**The effect of long-term moderate heat on sexual reproduction in *solanum carolinense* (horsenettle) and implications for evolutionary responses to environmental change**

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Abstract

Temperatures worldwide are gradually increasing due to climate change. *Solanum carolinense* has arange 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* frompopulations 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 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 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. 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 K3PO4 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 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(NO3)2, MgSO4, KNO3, H3BO3) 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.

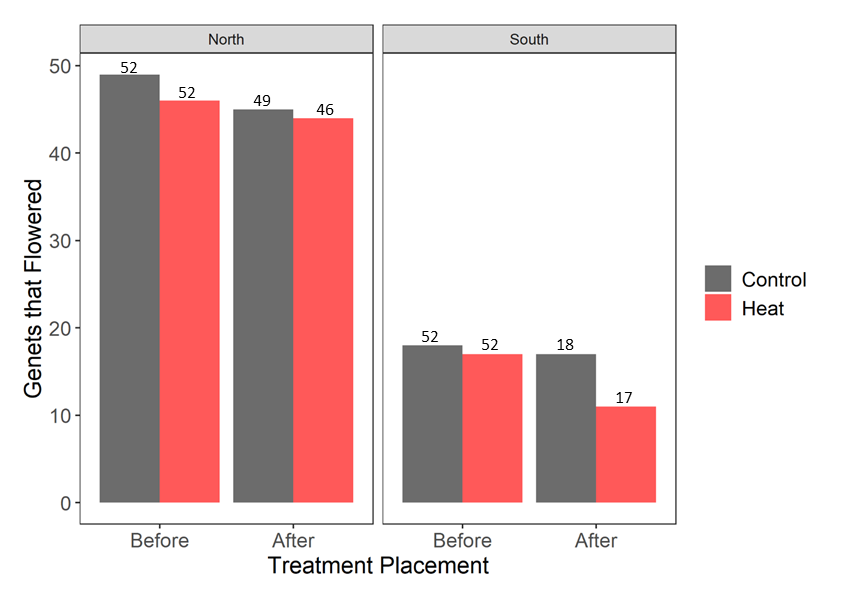


Figure 2.1. The number of genets that flowered in the control and heat treatments before and after they were placed in the treatments. Counts for the northern and southern regions are shown independently. Numbers above the bars represent the number of plants within each group that were initially placed 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 | χ2 | 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.

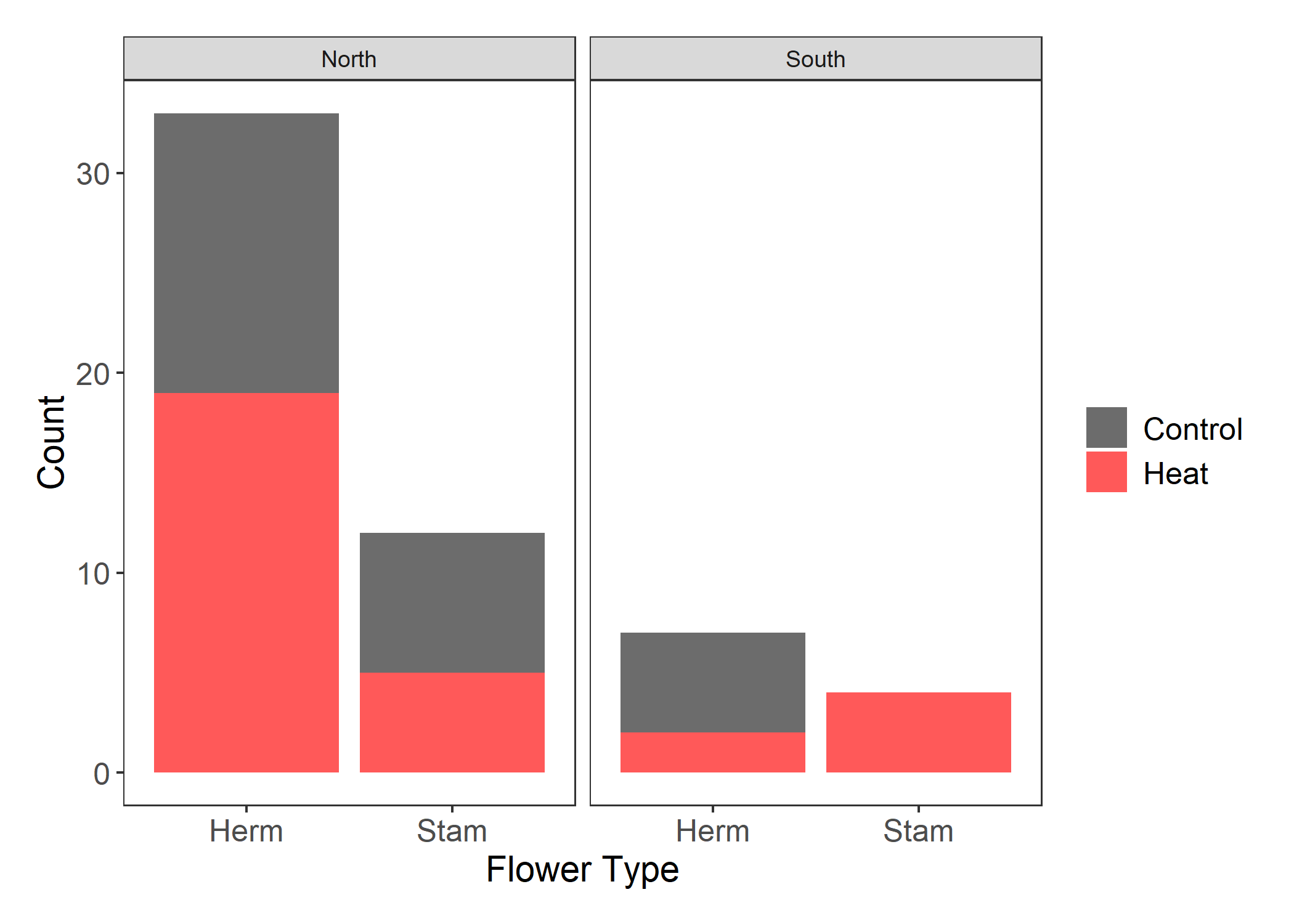


Figure 2.2. Number of plants with hermaphroditic and staminate flowers for the treatment groups. Counts for northern and southern plants displayed independently.

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 | |
| **Variable** | Region | | | Population | | | Population:Genet | Population |
| 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** | - |

Chart, box and whisker chart

Description automatically generated

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 (F25 = 4.453, p = 0.045) and anther length (F25 = 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 K2 = 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 | |
| **Variable** | Treatment | | | Population | | | Population:Genet | Population |
| 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** | - |

Chart, box and whisker chart

Description automatically generated

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 (F102 = 48.33, p = 3.49-10) and anther length (F109 = 67.85, p = 4.33e-13).

Chart, box and whisker chart

Description automatically generated

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 K2 = 14.51, p = 1.40e-04).

Chart, scatter chart

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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).

Chart, box and whisker chart

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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 (F34 = 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.

Chart, box and whisker chart

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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 (F46 = 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.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Variable |  | Region | | Treatment | |
| df | F | p | F | p |
| Pollen Germination (40°C) | 46 | **9.180** | **0.004** | 3.916 | 0.054 |

Chart, bar chart

Description automatically generated

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.

Chart, box and whisker chart

Description automatically generated

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 (F46 = 4.587, p = 0.038) and viable seeds (F46 = 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 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.

Chart, line chart

Description automatically generated

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.

Diagram

Description automatically generated

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 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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