**Intraspecific variation in responses to extreme and moderate temperature stress in the wild species, *Solanum carolinense* (Solanaceae)**

Abstract

Adaptation or acclimation to local temperature regimes has often been used as a proxy for predicting how plant populations will respond to impending novel conditions driven by human-caused climate change. To understand how plants may successfully respond to increasing air temperatures in the future, we explored how populations of *Solanum carolinense* in northern (MN) and southern (TX) regions of the continental United States differ in temperature tolerance traits in a two-experiment study. In the first experiment, we compared the heat and cold tolerance in vegetative (sporophyte) and reproductive (male gametophyte) traits. In the second experiment, we asked if long-term heat influences plant development by examining how development in moderate heat affected reproductive structures and reproductive success. We found that temperature-sensitivity differed between southern populations that regularly experience extreme heat and northern populations that do not. In contrast to our expectations, northern populations appeared more heat-tolerant than southern populations for vegetative traits such as chlorophyl stability and reproductive traits such as pollen germination and vice versa. Our results are consistent with a heat-avoidance, rather than tolerance, mechanism to mitigate extreme heat in pollen germination. In the second experiment, plants under the moderate heat treatment had significantly smaller reproductive structures and reduced seed production (27% fewer seeds on average than in the control treatment). Reproductive structures that developed in moderate heat were also reduced in size, particularly in the northern populations relative to populations from the south. We conclude that rising temperatures have the potential to incur substantial negative consequences to the reproductive success of individuals in this species and that some populations already mitigate stressful temperature conditions as a result of local adaptation and phenotypic plasticty.

**Introduction**

Climate change is rapidly altering environmental conditions at regional and local scales, leading to shifts in temperature regimes, precipitation patterns, and the severity of weather events (Seneviratne et al. 2021). As a result, there is widespread interest in understanding how plants, a mostly sessile taxonomic group, will cope with these rapid changes (Doak and Morris 2010; Molina-Montenegro and Naya 2012; Valladares et al. 2014; Demarche et al. 2018). Plants respond to environmental change in three ways while avoiding extinction; adapt relatively rapidly, acclimate to tolerate new conditions by shifting phenotypes that are plastic, or by shifting ranges (Janzen 1967; Molina-Montenegro and Naya 2012; Schlichting 1986). Because environmental conditions across a species’ range are often heterogeneous, in particular for species with large ranges, selective pressures are likely to differ among populations. Divergent selection in two regions can result in differing trait optima in the separate populations through local adaptation (Kawecki and Ebert 2004). Alternatively, plants in populations with different environments may be phenotypically plastic in their response to environmental conditions, resulting in populations with divergent traits, but little genetic divergence (Stotz et al. 2021, Nicotra et al. 2010). A current area of interest is the extent by which phenotypic plasticity is adaptive or maladaptive in response to climate change (Ghalambor et al. 2007; Nicotra et al. 2010; Dupont et al. 2024). We address this area of inquiry by comparing the phenotypes of floral structures and reproductive success when in moderate heat to ambient temperatures. Temperature is a variable that varies greatly in both severity and consistency with geographic region and often determines a species’ distribution (Von Büren and Hiltbrunner 2022).

Based on the IPCC Sixth Assessment Report (Seneviratne et al. 2021), temperatures are changing at unprecedented rates throughout the world. The National Climate Assessment (USGCRP 2018) reported that temperatures in the Midwestern and Southeastern United States have been steadily rising since the 1970’s. Changes to temperature regimes are expected to ultimately lead to temperatures that are above what is currently optimal for plant cellular processes, especially those involved in reproductive success (Sato et al. 2006; Müller et al. 2016; Xu et al. 2017; Jiang et al. 2019). Researchers have experimentally established that development in moderately high temperatures negatively affects floral morphology (Charles and Harris 1972, Sato et al. 2006, Müller et al. 2016, Xu et al. 2016), ovule viability (Xu et al. 2017), pollen viability (Sato, Kamiyama et al. 2006, Din, Khan et al. 2015, Müller et al. 2016, Xu et al. 2017, Poudyal et al. 2019), fruit set (Charles and Harris 1972, Sato et al. 2006, Din, Khan et al. 2015), and seed set (Din et al. 2015) in crop species. For many of these studies, heat was detrimental to development and reproduction. 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 moderate 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 tomatoes. Lastly, Muller et al. (2016) found that long-term moderate heat resulted in floral deformations and low pollen viability in tomatoes. While researchers have repeatedly shown that heat has negative effects on reproductive traits, few studies have examined how local adaptation may play a role in intraspecific variation in the responses to development in heat.

While many in this field have established that heat or temperature stress in general is detrimental to vegetative and reproductive traits in domesticated species, the question remains: can plants evolve tolerance or other strategies to mitigate temperature stress quickly enough to track climate change? First, selection for further trait divergence might not occur if species can acclimate to novel temperatures through phenotypic plasticity (Nicotra et al. 2010; Stotz et al. 2021). However, acclimation would require a species to have evolved appropriate levels of phenotypic plasticity and the responses to cues that improve or maintain fitness (Ghalambor et al. 2007; Molina-Montenegro and Naya 2013). In the case that a species cannot acclimate, populations in areas with temperature stress may face extirpation unless they have the potential to migrate to more favorable habitat. Lastly, local conditions introduce the possibility of divergent selection to act on the genetic diversity already within the population. Adaptation would involve a shift in tolerance by evolution of traits that improve the chances of survival or reproduction for individuals in a population experiencing thermal stress. In a place for time substitution, local adaptation to region-specific climate conditions can be studied as a proxy for how populations in areas of warming could respond to changes in climate.

We present the results of two experimental studies, where we sought to compare heat and cold tolerant plants from different latitudes and ultimately inform predictions of plant evolution in a warming environment. Our objectives were to (1) determine if local thermal conditions have divergently selected for temperature tolerance traits between northern and southern latitudes or not and (2) experimentally test the effects of moderate heat during flower and fruit development on phenotypic expression of pre- and post-pollination reproductive traits. We hypothesized that southern populations of *S. carolinense* evolved greater heat tolerance in vegetative and reproductive stages, relative to northern populations, because heat extremes in the south select for thermotolerance. Conversely, we expected the opposite for plants from more northern populations – higher tolerance to extreme cold and lower tolerance to heat stress in general. We hypothesized that heat would reduce floral structures and reproductive success, but to a lesser extent for plants from the south compared to those in the north.

Methods

*Solanum carolinense* L. (Solanaceae), also known as horsenettle, is a weedy, andromonoecious perennial that originated in southeastern North America. All other species in this clade are neotropical, suggesting that this species likely arose through dispersal to North America and independent diversification (Wahlert et a. 2014). Once established in the southeast, *Solanum carolinense* utilized its natural adaptability and propensity to reproduce both sexually and asexually to expand its range north- and west-ward into colder and hotter environments (Figure 1).

Map

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Figure 1. Map of the distribution of *Solanum carolinense* (EDDMapS 2022; 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.

We collected *Solanum carolinense* plants from two populations in Minnesota and three populations in 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 where these plants were collected, the average daily temperatures vary from a low of -9°C to a high of 22°C over the course of the year. The Texas plants together will be referred to as the southern plants. All three Texas 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, where these plants were collected, the average daily temperatures vary from a low of 6°C to a high of 29°C over the course of the year (Figure 2).

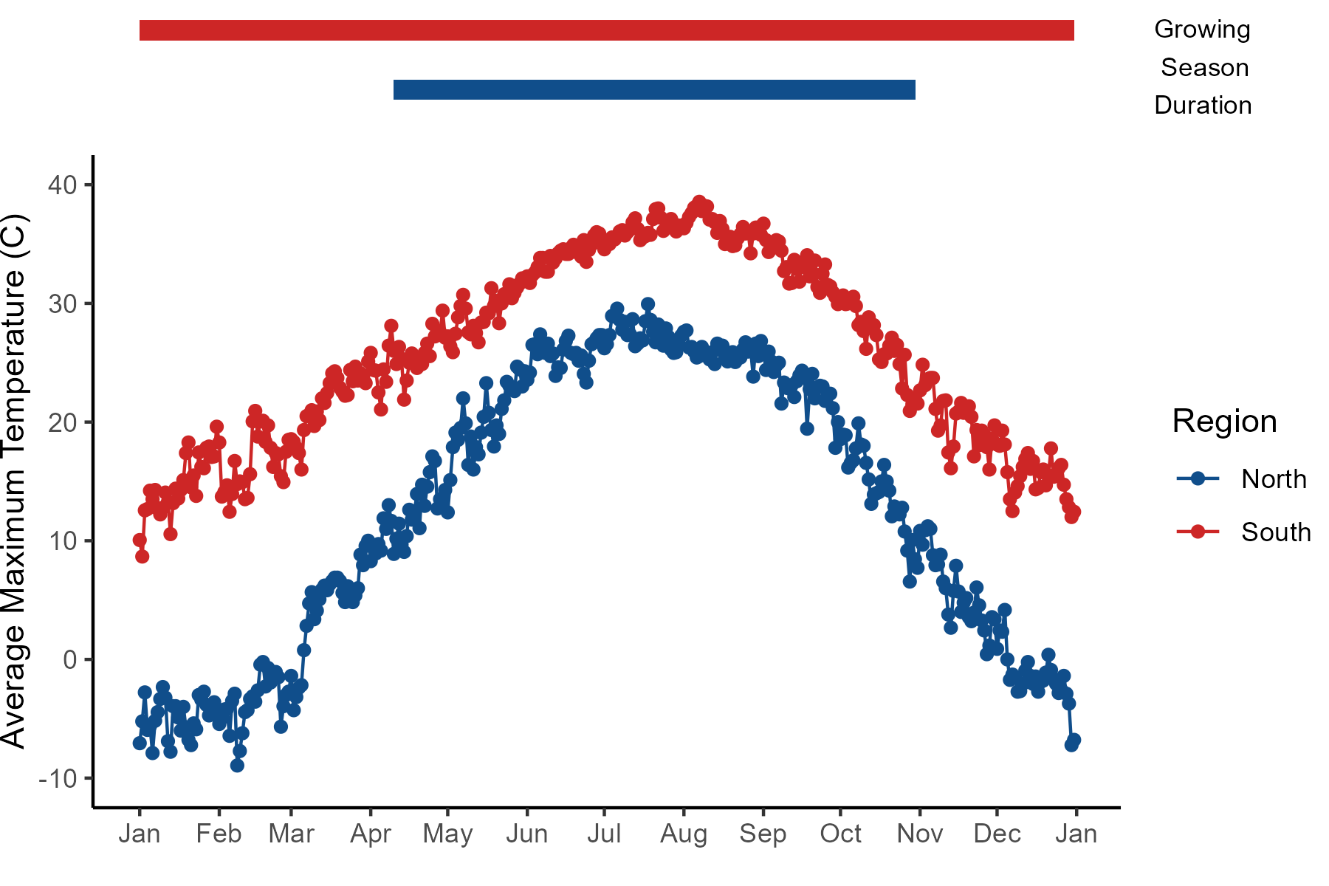


Figure 2. Average daily maximum temperature for the years 2011-2020 in Huston County, MN (North; blue) and Collin County, TX (South; red). The bars above the plot indicate the duration of the growing season or the periods at which temperatures are consecutively above 0°C*.*

We harvested plants by removing rhizomes of at least 10 cm from mature plants in the field and placing them in ziplock bags. We assumed that each rhizome represented a unique genotype (genet) because we maintained an interval of at least 1 meter between collections. The sample size was 42 genets from MN and 26 genets from TX.

The field-collected rhizomes were shipped to Fargo, ND, and stored in a 4°C refrigerator prior to a growth and dormancy period to establish an experimental population. After one to several weeks in the refrigerator, the rhizomes were potted in 3.8 L containers with a standard potting mix, allowing above and belowground material to grow for several months in a greenhouse. The above ground material was then cut, and the pots were again stored in a 4°C refrigerator to induce a period of dormancy.

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 into four equal-sized pieces. These were ultimately used to grow genetically identical plants (ramets) from each genet at different times (temporal blocks A, B, C and D; Figure 3) because of a lack of sufficient space to grow them all at once. (10-12 plants per week) Of the ramets planted each week, half were from the southern region and half were from the northern region. Since we had a total of 26 genets from the south, we randomly selected 26 of the 42 genets from the northern populations using a random number generator. Each northern plant was paired with a southern plant spatially on the greenhouse benches. originally 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.

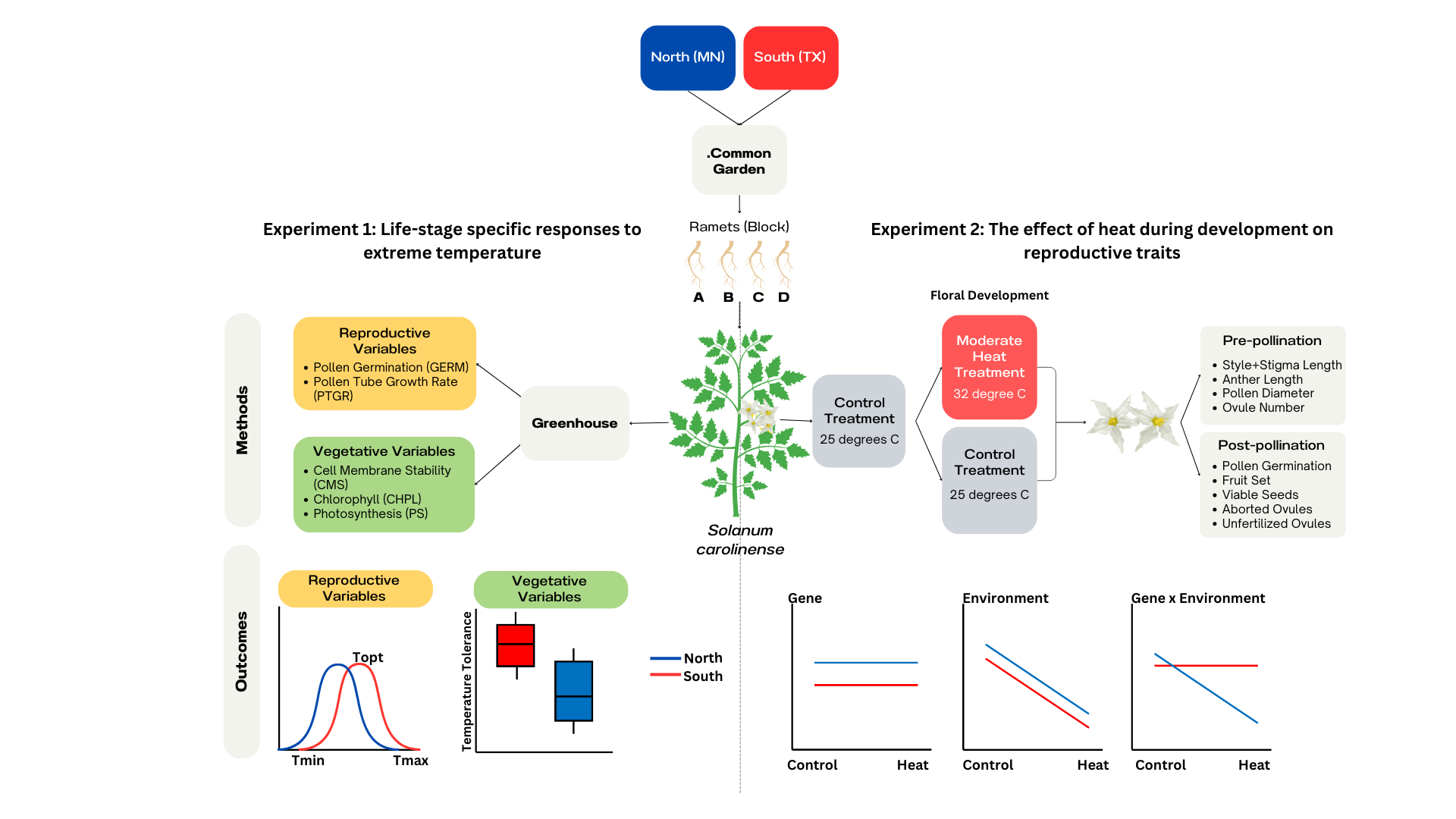


Figure 3. Conceptual diagram of methods and potential outcomes for Experiment 1 and Experiment 2.

**Experiment 1: Life-stage specific responses to extreme temperature**

To assess the temperature tolerance limits on plants, we measured three vegetative variables (cell membrane stability (CMS), chlorophyll content (CHPL), and net photosynthetic rate (PS)) and two reproductive variables (the propensity for pollen grains to germinate (GERM) and the growth rate of pollen tubes (PTGR)). We measured each variable on each plant for two temperature treatments always relative to a control treatment: hot treatment (acronym preceded by “H”) and extreme cold treatment (acronym preceded by “C”).

Vegetative temperature tolerance variables

CMS was calculated according to the protocol from Gajanayake et al. (2011) and Fang and To (2016). Ion leakage from leaf material exposed to either heat (HCMS: 55°C water bath for ten minutes) or cold (CCMS: -18°C) was measured using an electrical conductivity probe and compared to the conductivity of leaf material in control (27°C) and maximum damage (98°C) treatments. The heat treatment (55°C) used was based on the protocol from Fang and To (2016). The cold treatment (-18°C) was selected based on the methods used in Mishra et al. (2011), the potential temperatures *S. carolinense* experiences in MN, and the limited equipment options available to us for this study. Difference in chlorophyll content (CHPL) of leaves was estimated, as in Gitelson et al., (1998) for material exposed to either a hot temperature treatment (HCHPL: 60°C for 1 hr) or a cold temperature treatment (CCHPL: 4°C for 1 hr followed by -18°C for 1 hr) using a chlorophyll meter (Opti-Sciences CCM-300). The chlorophyll meter measures the fluorescence emitted at 735nm/700nm for a constant leaf area. Chlorophyll content before and after treatments was used to estimate the difference in chlorophyll content in mg/m2. The heat treatment was selected based on PS was a measure of the effects of temperature treatments on the photosynthetic capabilities of leaves. PS was estimated as the ratio of net photosynthetic rates before and after a temperature treatment (HPS: 33°C, CPS: 10°C for 48 hrs) based on leaf measurements made with a Licor. Temperature treatments were selected considering treatments used in previous studies (Sherzod et al. 2019, Xu et al. 2014, Zhu et al. 2018, Poudyal et al. 2019) and the temperature limits of the environmental chambers used for this study. More detailed methods are available in the Supporting Information.

Reproductive temperature tolerance variables

We focused on two pollen traits as reproductive variables for estimates of male thermotolerance during the reproductive stage: 1) the propensity for pollen grains to germinate (GERM) and 2) the growth rate of pollen tubes while exposed to a range of temperatures (PTGR). We paired measurements of pollen traits from plants in the north and south by sampling mature anthers of plants flowering simultaneously. Kakani et al. (2002) Pollen from one flower per plant was dispersed over five petri dishes containing the mixture described in Reddy and Kakani (2007; 3% Bacto-Agar based growth medium (sucrose, Ca(NO3)2, MgSO4, KNO3, H3BO3)). 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. Temperature treatments were selected based on the protocol used in previous studies (Fang and To 2016, Singh et al. 2008, Reddy and Kakani 2007, Kakani et al. 2002). Four pictures of each plate were taken using a compound microscope (Leica DM500 microscope, Leica ICC50 HD camera) and the LAS EZ 2.1.0 software. We measured pollen germination (Germ) for each plate by counting the number of pollen grains per image that had produced pollen tubes and dividing that count 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 et al. 2012) by the time allowed for growth (16 hours). Detailed methods provided in the Supporting Information. Each experimental plant was cut back to soil level and rhizomes were stored at 4°C for 3-9 months.

**Experiment 2: The effect of heat during development on reproductive traits**

After Experiment 1, we removed the aboveground portions of each plant and stored them at 4° C for a final dormancy period of 3-9 months (depending on Experiment 1 temporal blocking). Two ramets of all 26 genets from the north and south were placed in a randomized grid pattern in a growth chamber (Conviron PGC-FLEX). Due to space constraints in the environmental chambers, only A and B ramets were grown initially. Ramets C and D were placed in the chambers six months later. For initial growth, all plants were exposed to “control” conditions (25°C day/25°C night; fluorescent and incandescent lighting for 14 hours per day. Plants were fertilized once every two weeks with a high phosphorus fertilizer (12-55-6) to promote flower production (Super Bloom, Scotts).

Upon flowering, two ramets per genet were randomly assigned to the control conditions (25°C day/25°C night; 14hr/10hr) and the other two to the heat treatment conditions (32°C day/25°C night; 14hr/10hr; Figure 3). By necessity, these were in different Conviron chamber models (control: Conviron PGC-FLEX; heat treatment: Conviron E7/2). Plants were watered daily. Subsequent flowers and fruits developed at either elevated temperatures (32°C) or control temperatures (25°C). The moderate heat treatment of 32°C was selected based on the temperatures used in previous studies with moderate heat treatments in tomatoes (Sato, Kamiyama et al. 2006, Müller et al. 2016, Xu et al. 2017).

Pre-Pollination Phase Variables

The first three hermaphroditic flowers per plant that developed in the respective treatments were collected in ethanol and used for flower morphology measurements, ovule counts, and pollen size measurements. Floral morphology traits (length of the style+stigma and one anther) were measured under a dissecting scope. The number of ovules in each ovary was counted following a modified staining protocol adapted from Diaz and Macnair (1999). Pollen diameters of approximately 100 grains for one flower per genet were 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 Phase Variables

We pollinated three additional hermaphrodite flowers and collected the pollen of one more flower to measure subsequent female and male reproductive traits. Mature flowers were pollinated with a mix of pollen from 2 to 5 flowers (depending on the number of plants flowering at the time) that developed in the control conditions, with at least one flower from a southern plant and one flower from a northern plant represented in the pollen mix. Pollinations were accomplished by applying a mixture of pollen on the stigma with a dissection probe. Each pollinated flower was labelled with a jewelry tag. After pollination, the plants remained in their respective treatments for one week before we moved them into a greenhouse for the remainder of fruit maturation (Average Daily Temperatures 25.1°C day / 21.3°C night). Once fruits were ripe (at least one month old), they were harvested. We measured fruit set (number of fruits produced / three flowers pollinated) and the seed set. The number of viable seeds, aborted seeds, and unfertilized ovules were counted under a dissecting scope. Seeds that were visibly flat were considered aborded. Small black or brown spots visible under a dissecting microscope were considered unfertilized ovules.

In-vitro pollen germination at 40°C was used as a proxy for male reproductive success in high temperatures. As described in experiment 1, pollen from each plant was dispersed over a petri dish containing 3% Bacto-Agar based growth medium (sucrose, Ca(NO3)2, MgSO4, KNO3, H3BO3)and incubated at 40°C for 16 hours. Four pictures of each plate were taken using a compound microscope (Leica DM500 microscope, Leica ICC50 HD camera) and the LAS EZ 2.1.0 software. We measured pollen germination (Germ) for each plate by counting the number of pollen grains per image that had produced pollen tubes and dividing that by the total number of pollen grains observed.

VARIABLES AND EXPERIMENTAL DESIGN??? Altogether, we examined how the development of reproductive structures in heat affect the pre-pollination morphology including the length of style, stigma, and anthers, the number of ovules, and the size of pollen grains and post-pollination processes including fruit set, seed set, seed condition, and pollen germination rates.

Data Analysis

Experiment 1: Life-stage specific responses to extreme temperature

To analyze differences in vegetative traits between plant origins and among genets, we fit linear mixed effects models using the lmer function from the *lme4* package (Kuznetsova et al. 2017). Region (north vs. south) was considered the fixed effect and block (A, B, C, D) and genet were random intercepts. 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 (function t.test).

For the reproductive variables (GERM, PTGR), 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 (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 (function cor) to identify associations between vegetative and reproductive variables. The Holm-Bonferroni method (function p.adjust) was used to adjust p-values to account for multiple correlations (Holm 1979). All data were analyzed in R 4.1.2 (R Core Team 2020).

Experiment 2: The effect of heat during development on reproductive traits

All pre and post pollination traits were analyzed with different versions of mixed effects models using the *lme4* package (Bates et al. 2015) 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+stigma length. Since the we used the mean pollen diameter for one flower per block per genet and several genets only had one ramet (block) flower, we omitted genet as a random effect and used a general linear model (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. To examine significant interactions between region and treatment, we did a posthoc analysis using the *emmeans* package (Lenth 2023; function emmeans)We conducted correlation analysis for mean anther and mean style+stigma lengths (function cor.test).Fruit set was analyzed using a chi-squared test (function chisq.test).

Results

**Experiment 1: Life-stage specific responses to extreme temperature**

Vegetative traits

Of the six vegetative traits measured in this experiment, three differed between regions. 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. In contrast, southern plants had a 5% higher cell membrane stability in the extreme cold treatment than did northern plants (CCMS: F1,191=66.369, p <0.001; Table 1). There was a significant block effect for CCMS (Likelihood Ratio = 15.731, p <0.001; Supporting Information Fig. S3).

There was no overall significant difference between regions for HCMS, but there was a significant block effect (Likelihood Ratio = 4.728, p = 0.030). In block A, northern plants had a higher HCMS than southern plants (t = -2.910, p = 0.015), but this difference degraded in the later blocks (Supporting Information Fig. S3). Finally, there were no regional effects on photosynthetic rate in response to either cold or heat.

Table 1. Vegetative and reproductive 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 reproductive). Due to overfitting the model genet was omitted from CCMS, HPS, and Tmin PTGR. Block was not included in the analysis for reproductive traits and CPS. Statistics included in the table include the degrees of freedom (dF), F statistic (F), and p-value (p). 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 |
| Vegetative | 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 |
| Reproductive | 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).

*A diagram of a temperature

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Figure 4. Pollen germination results from Experiment 2. (A) Percent pollen germination per genet (points) and regional mean (bolded lines: blue=northern, red=southern) . (B) Mean (±se) Tmax for northern (blue) and southern (red) genets. Tmax is the upper x-intercept of the quadratic fit for temperature performance curves for each individual (i.e., the highest temperature predicted with pollen germinatipn).

Reproductive traits

There was a significant difference between regions for Tmax (Figure 4, Table 1) and Topt (Table 1). Pollen from plants from the north were more likely to germinate at high temperatures (Tmax: F1,26=12, p =0.002) and had higher thermal optima (Topt: F1,24=11, p =0.003) than pollen from 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. There were no significant differences in pollen tube growth rate between plants from the north and south for Tmax, Topt or Tmin.

Vegetative and Reproductive Tolerance correlations

After a Holm-Bonferroni correction for multiple correlations, there were no significant correlations between the vegetative variables and any reproductive and vegetative variables. However, there were two significant correlation coefficients between reproductive variables. Tmax and Tmin of pollen tube growth rates were positively correlated (r = 0.46). There was also a significant correlation between Tmax for pollen tube growth rate and for pollen germination (r = 0.3).

**Experiment 1: The effect of heat during development on reproductive traits**

Pre-pollination

We found that long-term moderate heat negatively impacted style+stigma length, anther length, pollen grain diameter, and ovule number (Table 2, Figure 5). On average, flowers that developed in the heat treatment had smaller floral structures. Style+stigma length decreased by 14% (Χ2=240.11, p<0.001) and anther length decreased by 11% (Χ2=183.57, p<0.001) in long term moderate heat conditions relative to the control. Style+stigma length also differed by region of origin. Plants from Texas on average had 5% longer style+stigma than plants from Minnesota (Χ2=10.50, p=0.001). The relationship between anther and style+stigma length changed with development in heat. Mean anther length and style+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; Figure 6). Development in heat increased the average number of ovules by approximately 1 ovule (Χ2=10.93, p=0.001) and reduced pollen size by 10% (F1,100=82.27, p<0.001). Neither ovule count or pollen size differed by region. We found significant interactions between treatment and region in style+stigma length (Χ2=6.00, p=0.014), anther length (Χ2=9.29, p=0.002), and ovule number (Χ2=52.87, p<0.001; Figure 7).

Post-pollination

Pollen development in long-term moderate heat did not affect germination at high temperatures and germination did not differ between regions (Table 2). Fruit set was also not affected by the heat treatment. The number of viable seeds was affected by heat (Χ2=99.71, p<0.001) and on average heat decreased seed set by 16 seeds. The number of unfertilized ovules increased by six in the heat treatment compared to the control (Χ2=11.34, p<0.001) and the number of aborted seeds increased by about 1.64 seeds on average (Χ2=41.77, 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=6.41, p=0.011). There was a significant interaction between the treatment and region for the number of unfertilized ovules (Χ2=64.16, p<0.001) and aborted seeds (Χ2=11.62, p<0.001; Figure 7).

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).

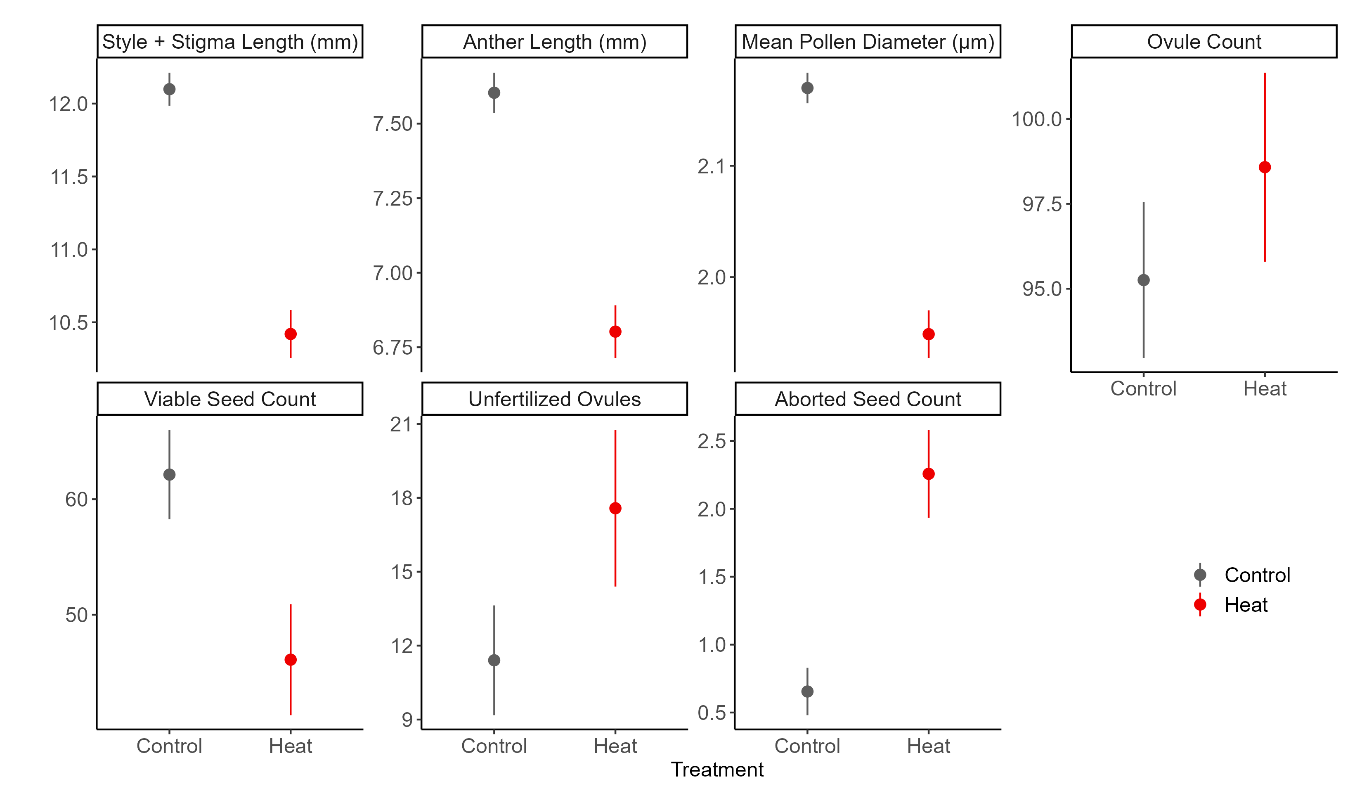


Figure 5. The effects of long-term moderate heat on morphological traits and seed set.

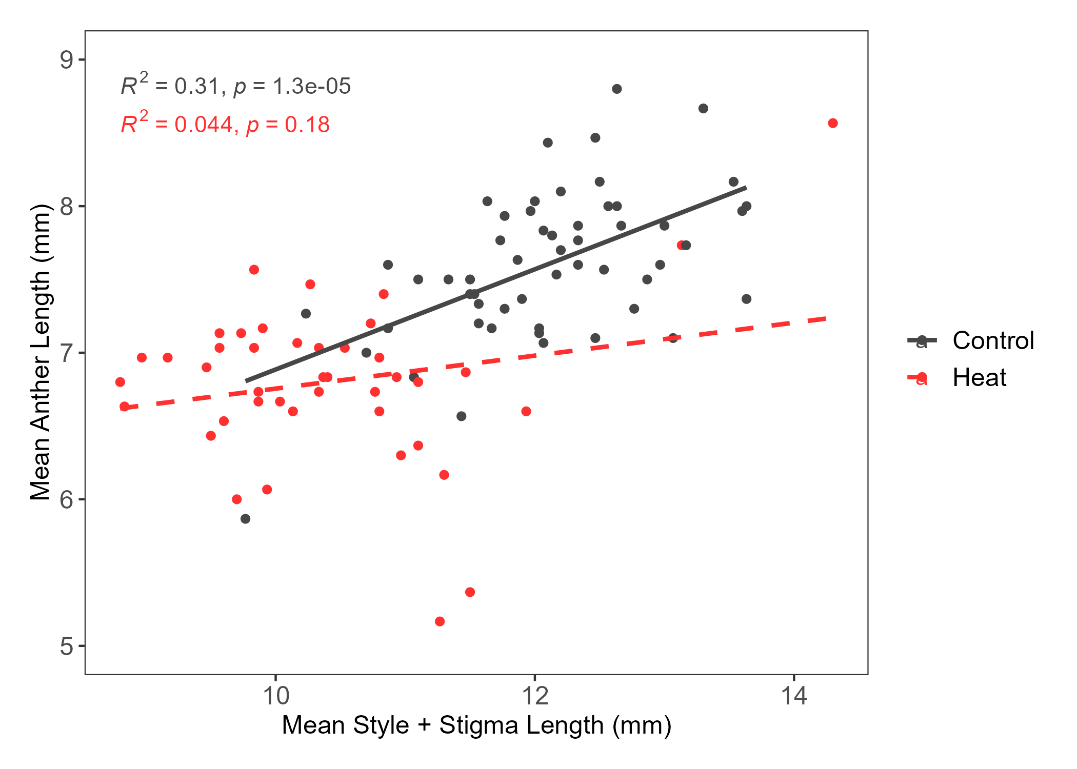


Figure 6. Correlation of Anther and Style+Stigma length averaged across genets in Experiment 1. Control shown in dark grey and heat treatment in red.

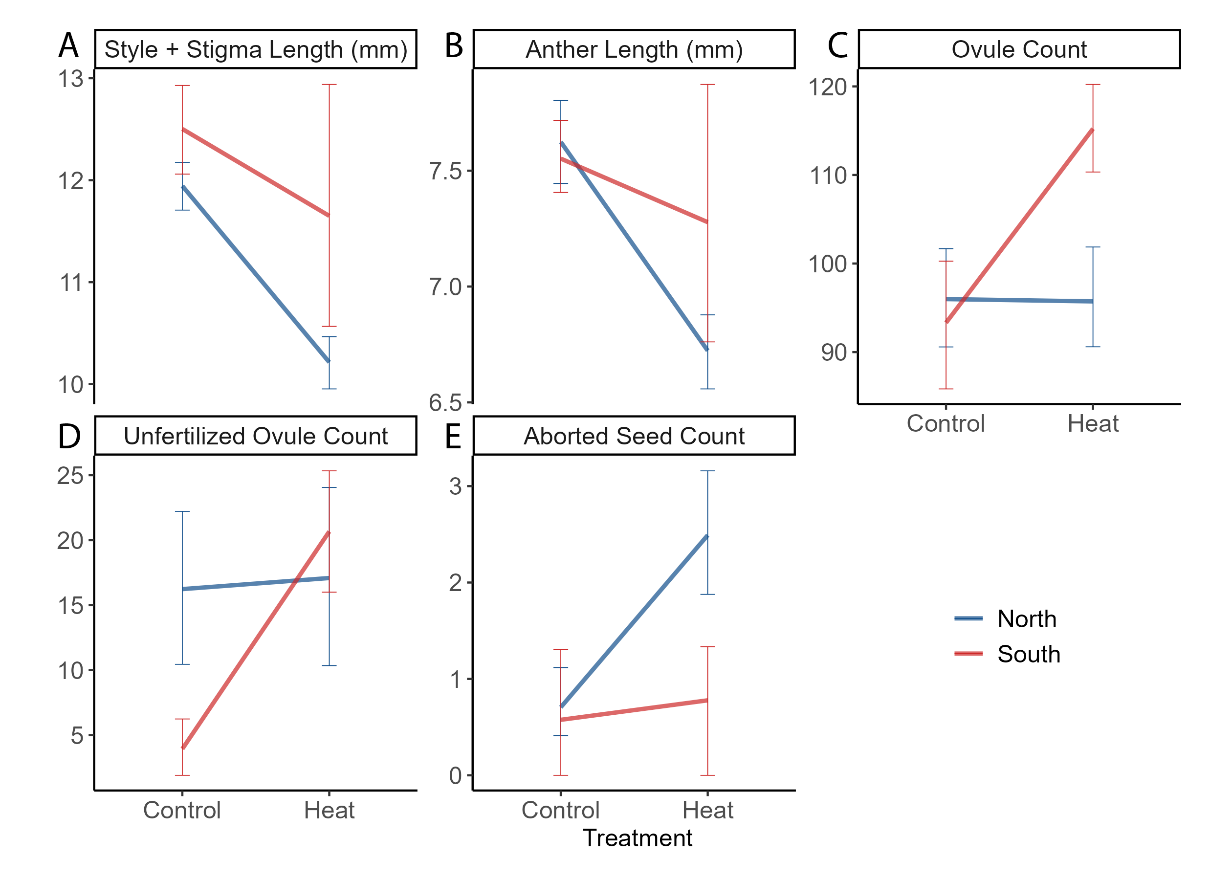


Figure 7. Statistically significant interactions in Experiment 1 between heat treatment (heat and control) and region (North; blue and South; red). Error bars indicate the standard error. In a posthoc analysis, Style+Stigma Length (A) interactions significantly differed for control and heat in the north (p < 0.001) and south (p < 0.001) and north in heat vs south in the control (p < 0.001). Anther length (B) interactions significantly differed for control vs heat in the north and north in heat vs south (p < 0.001) in control (p < 0.001). The only significant ovule (C) and unfertilized ovule count (D) interaction was control vs heat for southern plants (p < 0.001 for both). For aborted seed counts (E), heat vs control in norther plants (< 0.001) and north in the heat treatment vs south in the control (p < 0.001) were significant interactions.



Discussion

Regional differences

Despite the fact that all of the plants used in our experiments were the same species and were grown in approximately the same conditions, there were significant differences between the responses to hot temperatures between plants from northern versus southern latitudes (Figure 7). The floral morphology reductions in response to heat described previously, were not consistent between northern and southern plants. Reductions in the length of female and male floral structures were significantly more pronounced in plants from northern populations relative to southern populations (Table 2 – treatment x region effects, Figure 7). These patterns and the increase in ovule counts for southern plants suggest that they will maintain allocations of energy to floral structures despite the heat stress. In contrast, northern plants may reduce their energy allocation to floral structures as an alternative strategy for tolerating heat stress. These differences are consistent with long-term local adaptation of plants to the thermal patterns and environments in the two different places (MN and TX).

Another key difference between northern and southern plants was how heat, during pollination and fruit development, influenced the number of aborted and unfertilized ovules. In plants from southern regions, heat led to increases in the number of ovules that were unfertilized, while in plants from the north, heat led to increases in the number of aborted ovules. In control conditions, southern plants had a much lower percentage of ovules that were unfertilized than northern plants. In heat, the percentage was about the same for the two regions. The reduced efficiency of fertilization in southern plants when exposed to heat may be the result of two separate phenomena. First, in heat, southern plants produced more ovules and generally had longer styles than northern plants. The increased number of unfertilized ovules may be a result of southern plants producing a larger number of ovules during development and the inability of a full pollen load to fertilize the excess ovules. Second, prolonged heat exposure and termination of pollen tube growth in the longer styles of flowers on southern plants could also contribute to the number of ovules that went unfertilized in heat. Both of the above phenomena and the generally low seed abortion rate suggest that pollen is the limiting factor.

A similar result to ours was attained by Jiang (2019), who found, in peas, that ovules maintained viability in heat stress, but pollen viability decreased. Indeed, pollen germination has been shown to be negatively affected by heat in many studies (Sato et al. 2006; Müller et al. 2016; Xu et al. 2017; Jiang et al. 2019; Poudyal et al. 2019). In Experiment 2, we found no evidence that pollen development in heat reduces pollen germination (Table 1), but in Experiment 1, we established that heat generally reduces pollen germination for pollen from northern and to a greater extent southern plants.

A second set of evidence that regional differences in *Solanum carolinense* have evolved over time comes from the divergent patterns of tolerance to extreme temperatures between northern and southern plants in Experiment 1 (Table 1). Contrary to our expectations, we found that in multiple life stages, northern plants were more tolerant of extreme heat than are southern plants and southern plants were more tolerant of cold in terms of membrane stability. Typically, heat tolerance is measured by exposing plant material to heat and quantifying cell membrane stability and chloroplast integrity under high relative to control levels of heat (Murty and Majumder 1962; Martineau et al. 1979; Gajanayake et al. 2011; Fang and To 2016). In Experiment 1, we found northern plants had higher chlorophyll content (HCHPL) and baseline cell membrane stability (HCMS) under hot conditions, as well as lower membrane stability under cold conditions in comparison to southern plants (Table 1, Figure 5). There was a significant block effect for CMS in both the heat and cold treatment. The block effect on CMS may be due to the capacity of *S. carolinense* to induce temperature tolerance and acclimate to environmental conditions (Clarke et al. 2004). Temperatures in the greenhouse progressively rose throughout the spring and summer (Supporting Information Fig. S2), potentially inducing heat tolerance and pre-conditioning out plants for the temperature manipulations.

Plants from the north had more stable chlorophyll content in both the hot (HCHPL) and cold treatments (CCHPL; Table 1). The capacity of chloroplasts in northern plants to outperform southern plants in both extreme cold and heat might be due to northern plants experiencing a larger range of temperatures and broad adaptation to stress in general. 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). If responses in tolerance to extreme cold in the north, where wintering rhizomes remain in frozen ground for months, yields general physiological tolerance to any temperature extremes, then northern plants should be more tolerant than southern plants. Instead, we found that chloroplasts seem to be more tolerant in the north but not cell membranes. Heat shock proteins, that play an important role in maintaining tolerance to heat in plant cells (Feder and Hofmann 1999; Frank et al. 2009; Heckathorn et al. 1998), can also confer tolerance to cold by stabilizing protein configurations and functions in cells at stressful temperatures (Neta-Sharir et al. 2005). Therefore, selection for extreme temperature tolerance may be more common in northern latitudes. Northern plants will suffer severe fitness consequences if they do not maximize growth and reproduction during the relatively short growing season (Fig.2), whereas southern plants can avoid growing and reproducing during the hottest portions of summer and still have months of mild temperatures available. Our results suggest that increased thermal tolerance applies to chloroplasts in the north but not to cell membranes.

The results from reproductive trait comparisons also countered our expectations for the direction of temperature tolerance. Pollen from the north had a higher propensity to produce pollen tubes (Germ) at high temperatures than their southern counterparts (Table 1, Figure 5). One possible explanation for these results is that there is an avoidance strategy in southern populations where maximum summer temperatures can reach over 38°C consistently. Under these conditions, there could well be a selective advantage to pollen remaining dormant rather than germinating at high temperatures. In contrast, there may be no selection for dormancy at relatively high temperatures in the north. This explanation is supported by a theory regarding pollen dormancy developed in Rutley et al. (2022). They proposed the “two baskets model” categorizing pollen and stating that there are active (high-ROS) and backup (low-ROS) subpopulations of pollen within anthers of flowering species. Active pollen readily germinates and has fast metabolisms, increasing pollen tube growth rates, and typically outcompete the smaller, partially dehydrated backup pollen with low metabolisms. The two subpopulations of pollen are adaptive 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 carolinense*, there have been studies demonstrating these two pollen types in *Solanum lycopersicum*, tomato (Jegadeesan et al. 2018; Keller and Simm 2018; Luria et al. 2019).

Predictions about how species will be affected by climate change can be improved with a better understanding of how different populations of the same species differ in their responses to heat now but at different latitudes with different thermal patterns. The future climate experienced by plants in Minnesota is predicted to more closely resemble that experienced by plants in Texas now and in the past including both higher average temperatures in summer months and higher maximum daily temperatures (IPCC 2014). Growing seasons are already getting longer in northern populations (Badh et al. 2009; Dunnell and Travers 2011). Given the responses to heat by plants in our experiments in the form of relatively moderate heat (32°C) during floral development, pollen tube growth and fruit maturation and extreme heat (40-60°C) in acute doses, we suggest that plants in the two regions we studied have evolved some differences that represent differing strategies for surviving thermal stress. There is little evidence that southern plants have evolved greater cellular tolerances to extreme heat despite growing in an environment that can have daily maximum temperatures above 40.5° C. The stability of chloroplasts and pollen germination capabilities were reduced after exposure to extreme heat in southern relative to northern plants (Table 1, Figure 5). If plants in the south have shifted to an avoidance strategy where the temperature extremes of summer months are avoided by dormancy of pollen or flowering patterns shifted earlier or later, then selection for tolerance of high heat may not occur. In contrast, northern plants that experience relatively short seasons when growth and flowering are possible will need to flower and develop fruit during the hottest times of the year to produce viable seed. However, as climate change leads to longer, hotter growing seasons, our expectation is that an upper limit to tolerance of heat will ultimately lead to different phenological patterns and perhaps dormancy. Thus, the evolutionary consequences of climate change for flowering plants is likely to be a complex shift in phenological and physiological patterns.

Responses to heat

As in other studies (Muller et al. 2016, Fahad et al. 2017), we found that exposure to higher temperatures during plant and floral growth led to negative effects on traits tied to successful reproduction. In Experiment 2, where plants were exposed to moderate heat (32 °C) and control conditions (25 °C) during floral development, there was a significant treatment effect on 7 of the 8 characteristics we measured (Table 2, Figure 5) including floral morphology measurements, pollen size and ovule fate (viable, aborted, unfertilized). Regardless of where they were from, flowers were smaller and the number of viable seeds decreased in hot conditions.

foundFigure 6Several other studies found that heat affects the floral structures in other taxa, but not necessarily in the same way (Lyrene 1994). Muller et al. (2016) found anther deformations when tomato flowers developed in moderate heat (32°C/26°C). Charles and Harris (1972) found that as temperatures increased, the distance between the antheridial cone and the stigma (herkogamy) in tomatoes decreased (longer pistil or shorter stamen). Further investigations would be useful to determine if observed changes to positions of integral reproductive structures in heat affect rates of self-pollination and inbreeding in *S. carolinense*.

The effect of heat on viable seed production and pollen size in our study represent important responses to temperature stress that could have fitness consequences. Pollen diameter dropped significantly in Experiment 2, when flowers developed in hotter conditions (Figure 5). 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 reduced the number of viable seeds. There is contrasting support for this result in the literature for the close relative, tomato. Xu et al. (2017) found that heat had little influence on seed number compared to other reproductive traits, but Din et al. (2015) found that seed set was reduced in heat, especially in more temperature sensitive accessions. They attributed this difference to heat reducing pollen viability, or pollen tube growth in the style. *S. carolinense* is likely responding to heat in the same way as tomato and may be even more sensitive to heat than some accessions of tomato. In sum, these results suggest that the stress of warmer temperatures during floral development can have important negative effects with potential evolutionary consequences.

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