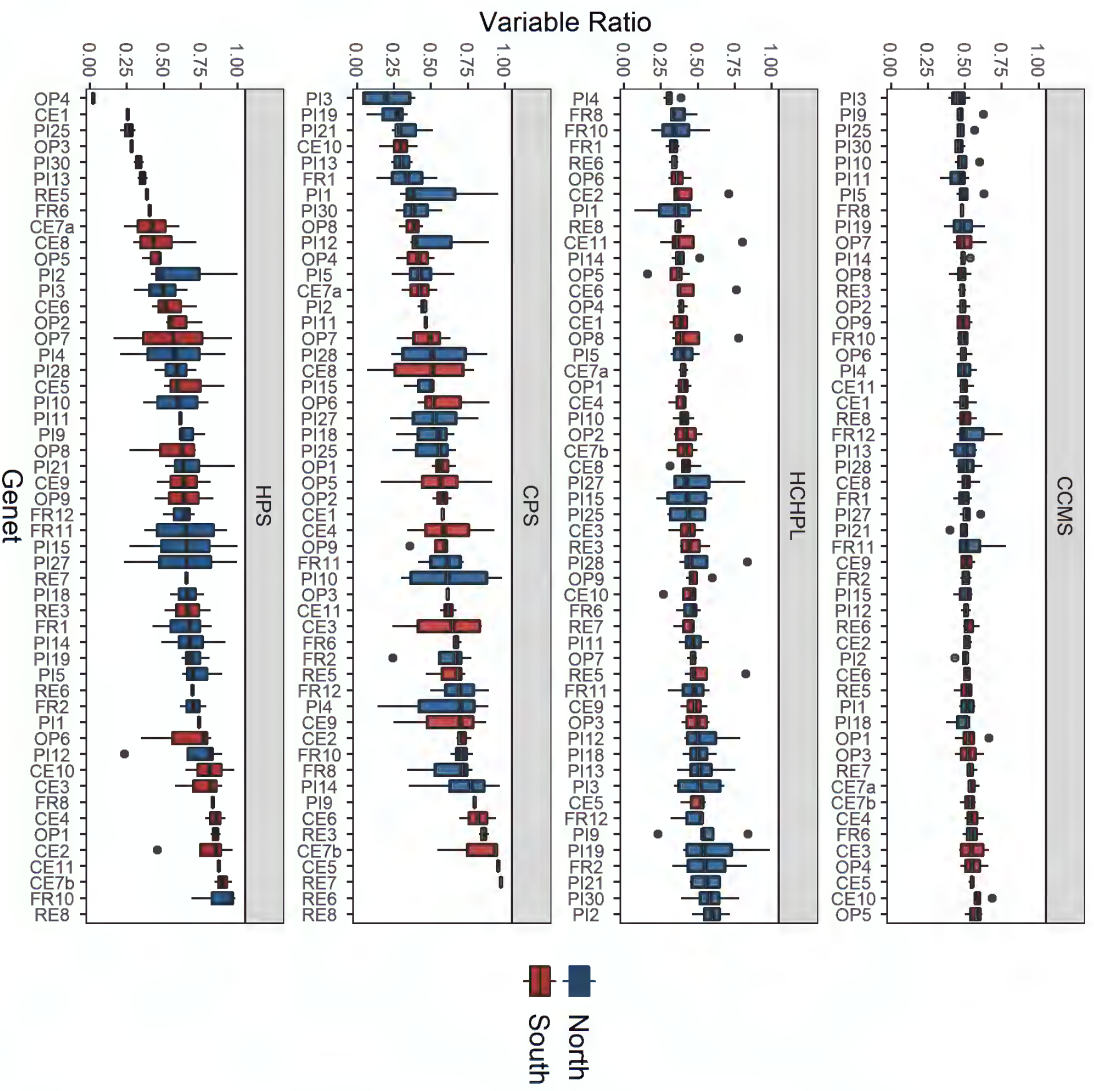
Figure A3. Genotype differences for temperature tolerance traits including cold cell membrane stability (CCMS), hot chlorophyll content (HCHPL), cold photosynthesis (CPS), and hot photosynthesis (HPS). Genets ordered by the sum of median ratios for HCMS and CCHPL. There are no significant differences between the variables shown here.

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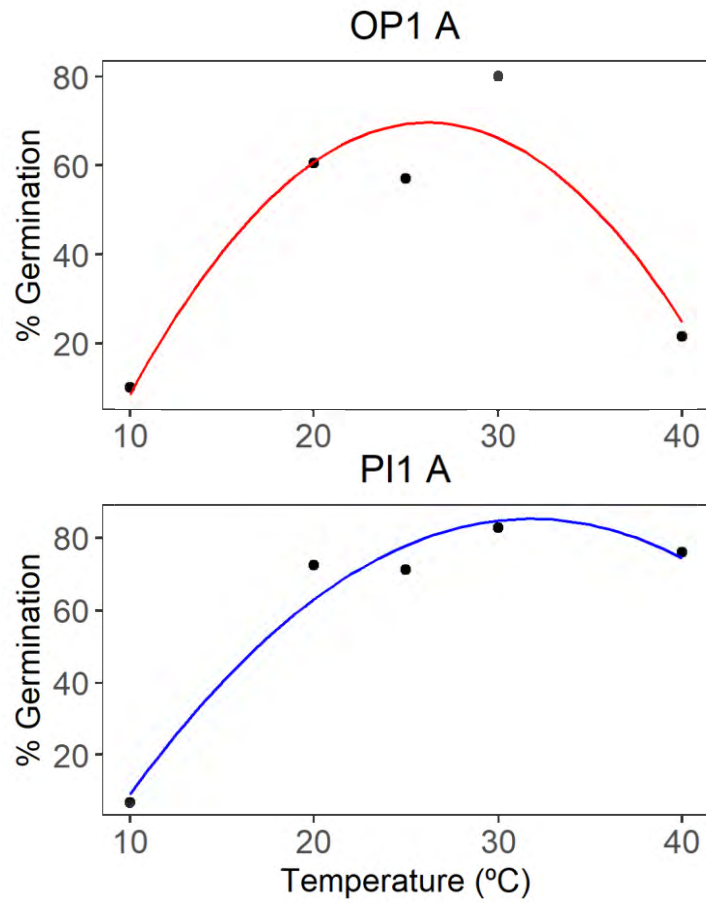
Figure A4. Examples of quadratic fit curve for pollen germination of one genet from the southern region (OP1 A, red) and one genet from the northern region (PI1 A, blue).
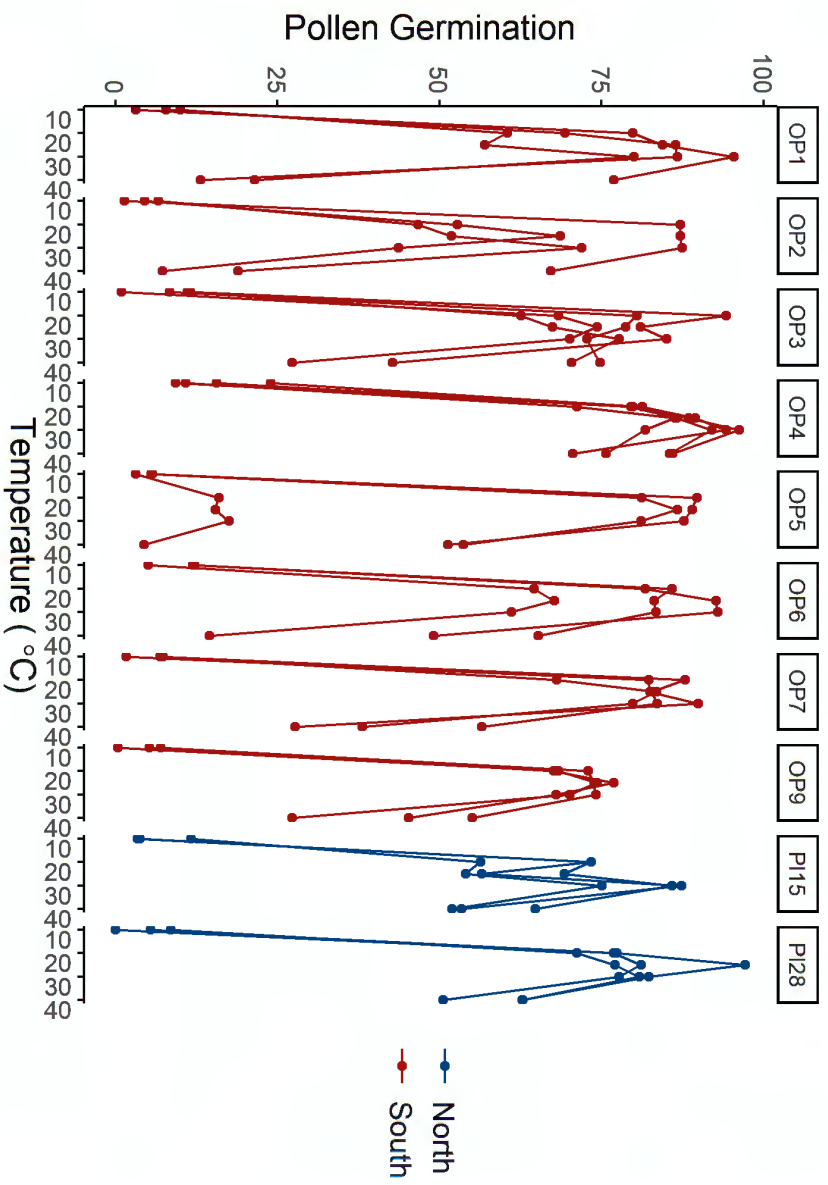
Figure A5. Pollen germination profiles for genets with at least 3 ramets that flowered. Color indicates region of origin.
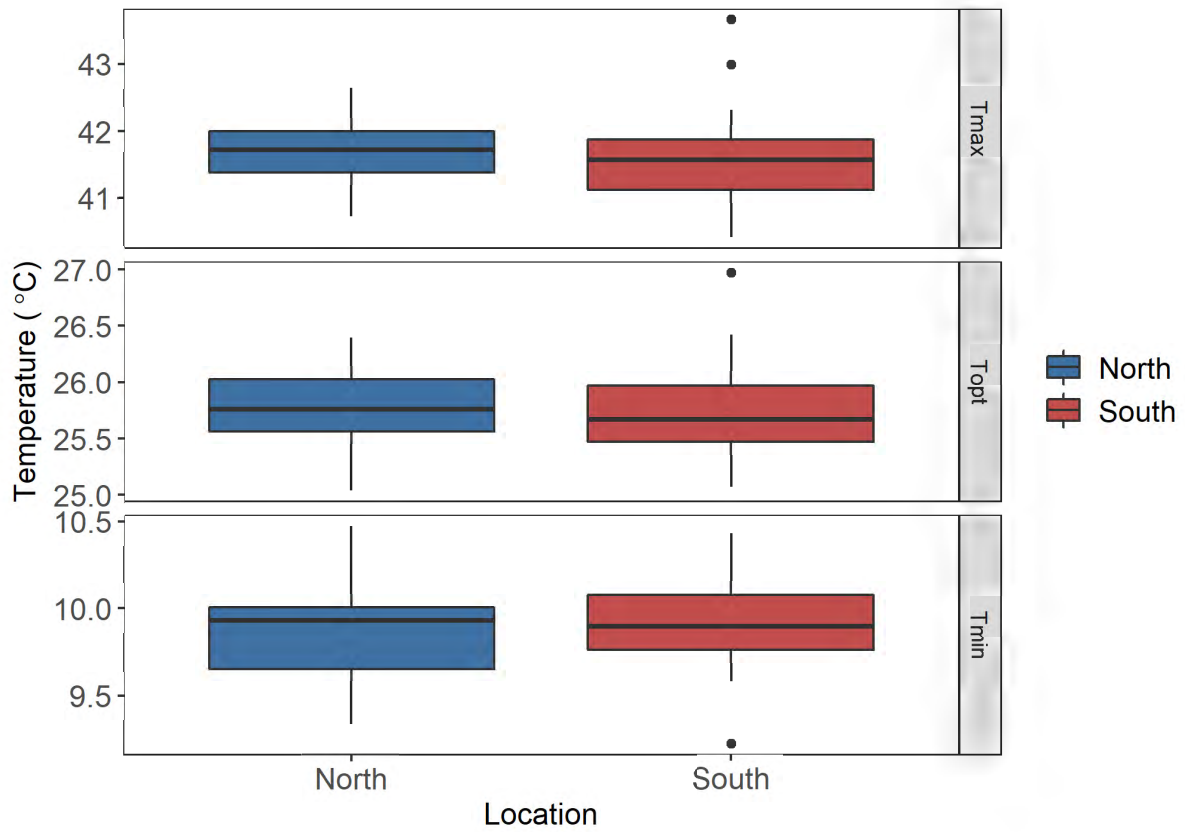
Figure A6. Pollen tube growth rate values extracted from a quadratic fit for the maximum, optimal, and minimum temperatures.
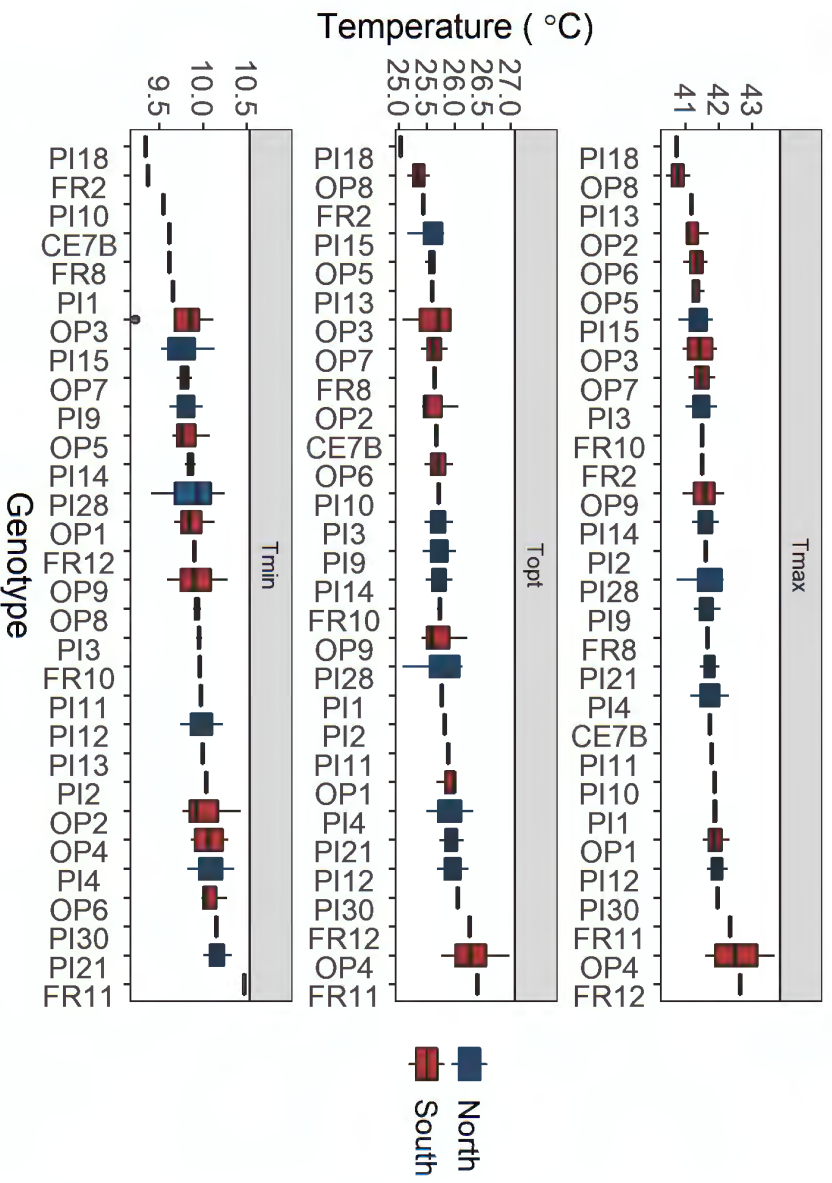
Figure A7. Genotype differences for the maximum (Tmax), optimal (Topt), and minimum (Tmin) pollen tube growth rate temperatures extracted form the quadratic fits of the pollen tube growth rate data for each individual. There were no significant differences among the genets for pollen tube growth rate. Each plot is in order of increasing PTGR.

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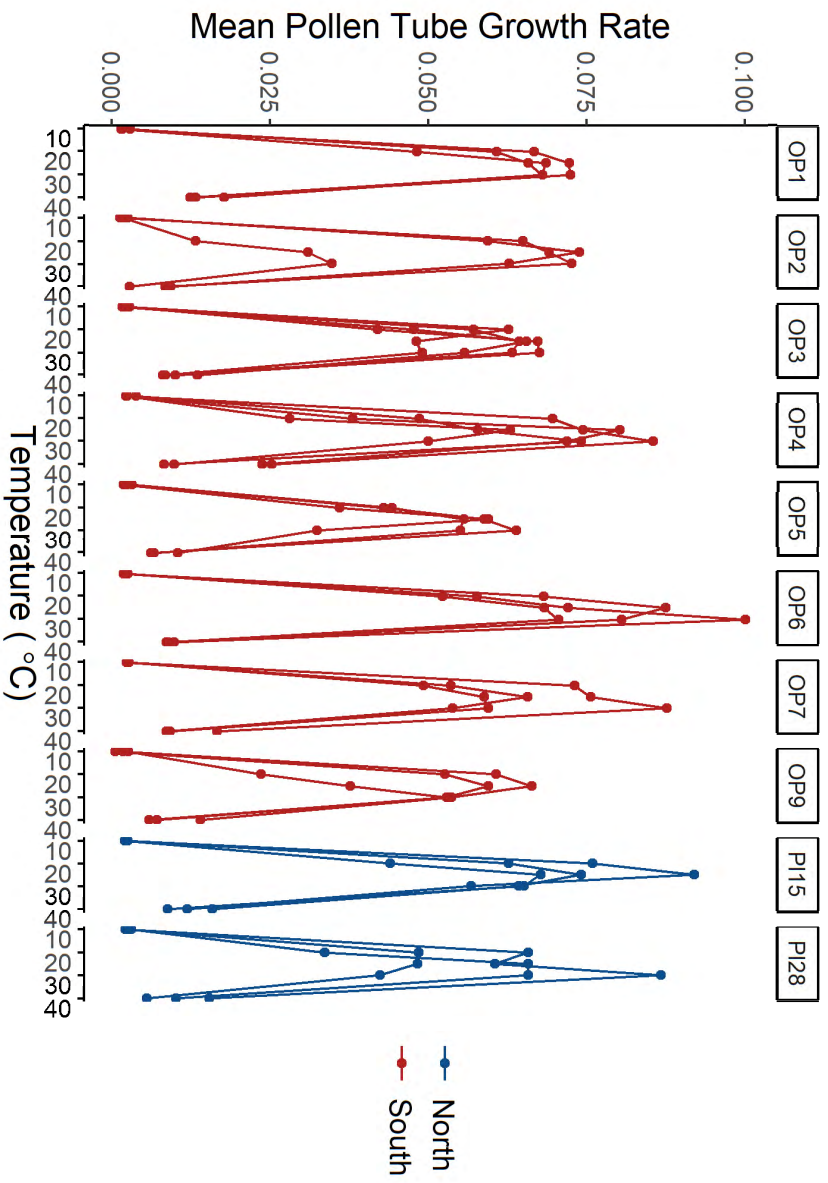
Figure A8. Pollen tube growth rate profiles for genets with at least 3 ramets that flowered. Color indicates region of origin.

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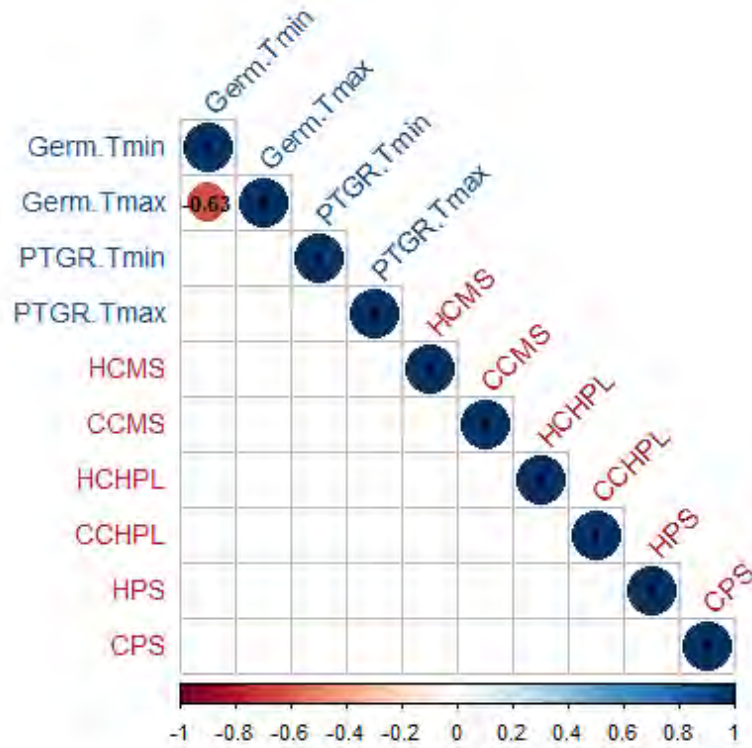
Figure A9. Correlation matrix of southern plants with significant Pearson's correlations. Sporophytic (red font) and gametophytic variables (blue font) included. Blue colors indicate positive correlations and red colors indicate negative correlations.
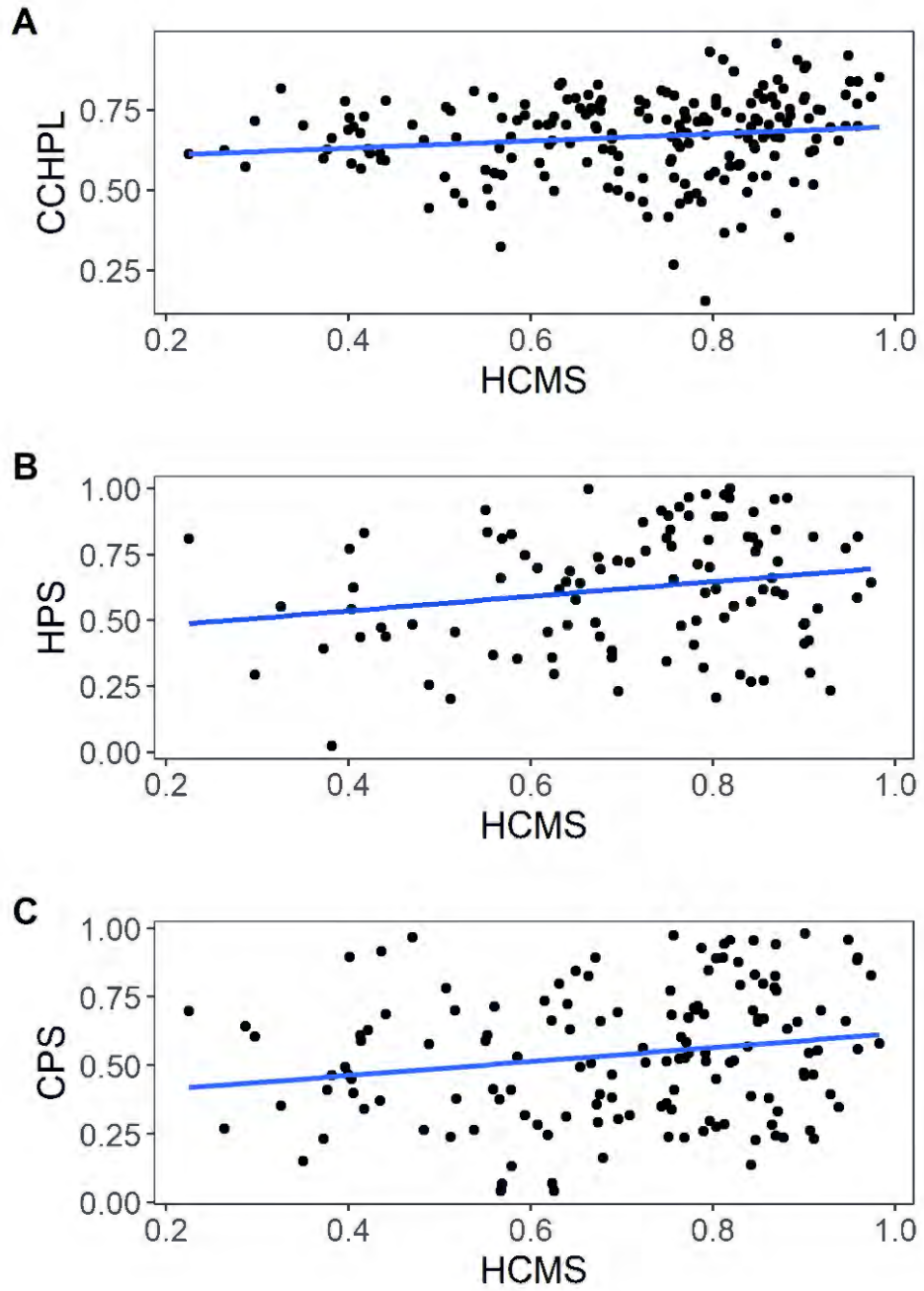
Figure A10. Scatter plot of the significant correlations between sporophytic variables including plants from the northern and southern regions.
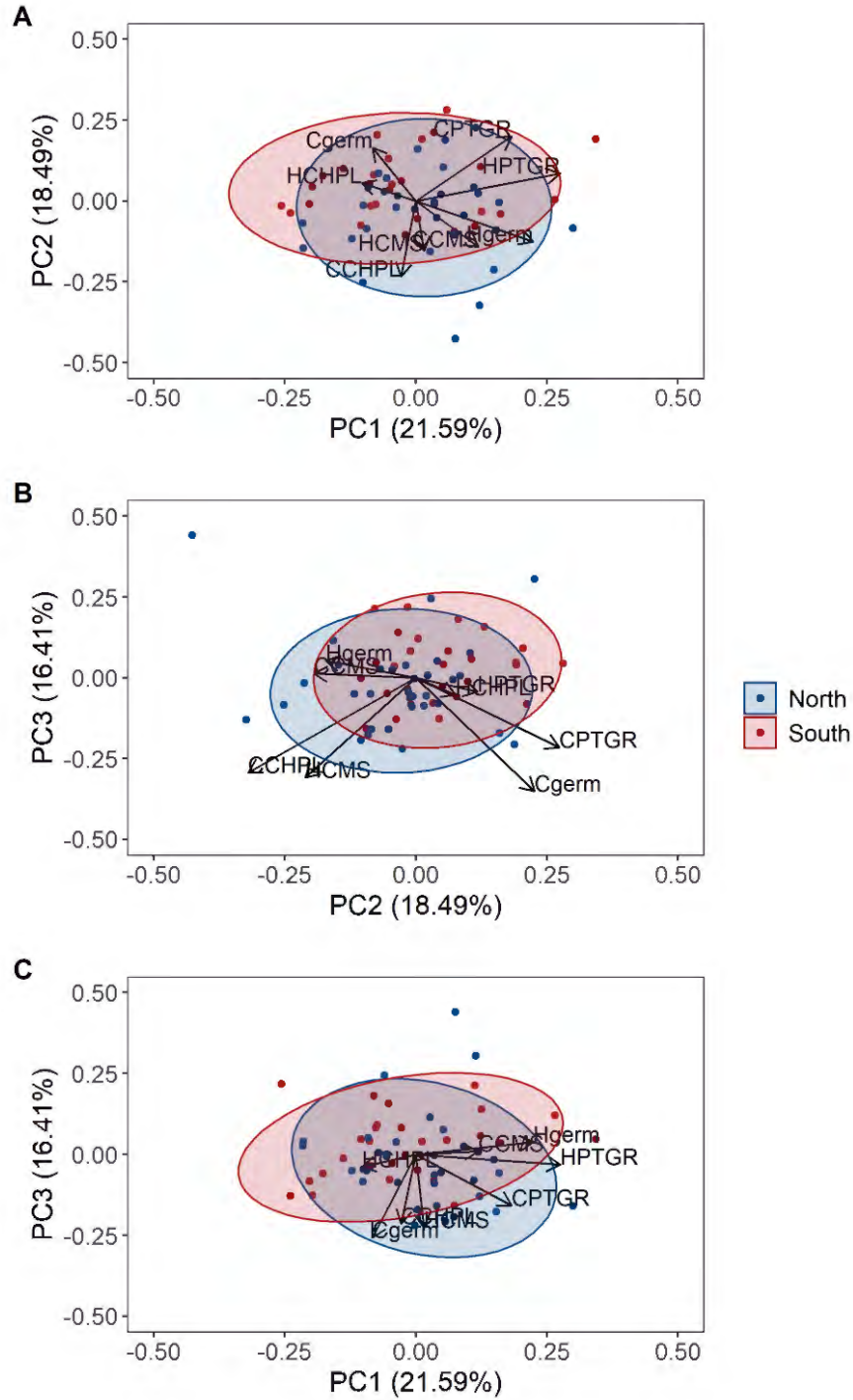
Figure A11. Principal component analysis with sporophytic and gametophytic variables, excluding photosynthesis. A) PC1 and PC2, B) PC2 and PC3, C) PC1 and PC3. Ellipsoid indicating 95% confidence interval.
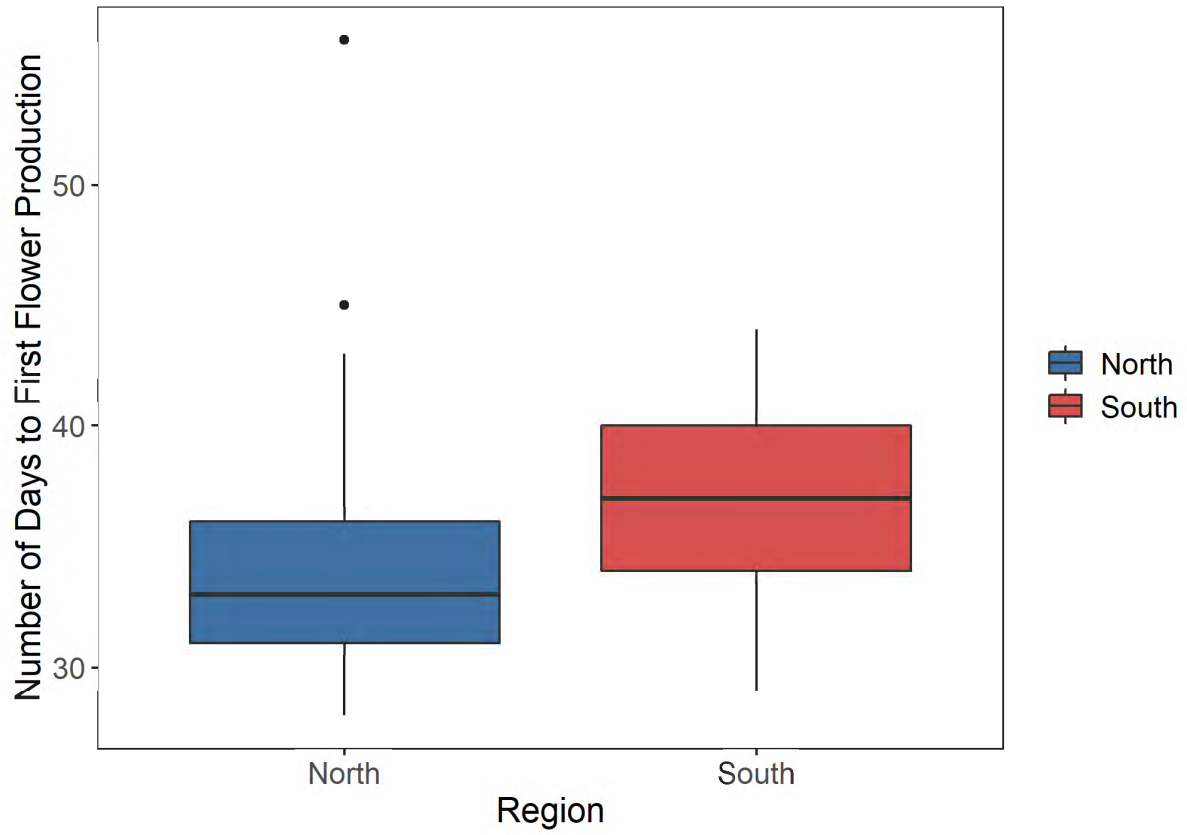
Figure B1. Regional differences for the number of days to the first flower production after growth initiation. Midline indicates the median trait value for the region.
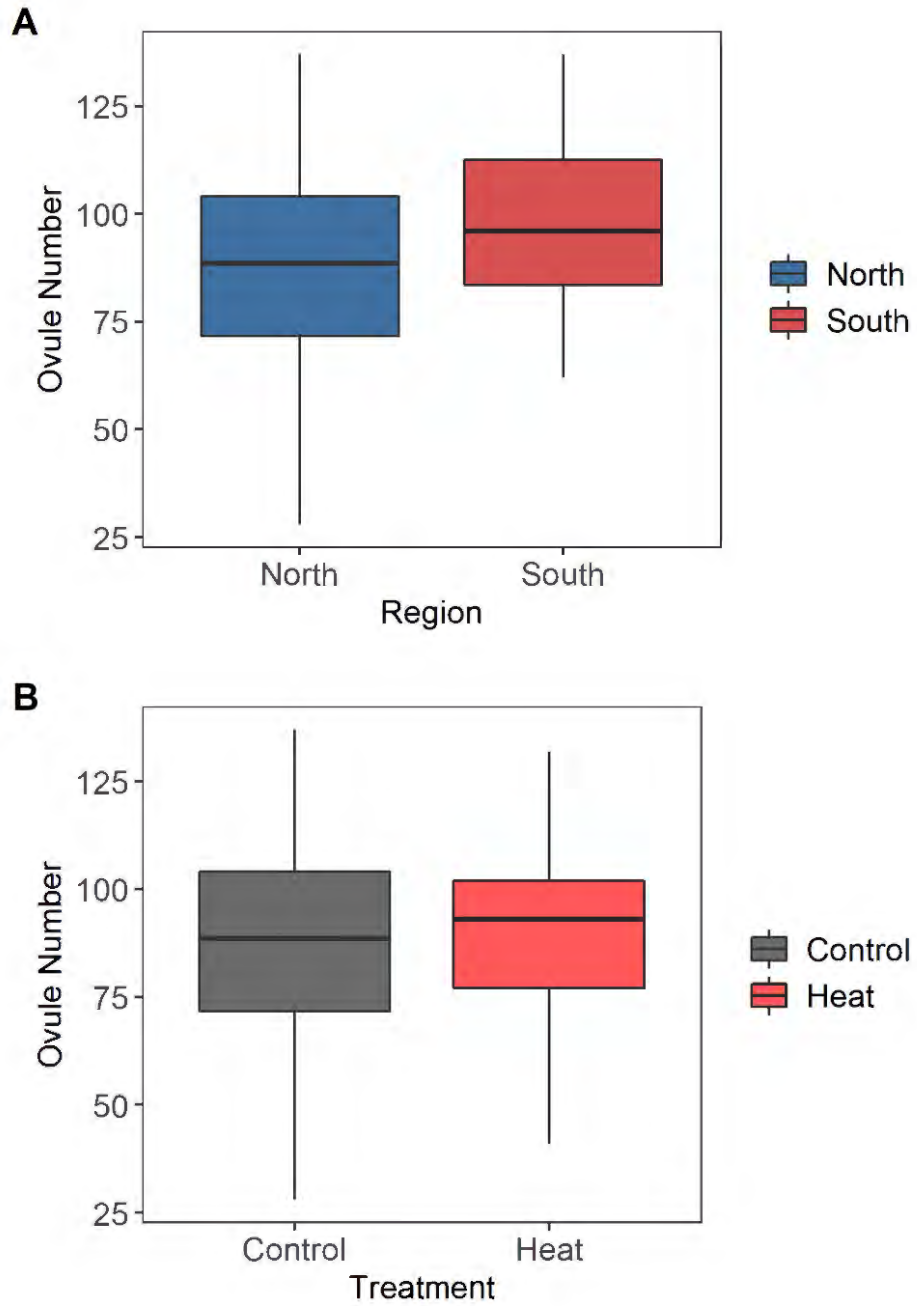
Figure B2. Differences between regions (A) and treatments (B) for ovule number. Midline indicates the median trait value for the region or treatment group.
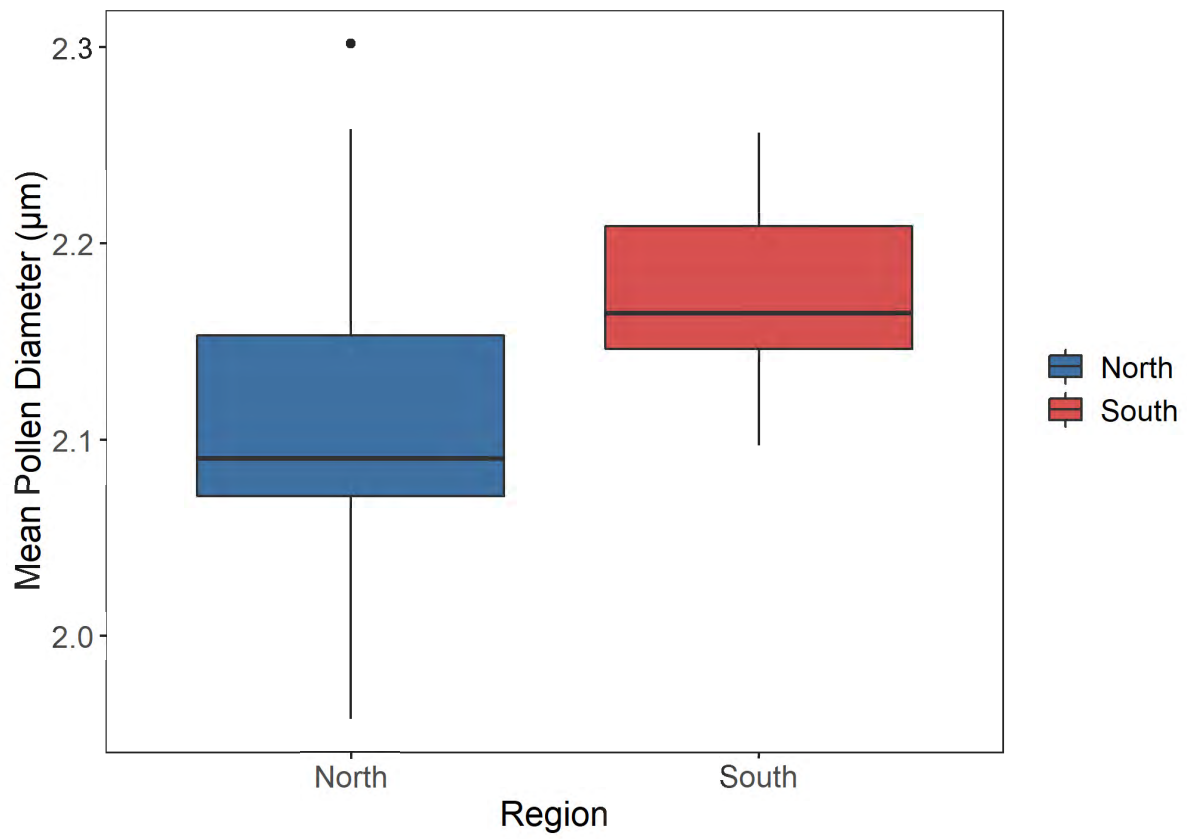
Figure B3. Regional differences for mean pollen diameter. Midline indicates the median trait value for the region
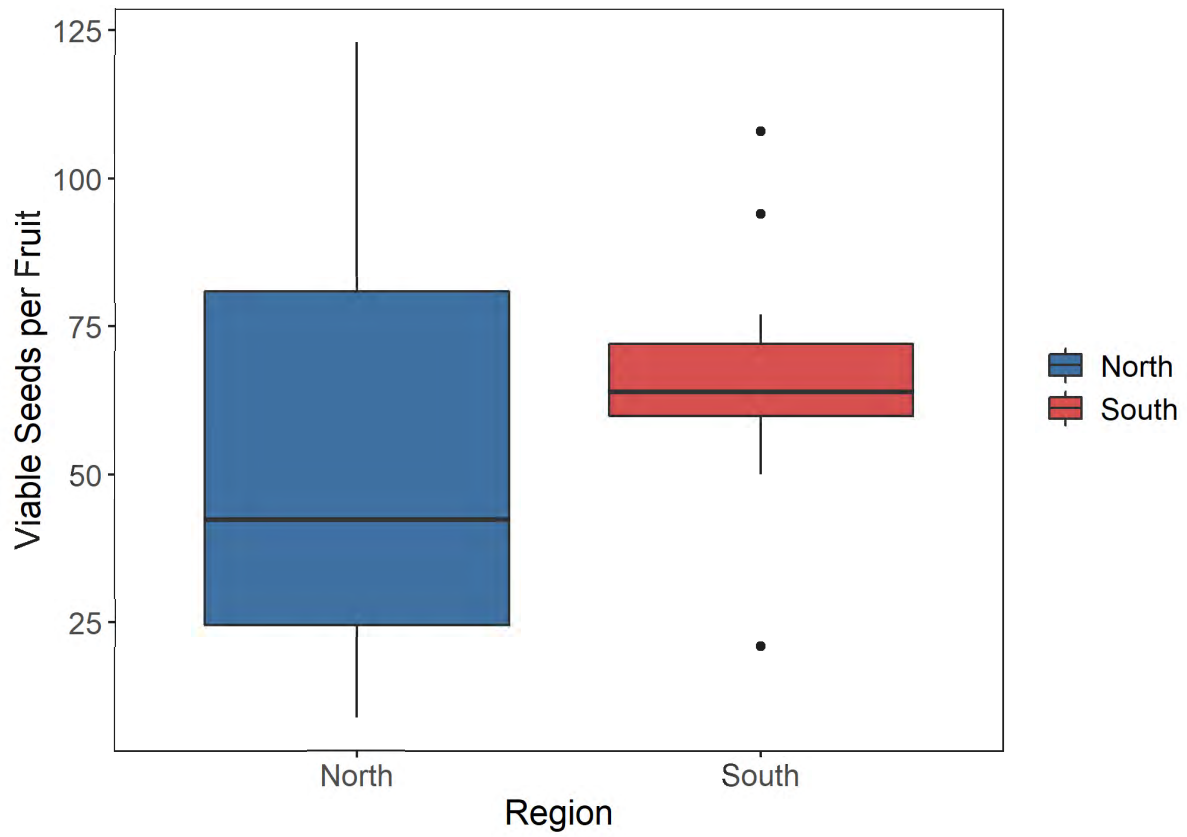
Figure B4. Regional differences for viable seed number per fruit when flowers and fruit developed in the control treatment.