**Chapter 1 Intro**

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? With the uprise of novel 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, Schlichting 1986, Molina-Montenegro and Naya 2012).

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 locations can result in differing trait optima in the separate populations, leading to local adaptation (Kawecki and Ebert 2004). Specifically, temperature is a variable that can determine species distributions and can vary greatly in both severity and consistency with geographic region (Von Büren and Hiltbrunner 2022). Based on the IPCC Sixth Assessment Report (Seneviratne, Xuebin. et al. 2021), temperatures are changing at unprecedented rates throughout the world. Spatial disparities in local conditions and past population-level responses can provide a clue to how a species might respond as global warming changes local conditions. Therefore, we seek to answer the questions: 1) how do populations of the same species persist in different temperature regimes and 2) how would plants preadapted to local conditions respond to more severe temperature conditions? In this two-part study, we examined plant (sporophytic and gametophytic) responses to extreme heat and cold and we studied the effect of long-term moderate heat on reproductive traits.

We compared plants from Minnesota and Texas and estimated 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 (net photosynthesis, chlorophyll content stability, and cell membrane stability) and the gametophytic variables were measured using pollen (pollen germination and pollen tube growth rate).

The first objective was to determine if local thermal conditions have divergently selected for temperature tolerance traits and led to adaptations reflecting regional climate regimes. We hypothesized that if there was 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 is the potential for a positive correlation of temperature tolerance in the two life stages.

**Chapter 2 Intro**

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 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 (Conner and Zangori 1998, Haileselassie, Mollel et al. 2005, Burkle and Irwin 2009), moisture (Galen 2000, Fang, Turner et al. 2010), and heat (Xu, Wolters-Arts et al. 2017). Male reproductive success is also dependent on environmental conditions. Pollen viability decreases with high temperatures (Sato, Kamiyama et al. 2006, Din, Khan et al. 2015, Müller, Xu et al. 2016, Xu, Wolters-Arts et al. 2017, Poudyal, Rosenqvist et al. 2019) and drought stress (Fang, Turner 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 (Sato, Kamiyama et al. 2006, Müller, Xu et al. 2016, Xu, Wolters-Arts et al. 2017, Jiang, Lahlali et al. 2019). 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, Sato, Kamiyama et al. 2006, Müller, Xu et al. 2016), ovule viability (Xu, Wolters-Arts et al. 2017), pollen viability (Sato, Kamiyama et al. 2006, Din, Khan et al. 2015, Müller, Xu et al. 2016, Xu, Wolters-Arts et al. 2017, Poudyal, Rosenqvist et al. 2019), fruit set (Charles and Harris 1972, Sato, Kamiyama et al. 2006, Din, Khan et al. 2015), and seed set (Din, Khan 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, Singh 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 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.

Combined Objective:

1. Is there a correlation between sporophytic and gametophytic temperature tolerance in extreme, short-term temperature treatments?
2. Is there an effect of long-term moderate heat on pre and post pollination reproductive traits?
3. Do responses to temperature treatments differ between plants from Minnesota and Texas?

Methods

Species Description

*Solanum carolinense* L. (Solanaceae), also known as horsenettle, is a weedy, herbaceous perennial that originated in southeastern North America. *Solanum carolinense* has spines that line the stem and midrib of the variably lobed leaves, which is characteristic of the Carolinense clade of the subgroup Leptostemonum (Wahlert, Chiarini et al. 2014). Since all other species in this clade are neotropical, this species likely arose through dispersal to North America and independent diversification. *Solanum carolinense* reproduces both sexually and asexually. Asexually, this species utilizes clonal recruitment by growth from rhizomes. *Solanum carolinense* grows indeterminately and is andromonoecious, meaning that both staminate and hermaphroditic flowers are produced. 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, Keser et al. 2009). However, as flowers age, the SI system deteriorates and the potential for successful self-fertilization with fruit production increases (Travers, Mena-Ali et al. 2004). The fruit are small yellow to green, tomato-like berries that are dispersed by small mammals and birds (Cipollini and Levey 1997).

Map

Description automatically generated

Figure 1. Map showing the distribution of *Solanum carolinense* (grey dots), northern (blue dots) and southern regions (red dots), and populations of origin for plants in this study. The populations Frontenac (top blow-up, blue) and Prairie Island (top blow-up, purple) were in the northern region and the populations Cemetery (bottom blow-up, red), Oil Patch (bottom blow-up, orange), and Reserve (bottom blow-up, green) were located in the southern region.

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 (Figure 1). 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 (Figure 1). In Houston County, MN, the mean daily low temperature is -14°C and the mean daily high is 29°C. 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 and the mean daily high is 43°C. While in close proximity, the southern populations had distinct morphological characteristics, such as plant size and leaf shape, that distinguished them as separate populations.

Collections involved digging up and cutting rhizomes 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, ND, 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.

**Life-stage specific responses to extreme temperature**

In January 2021, 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 at separate times. 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. Northern plants were paired with a southern plant and these pairs were randomly located on greenhouse benches. The plants were fertilized every other week with 10-10-10 fertilizer and transplanted to larger, 4.5 L containers when they outgrew the small cone-shaped containers. After approximately 10 weeks, we began collecting sporophytic measurements from one sub-block per week. Gametophytic data were measured when plants began flowering.

Temperature tolerance variables

To test the relationship of temperature tolerance between the sporophyte and gametophyte we used three sporophytic variables and two gametophytic variables. The sporophytic variables included cell membrane stability (CMS), chlorophyll content (CHPL), and net photosynthetic rate (PS). For each variable, there was an extreme hot treatment (acronym preceded by “H”) and an extreme cold treatment (acronym preceded by “C”).

CMS was calculated using the conductivity measurement of deionized water with 10 leaf rounds after a temperature treatment (HCMS: 55°C, CCMS: -18°C) and after a max damage treatment (98°C) relative to the control conductivity at room temperature (protocol from Gajanayake et al. (2011) and Fang and To (2016)). CHPL was the chlorophyll content estimated using a chlorophyll meter (Opti-Sciences CCM-300) before and after a temperature treatment (HCHPL: 60°C, CCHPL: -18°C) relative to control measurements of a treatment at room temperature. The chlorophyll meter measures the fluorescence emitted at 735nm/700nm for a constant leaf area and uses a ratio based on experiments by Gittelson et al. (1998) to estimate chlorophyll content in mg/m2. PS was the ratio of net photosynthetic rates before and after a temperature treatment (HPS: 33°C, CPS: 10°C). More detailed methods are available in the Supporting Information.

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, we removed a mature flower from both plants. Pollen from each flower was dispersed over five petri dishes containing 3% Bacto-Agar based growth medium (sucrose, Ca(NO3)2, MgSO4, KNO3, H3BO3; protocol from Reddy and Kakani (2007)). The dusted plates were each placed at one of the five temperature treatments (10°C, 20°C, 25°C, 30°C, 40°C) for 16 hours. 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 germination (Germ) was measured by counting the number of pollen grains that produced pollen tubes and dividing that by the total number of pollen grains observed. Pollen tube growth rate (PTGR) was calculated by dividing the length of the 20 longest pollen tubes measured using ImageJ (Schneider, Rasband et al. 2012) by the time allowed for growth (16 hours). Detailed methods provided in the Supporting Information.

**The effect of long-term moderate heat on reproductive traits**

On January 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. Ramets C and D were placed in the chamber in May 2022. 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

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 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

The pollen germination percentage was calculated for grains on artificial media at 40°C. The same germination protocol mentioned in the greenhouse experiment was used.

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

*Life-stage specific responses to extreme temperature*

All data were analyzed in R 4.1.2 (R Core Team 2020). In order to measure differences in sporophytic traits between plant origins and among genets, we fit linear mixed effects models using the lmer function from the *lmerTest* package (Kuznetsova, Brockhoff et al. 2017). Region (north vs. south) was considered the fixed effect and block (A, B, C, D) and genet were random effects. Since there was a significant block effect in some of the variables, we compared plants from the north and south within blocks using a paired t-test (*stats*; function t.test).

For the gametophytic variables, we fit quadratic temperature performance curves (determined using model selection) 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). 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 the response curves differed between regions. 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 Pearson’s method for correlation analysis (*stats*; function cor) to identify associations between sporophytic and gametophytic variables. The Holm-Bonferroni method (*stats*; function p.adjust) was used to adjust p-values to account for multiple correlations.

*The effect of long-term moderate heat on reproductive traits*

Flower date was analyzed for regional differences using a linear mixed effects model in the *lme4* package (Bates, Mächler et al. 2014) with region as the fixed effect and genet as the random effect. Differences in flower type development between the treatments were analyzed using a chi-squared test in the *stats* package (R Core Team 2020). All pre and post pollination traits were analyzed with different versions of mixed effects models depending on the data type. The general structure for the model was region, treatment, and the interaction of region and treatment as fixed effects and genet as the random intercept. We used general linear mixed effects models (*lme4*; function lmer) for anther and style plus stigma length. To avoid overfitting the model for pollen diameter, we omitted genet as a random effect and used a general linear model (*stats*; function lm). We used generalized mixed effects models (*lme4*; function glmer) with a Poisson distribution for all count data, which included counts of ovules, viable seeds, unfertilized ovules, and aborted seeds. Since pollen germination at 40°C was a proportion, we used a generalized mixed effects model (*lme4*; function glmer) with a binomial distribution for analysis. We conducted correlation analysis for mean anther and mean style plus stigma lengths (*stats*; function cor.test).Fruit set was analyzed using a chi-squared test (*stats*; function chisq.test).

Results

**Life-stage specific responses to extreme temperature**

*Sporophyte*

Of the six sporophytic variables, three differed regionally. In extreme heat (HCHPL: F1,51=4.418, p =0.041) and cold (CCHPL: F1,50=66.369, p <0.001), northern plants retained chlorophyll content more effectively than southern plants (Table 1). The chlorophyll content of northern plants was 8% and 19% higher than southern plants for the heat and cold treatments respectively. Southern plants had a 5% higher cell membrane stability in the extreme cold treatment than northern plants (CCMS: F1,191=66.369, p <0.001; Table 1).

There was no significant difference between regions for HCMS, but heat tolerance did depend on region of origin 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. The block effect on CMS may be due to the capacity of *S. carolinense* to induce temperature tolerance and acclimate to environmental conditions (Clarke, Mur et al. 2004). In block A, northern plants had a higher HCMS, but this difference degraded in the later blocks during the times when greenhouse temperatures were higher during plant development. We considered block A values the baseline HCMS and determined that northern plant have higher baseline heat tolerance. Net photosynthetic rated did not depend on region of origin for both the hot and cold treatments. There were no statistically significant correlations among sporophytic traits.

Table 1. Sporophyte and gametophyte temperature tolerance results from mixed effects linear models with the fixed effect region (north vs south) and the random effects genet and block (omitted for gametophyte). Due to overfitting the model genet was omitted from CCMS, HPS, and Tmin PTGR. Block was not included in the analysis for gametophytes and CPS. Random effect statistical values reported in the Supporting Information (Table S2), as well as results from a mixed model using only control values (Supporting Information Table S3).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Variable | | Region | | | | |
| Expected | Observed | dF | F | p |
| Sporophyte | Cell Membrane Stability (Heat) | S > N | - | 1, 50 | 3.673 | 0.0610 |
| Cell Membrane Stability (Cold) | N > S | S > N | **1, 191** | **6.482** | **0.012** |
| Chlorophyll Content (Heat) | S > N | N > S | **1, 51** | **4.418** | **0.041** |
| Chlorophyll Content (Cold) | N > S | N > S | **1, 50** | **66.369** | **<0.001** |
| Photosynthetic Rate (Heat) | S > N | - | 1 | 0 | 0.997 |
| Photosynthetic Rate (Cold) | N > S | - | 1, 47 | 3.269 | 0.077 |
| Gametophyte | Pollen Germination (Tmax) | S > N | N > S | **1, 26** | **12.054** | **0.002** |
| Pollen Germination (Topt) | S > N | N > S | **1, 24** | **10.916** | **0.003** |
| Pollen Germination (Tmin)\* | S > N | - | 1, 21 | 0.151 | 0.702 |
| Pollen Tube Growth Rate (Tmax) | S > N | - | 1, 29 | 0.446 | 0.509 |
| Pollen Tube Growth Rate (Topt) | S > N | - | 1, 29 | 0.121 | 0.731 |
| Pollen Tube Growth Rate (Tmin) | S > N | - | 1, 59 | 0.168 | 0.683 |

\* Outlier removed. Bolded values: statistically significant (α=0.05).

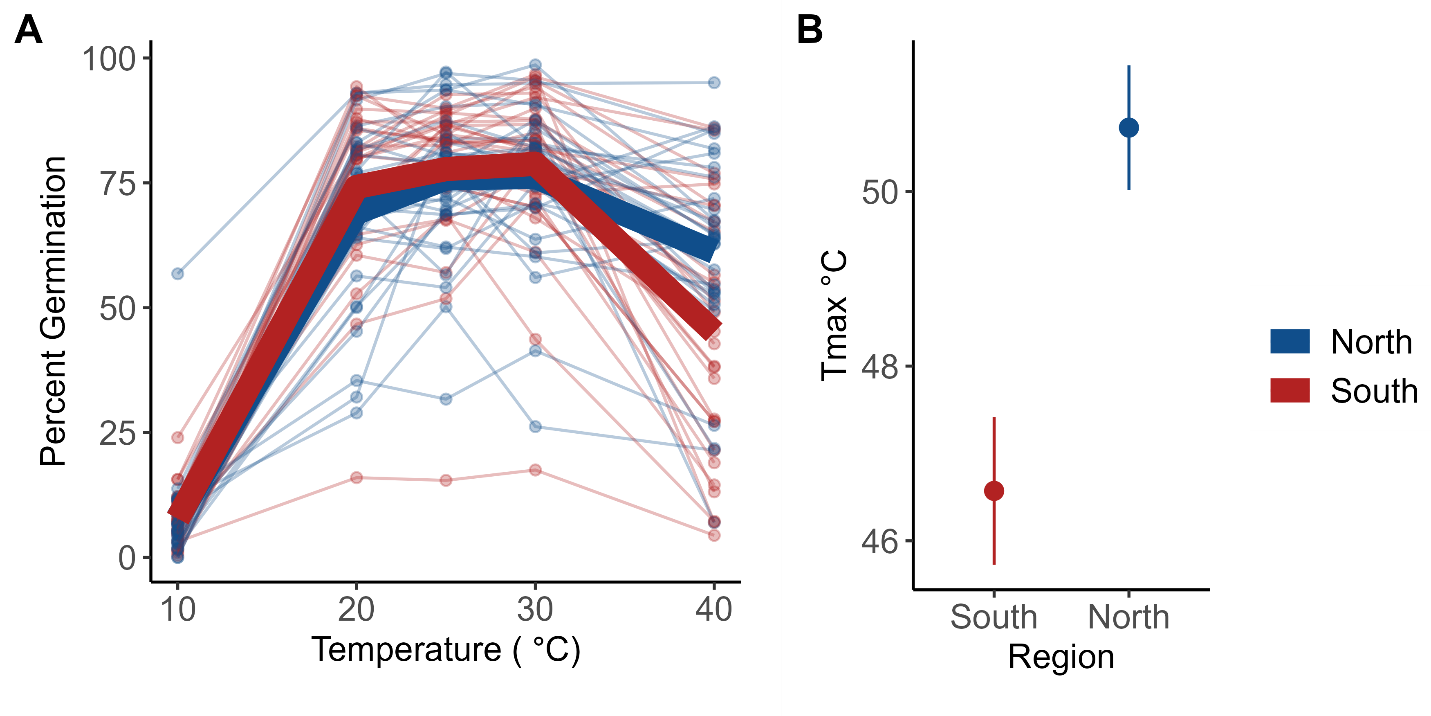
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Figure 1. Genet percent germination mean (points) and regional mean (bolded lines) colored by region (A). Mean (±se) Tmax for northern (blue) and southern (red) genets (B). Tmax was the x-intercept of the quadratic fit for each individual at the highest germination temperature predicted. Plants from the northern region germinate at higher temperatures (Tmax: F1,26=12, p =0.002).

*Gametophyte*

Of all genets included in this study, 20 genets 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. There was a significant difference between regions for Tmax (Fig. 1, Table 1) and Topt (Table 1). Plants from the north germinated more readily at high temperatures (Tmax: F1,26=12, p =0.002) and had higher thermal optima (Topt: F1,24=11, p =0.003) than plants from the south. There was no significant difference between the two regions for Tmin. One outlier was identified using the Grubbs’ test for outliers and subsequently dropped from the analysis. For pollen tube growth rates, there were no significant differences between plants from the north and south for any of the three variables.

We used correlation analysis to identify relationships between hot and cold tolerance for the sporophytic and gametophytic variables. There were no significant correlations between the sporophytic variables and between gametophytic and sporophytic variables. There were two significant correlation coefficients between gametophytic variables. Maximum and minimum pollen tube growth rates were positively correlated (r = 0.46). The second correlation between maximum pollen tube growth rate and maximum pollen germination was also positive (r = 0.3).

**The effect of long-term moderate heat on reproductive traits**

*Pre-pollination*

We found that long-term moderate heat predicted style plus stigma length, anther length, pollen grain diameter, and ovule number (Table 2, Fig. 2). Flowering time and first flower type (hermaphrodite and male) did not differ between the treatments or region of origin. On average, flowers that developed in the heat treatment had smaller floral structures. Style plus stigma length decreased by 14% (Χ2=240, p<0.001) and anther length decreased by 11% (Χ2=183, p<0.001) in long term moderate heat conditions relative to the control. Style plus stigma length also differed by region of origin. Plants from Texas on average had 5% longer style plus stigma than plants from Minnesota (Χ2=11, p=0.001). The relationship between anther and style plus stigma length also changed with development in heat. Mean anther length and style plus stigma length were correlated in the control treatment (r=0.55, t52=4.81, p<0.001), but not in the heat treatment (r=0.21, t40=1.35, p=0.184; Fig. 4). Development in heat increased the average number of ovules by about 1 ovule (Χ2=11, p=0.001) and reduced pollen size by 10% (F1,100=82, p<0.001). Neither trait differed by region. We found significant interactions between treatment and region in style plus stigma length (Χ2=6, p=0.014), anther length (Χ2=9, p=0.002), and ovule number (Χ2=53, p<0.001; Fig. 3).

*Post-pollination*

Pollen development in long-term moderate heat did not affect germination at high temperatures and germination did not differ between regions. Fruit set was also not affected by pollination and fertilization in the heat treatment. The number of viable seeds was affected by heat (Χ2=100, p<0.001) and on average decreased seed set by 16 seeds. The number of unfertilized ovules increased by six in the heat treatment compared to the control (Χ2=11, p<0.001) and the number of aborted seeds increased by about 1.64 seeds on average (Χ2=42, p<0.001). We note here that the average number of aborted seeds in the control group was relatively low with an average number of 0.63 seeds. The number of unfertilized ovules did differ by region (Χ2=6, p=0.011). There was a significant interaction between the treatment and region for the number of unfertilized ovules (Χ2=64, p<0.001) and aborted seeds (Χ2=12, p<0.001; Fig. 3).

Table 2. ANOVA results with the fixed effects temperature treatment (control and heat), region of origin (north and south), and the interaction between treatment and region. Genet was included as a random effect (excluded in pollen grain size due to overfitting the model).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Variable | Treatment | | | Region | | Treatment:Region | |
| dF | Χ2 | p | Χ2 | p | Χ2 | p |
| Style + Stigma Length (mm) | 1 | **240.11** | **<0.001** | **10.50** | **0.001** | **6.00** | **0.014** |
| Anther Length (mm) | 1 | **183.57** | **<0.001** | 0.27 | 0.605 | **9.29** | **0.002** |
| Ovule Number | 1 | **10.93** | **<0.001** | 0.036 | 0.849 | **52.87** | **<0.001** |
| Pollen Grain Size (μm) \* | 1,100 | **F=82.27** | **<0.001** | 0.00 | 0.979 | 0.00 | 0.981 |
| Pollen Germination (40°C) | 1 | 0.10 | 0.748 | 1.51 | 0.219 | 0.01 | 0.931 |
| Viable Seed | 1 | **99.71** | **<0.001** | 2.85 | 0.091 | 0.03 | 0.867 |
| Unfertilized Ovules | 1 | **11.34** | **<0.001** | **6.41** | **0.011** | **64.16** | **<0.001** |
| Aborted Seeds | 1 | **41.77** | **<0.001** | 1.99 | 0.158 | **11.62** | **<0.001** |

\*Model excluded genet random effect to avoid overfitting model. Bolded values: statistically significant (α=0.05).

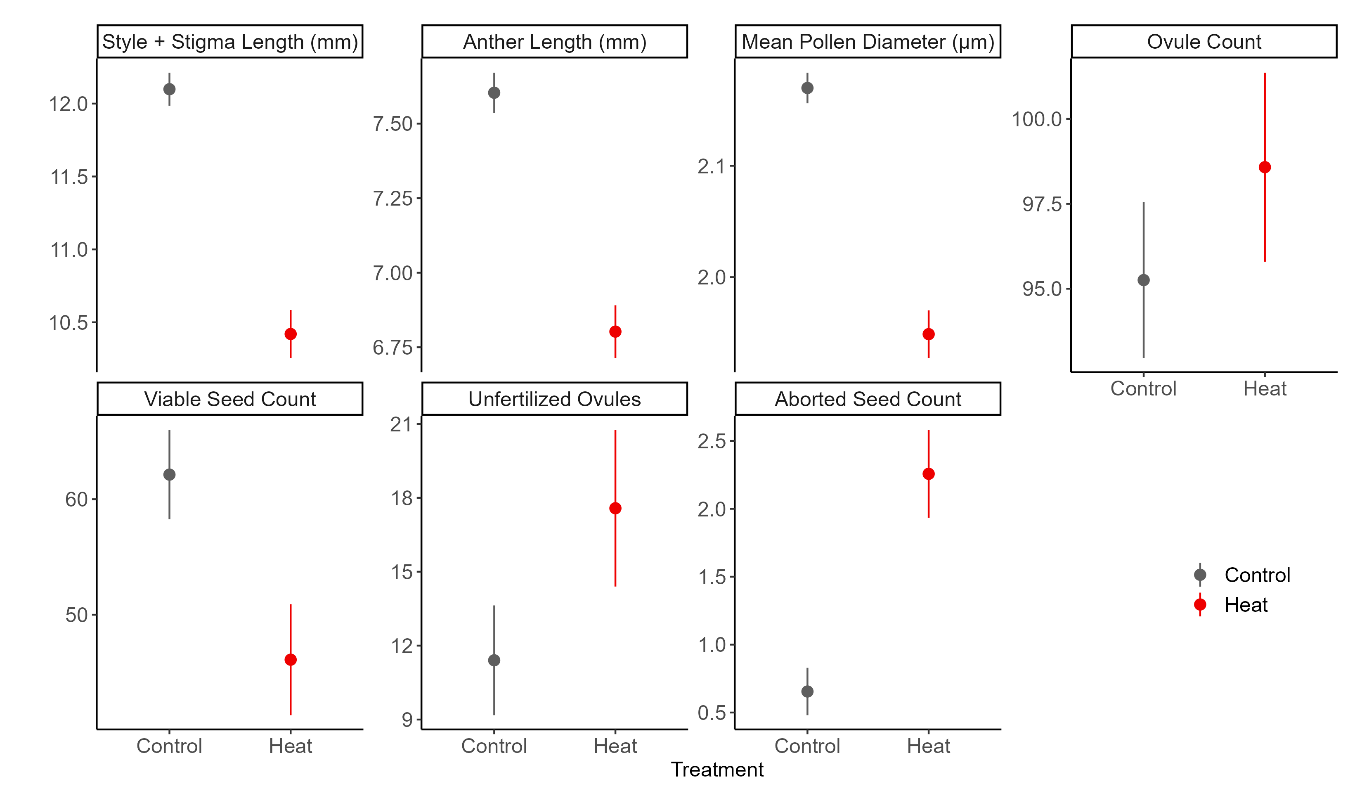


Figure 2. The effects of long-term moderate heat on morphological traits and seed set. Plant development in heat reduced the size of the stigma + style, anther, and pollen grains. The number of ovules increased. Development and fertilization in heat reduced the number of viable seeds per fruit. The number of unfertilized ovules and aborted seeds increased.

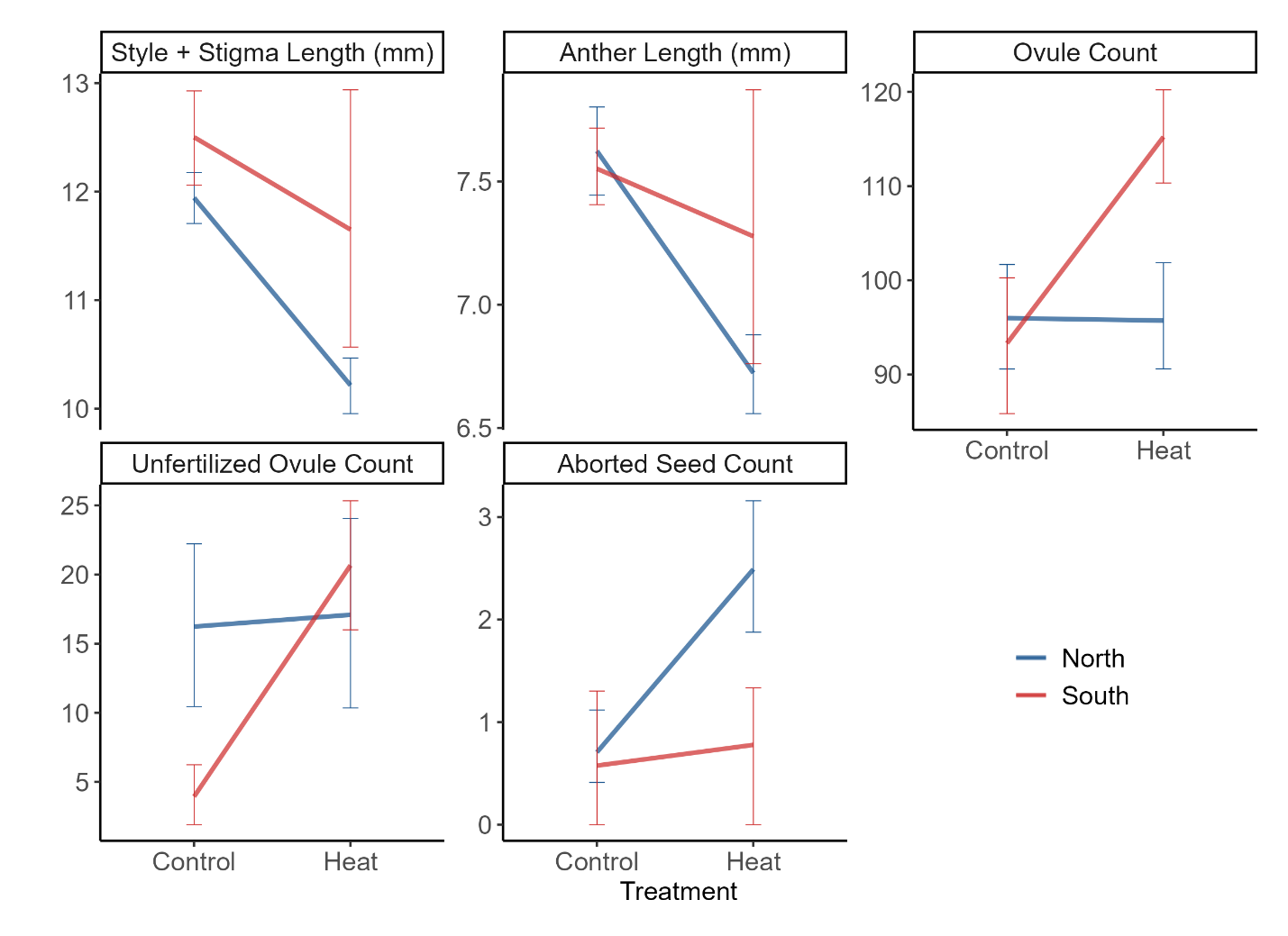


Figure 3. Interactions between treatment and region that were statistically significant.

Chart, scatter chart

Description automatically generated

Figure 4. Correlation of anther and style plus stigma length averaged across genets. Control shown in dark grey and heat treatment in red. Correlation between morphological traits (r=0.55, t52=4.81, p<0.001) deteriorated in the heat treatment (r=0.21, t40=1.35, p=0.184).

Discussion

**Life-stage specific responses to extreme temperature**

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. In contrast to our expectations, northern plants were generally more tolerant of extreme heat than southern plants or southern plants have adopted avoidance mechanisms that are adaptive in extreme heat.

Northern plants had higher chlorophyll content (HCHPL) and baseline cell membrane stability (HCMS; Fig. 4) under hot conditions, as well as higher maximum and optimal temperatures for pollen germination in comparison to southern plants (Table 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 reflect other mechanisms of adaptation that we did not consider, such as limitations of adaptation to extreme temperatures, exposure to variable temperature conditions, and avoidance strategies.

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 and selective pressure. Limitations of adaptation to extreme temperature could explain the counter gradient results we attained for CMS.

Plants from the north had more stable chlorophyll content in both the hot (HCHPL) and cold treatments (CCHPL; Table 1). The capacity of northern plants to outperform southern plants in both extreme cold and heat might be due to 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, Boote et al. 2011), populations in the north may have evolved to acclimate to temperature stress to a greater extent than southern plant.

We obtained further results that countered our expectations for the direction of temperature tolerance adaptation in the gametophyte. Pollen from the north had a higher propensity to produce pollen tubes (Germ) at high temperatures than their southern counterparts (Table 1, Fig. 1). The distinct difference between north and south suggests that *S. carolinense* pollen is sensitive to high temperatures, resulting in local adaptation. Since southern populations experience high temperatures more regularly than northern plants, there may be an avoidance strategy in southern populations whereby pollen grains remain dormant at high temperatures. In contrast, there is no selection for dormancy at high temperatures in the north.

Rutley et al. (2022) proposed the two baskets model categorizing pollen, which states that there are active (high-ROS) and backup (low-ROS) subpopulations of pollen within anthers of flowering species. Active pollen readily germinate and have fast metabolisms, increasing pollen tube growth rates, and usually outcompete the smaller, partially dehydrated backup pollen with low metabolisms. The two subpopulations of pollen are adaptive and beneficial under different conditions. In stressful environments, such as extreme heat or drought, asynchrony in pollen germination permits some pollen to remain dormant and grow pollen tubes later in more favorable conditions. In favorable conditions, active pollen tubes grow faster and are more likely to fertilize ovules than backup pollen. While the two-pollen system has not been established in *Solanum carolinenes*, there have been studies supporting two pollen types in *Solanum lycopersicum*,tomato (Jegadeesan, Chaturvedi et al. 2018, Keller and Simm 2018, Luria, Rutley et al. 2019).

We hypothesize that *Solanum carolinense* populations in the south have higher proportions of backup to active pollen grains than those in the north due to stronger selection from increased exposure to extreme heat in the south. Backup pollen that remains dormant would not be adaptive in northern populations, with little exposure to high temperature stress. This two-pollen model was partially supported by the negative correlation we found between Tmax and Tmin germination in southern plants. The negative correlation indicates that plants with pollen that germinate readily at high temperatures (high Tmax) also germinate at low temperatures (low Tmin). Active pollen would germinate in any condition (extreme heat and cold stress), while backup pollen would not germinate as freely during stressful conditions.

Tanksley et al. (1981) highlighted the association between selection in the gametophyte and sporophyte with the discovery of a correlation between allozyme genes expressed in both stages. Based on their findings and several studies that followed (Willing and Mascarenhas 1984, Pedersen, Simonsen et al. 1987, Hedhly, Hormaza et al. 2005, Poudyal, Rosenqvist et al. 2019), including studies on temperature tolerance (Hedhly, Hormaza et al. 2005, Poudyal, Rosenqvist 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, Rifkin 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 in our study, suggesting that there are different mechanisms mitigating temperature stress in the two stages. This is not the first study to find differences in patterns for extreme temperature 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.

Another explanation for the lack of coordinated response to temperature stress between the two life stages is that horsenettle has not been 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).

**The effect of long-term moderate heat on reproductive traits**

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 pre-pollination traits including flower morphology, pollen diameter, and ovule number, and post-pollination traits, such as the number of viable seeds, unfertilized ovules, and aborted seeds. In almost all traits where we found a treatment effect, heat reduced the size or number of reproductive structures.

Flowering was not affected by treatment, but we did have a disproportionate number of plants flower in the north compared to the south. In both heat and the control treatments, almost all northern plants flowered, while plants from only one southern population 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 the southern populations may be due to the location of these populations relative to the range margin for *Solanum carolinense*. Ecological pressures at the range margin may decrease sexual reproductive success and result in selection favoring clonal reproduction (Eckert 2001).Barrett (2015) 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.

Style plus stigma length and anther length were significantly smaller in the heat treatment than the control treatment. Several other studies have found that heat affects the same floral structures in other taxa, but not necessarily the same way (Lyrene 1994). Muller et al. (2016) found anther deformations when tomato flowers developed in mild heat (32°C/26°C ). 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 stigma does 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, Yetish et al. 2011) and the position of anthers relative to the stigma could affect the receipt of pollen from pollinators. We found that in the control treatment style plus stigma length was correlated with anther length, but the correlation breaks down in heat (Fig. 4). 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.*

Pollen grains that developed in long-term low heat were significantly smaller than those in controlled (25°C) conditions. There are potential fitness implications for changes in pollen size, and specifically reductions in pollen size. McCallum and Chang (2016) found evidence of pollen size influencing siring success; larger pollen grains were more competitive (sired more seeds) than smaller pollen grains in common morning glory.

We found that heat during the development of maternal tissues resulted in a slight increase in the number of ovules. However, fertilization in heat reduced the number of viable seeds per fruit (Fig. 2, Table 2) and increased the number of unfertilized ovules and aborted seeds. Previous studies have found mixed responses to heat in tomatoes. 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 the ovule number increased in heat, the decrease in viable seed number and increase in unfertilized ovules we attained, might be a product of low pollen viability at 32°C compared to 25°C. While the number of aborted seeds did increase in heat, there were very few aborted seeds counted in either treatment. This suggests that male viability and pollen tube growth may be the limiting factor at 32°C after pollen developed at 25°C, and not female viability. A similar result was attained in peas. Jiang (2019) found that ovules maintained viability in heat stress, while pollen viability decreased.

We found 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 reduced 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 (Sato, Kamiyama et al. 2006, Xu, Wolters-Arts et al. 2017, Jiang, Lahlali et al. 2019, Poudyal, Rosenqvist et al. 2019).

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 coupled with 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, Atkin et al. 2010). Regarding climate change specifically, phenotypic plasticity can be disadvantageous by decoupling the effect of environmental conditions on survival and reproduction of a particular genotype, thereby disrupting evolutionary tracking of the climate.

On the other hand, phenotypic plasticity itself can be an adaptive trait (Schlichting 1986). Molina-Montenegro and Naya (2012) found that phenotypic plasticity of several traits increased in populations with latitude of origin. 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 by allowing individuals in a population to acclimate to a larger range of conditions and persist in inconsistent and potentially unfavorable conditions. We were interested in gene x environment interactions in *Solanum carolinense* plants, and thus examined the interaction of the treatment and region of origin.

There were significant interactions between treatment and region for style plus stigma and anther length and the counts for ovule, unfertilized ovules, and aborted seeds. Style plus stigma and anther length responded similarly to heat in both the northern and southern populations. However, northern plants did respond more dramatically (increased slope) than southern plants, which aligns with our expectations of higher heat sensitivity in northern plants. Interestingly, the number of ovules and unfertilized ovules increased noticeably in heat for plants from southern populations but not in northern plants. Northern plants did however have increased seed abortion in heat, relative to southern plants. ANY SOURCES WHERE HEAT INCREASES OVULE NUMBER??

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. We did find some evidence of local adaptation in sporophytic traits, suggesting that northern plants are more robust to extreme temperatures on both ends of the spectrum. However, in the gametophyte, the responses of northern plants to extreme heat may not be adaptive. We found evidence suggesting that southern plants avoid pollen germination in high temperatures by increasing the proportion of backup to active pollen.

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. 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.

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 temperatures that are lacking in northern populations or vice versa.

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