1   **Intraspecific variation in responses to extreme and moderate temperature stress in the wild**

2   **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 over the long-term to increasing air temperatures in the future, we explored how northern (MN) and southern (TX) populations of the species, *Solanum carolinense*, differ in temperature tolerance traits in a two-part study. We first asked if long-term heat influences plant development by examining how development in moderate heat affected reproductive structures and reproductive success. We then compared how plants from the two regions respond to extreme heat and cold in both vegetative and reproductive traits. We found that moderate heat was generally detrimental to the development of reproductive structures and seed production. Plants in heat produced 27% fewer seeds on average than plants in the control. Reproductive structures that developed in heat were also reduced in size, particularly in the northern populations relative to populations from the south. In the second experiment, 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 certain traits and vice versa. Our results are consistent with an avoidance, rather than tolerance, mechanism to mitigate extreme heat in pollen germination. We conclude that rising temperatures have the potential to incur substantial consequences to the reproductive success of individuals in this species and that some populations already mitigate stressful temperature conditions as a result of evolutionary processes.

Introduction

Climate change is rapidly altering environmental conditions at regional and local scales, leading to relatively rapid shifts in temperature regimes, precipitation patterns, and the severity of weather events (Seneviratne, Xuebin. 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

34   particular for species with large ranges, selective pressures are likely to differ among

35   populations. Divergent selection in two regions can result in differing trait optima in the separate

36   populations through local adaptation (Kawecki and Ebert 2004). Alternatively, plants in

37   populations with different environments may be phenotypically plastic in their response to

38   environmental conditions, resulting in populations with divergent traits, but little genetic

39   divergence. Temperature is a variable that varies greatly in both severity and consistency with

40   geographic region and often determines a species' distribution (Von Büren and Hiltbrunner

41   2022). To understand how plants will respond to a warming world and test for local adaptation of

42   plants to regional thermal environments, we examined how heat and cold stress affected traits

43   across latitudes in a widespread weed, *Solanum carolinense* (Solanaceae).

44   Based on the IPCC Sixth Assessment Report (Seneviratne et al. 2021), temperatures are

45   changing at unprecedented rates throughout the world. The National Climate Assessment

46   (USGCRP 2018) reported that temperatures in the Midwestern and Southeastern United States

47   have been steadily rising since the 1970's. Changes to temperature regimes are expected to

48   ultimately lead to temperatures that are above what is currently optimal for plant cellular

49   processes, especially those involved in reproductive success (Sato et al. 2006; Müller et al. 2016;

50   Xu et al. 2017; Jiang et al. 2019). Researchers have experimentally established that development

51   in moderately high temperatures affects floral morphology (Charles and Harris 1972, Sato et al.

52   2006, Müller et al. 2016, Xu et al. 2016), ovule viability (Xu et al. 2017), pollen viability (Sato,

53   Kamiyama et al. 2006, Din, Khan et al. 2015, Müller et al. 2016, Xu et al. 2017, Poudyal et al.

54   2019), fruit set (Charles and Harris 1972, Sato et al. 2006, Din, Khan et al. 2015), and seed set

55   (Din et al. 2015) in crop species. For many of these studies, heat was detrimental to development

56   and reproduction. Sato et al. (2006) found that elevated temperatures decreased fruit set and

57   pollen viability as well as stamen height in tomato. Poudyal et al. (2019) found that pollen

58   viability decreased in heat, but more tolerant tomato accessions had higher pollen germination

59   than sensitive accessions. Xu et al. (2017) found that long-term mild heat decreased pollen

60   viability, pollen number, female fertility, and fruit set. Charles and Harris (1972) found that

61   flower production, fruit set, fruit size, pollen germination, and distance between the stigma and

62   antheridial cone all decreased at high temperatures in tomatoes. Lastly, Muller et al. (2016)

63   found that long-term mild heat resulted in floral deformations and low pollen viability in

64   tomatoes. Researchers have repeatedly shown that heat has negative effects on reproductive traits

65   and correlates of male and female reproductive success in crop species.

66   While many in this field have established that heat or temperature stress in general is

67   detrimental to vegetative and reproductive traits in domesticated species, the question remains:

68   can plants evolve tolerance or other strategies to mitigate temperature stress quickly enough to

69   track climate change? First, selection for further trait divergence might not occur if species can

70   acclimate to novel temperatures through phenotypic plasticity. However, acclimation would

71   require a species to have evolved appropriate levels of phenotypic plasticity and the responses to

72   cues that improve or maintain fitness. In the case that a species cannot acclimate, populations in

73   areas with temperature stress may face extirpation unless they have the potential to migrate to

74   more favorable habitat- range shifts. Lastly, local conditions introduce the possibility of

75   divergent selection to act on the genetic diversity already within the population. Adaptation

76   would involve a shift in tolerance by evolution of traits that improve the chances of survival or

77   reproduction for individuals in a population experiencing thermal stress. In a place for time

78   substitution, local adaptation to region-specific climate conditions can be studied as a proxy for

79   how populations in areas of warming could respond to changes in climate.

80   Here we present the results of two experimental studies on *Solanum carolinense*, where

81   we sought to compare heat and cold tolerant plants from different latitudes and ultimately inform

82   predictions of plant evolution in a warming environment. Our objectives were to (1) determine if

83   local thermal conditions have divergently selected for temperature tolerance traits between

84   northern and southern latitudes or not and (2) experimentally test the effects of moderate heat

85   (32°C) versus control (25°C) temperatures during flower and fruit development on phenotypic

86   expression of pre- and post-pollination reproductive traits. We hypothesized that southern

87   populations of *Solanum carolinense* evolved greater tolerance to moderate and extreme heat in

88   reproductive and vegetative stages, relative to northern populations, because heat extremes in the

89   south select for thermotolerance. Conversely, we expected the opposite for plants from more

90   northern populations – higher tolerance to extreme cold and lower tolerance to heat stress in

91   general.

92   **Methods**

93   *Solanum carolinense* L. (Solanaceae), also known as horsenettle, is a weedy,

94   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
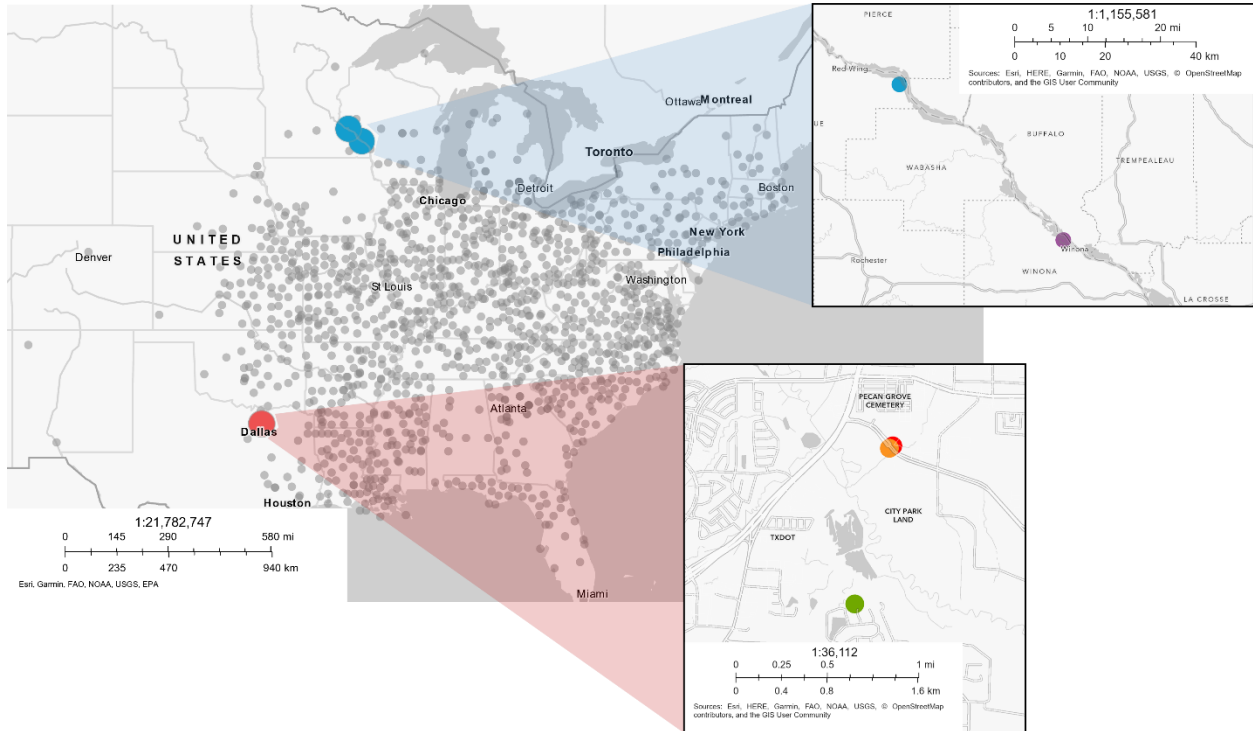
(Figure 3).

Figure 1. Map of the distribution of *Solanum carolinense* (grey dots), northern (blue dots) and

southern regions (red dots), and populations of origin for plants in this study. The populations

Frontenac (top blow-up, blue) and Prairie Island (top blow-up, purple) were in the northern

region and the populations Cemetery (bottom blow-up, red), Oil Patch (bottom blow-up, orange),

and Reserve (bottom blow-up, green) were located in the southern region.

      We collected *Solanum carolinense* plants from multiple populations in Minnesota and

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

113   were located within a circle with a 1.5 Km radius near McKinney TX (Oil Patch: 33.173465 N, -

114   96.615402 W; Reserve: 33.159962 N, -96.619011 W; and Cemetery: 33.173672 N, -96.615096

115   W). In Colin County TX, where these plants were collected, the average daily temperatures vary

116   from a low of 6°C to a high of 29°C over the course of the year (Figure 2).
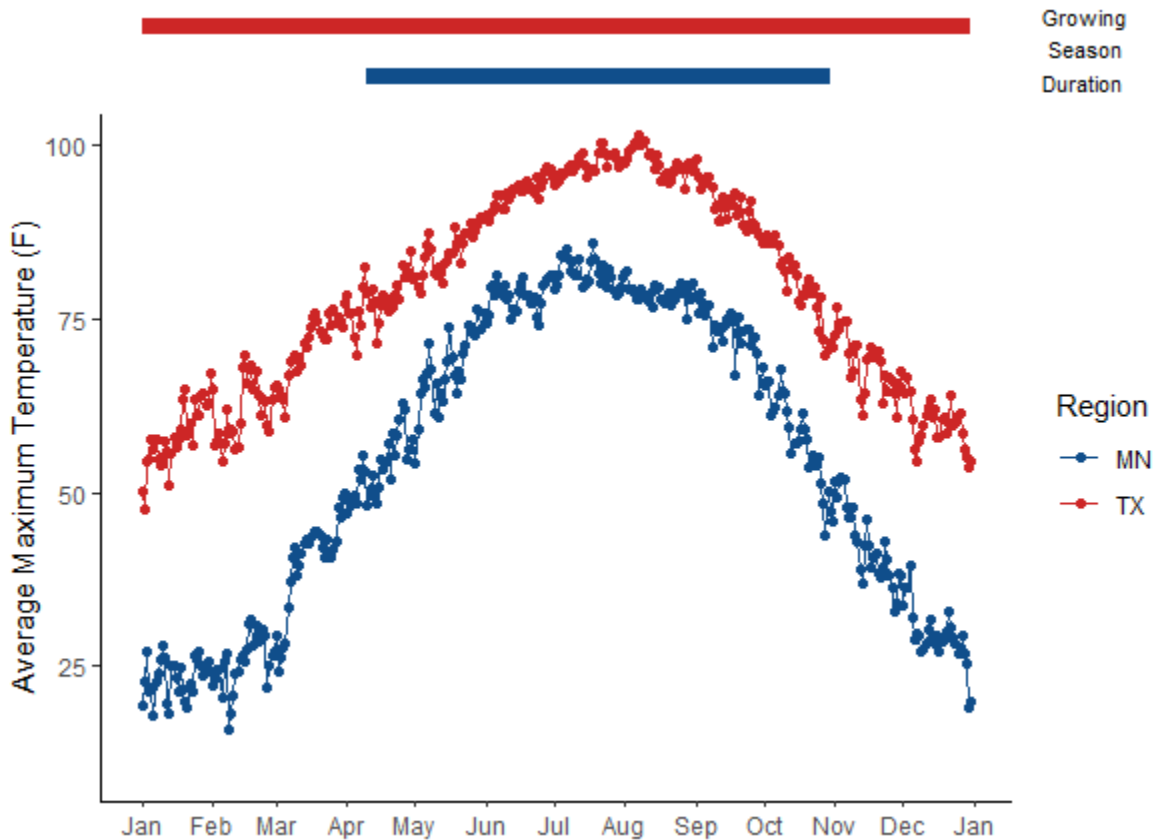

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118   Figure 2. Average daily maximum temperature for the years 2011-2020 in Huston County, MN

119   and Collin County, TX. The bars above the plot indicate the duration of the growing season or

120   the periods at which temperatures are consecutively above 32°F (0°C).

121   We collected plants by removing rhizomes of at least 10 cm from mature plants in the

122   field and placing them in ziplock bags. We assumed that each rhizome represented a unique

123   genotype (genet) because we maintained an interval of at least 1 meter between collections. The

124   number of rhizomes (genets) collected at each population were as follows: Prairie Island (n= 29);

125   Frontenac (n= 13); Oil Patch (n= 9); Reserve (n= 6); and Cemetery (n= 11).

126   The field-collected rhizomes were shipped to Fargo, ND, and stored in a 4°C refrigerator

127   prior to a growth and dormancy period to establish an experimental population. After one to

128 several weeks in the refrigerator, the rhizomes were potted in one-gallon containers with a

129 standard potting mix, allowing above and belowground material to grow for several months in a

130 greenhouse. The above ground material was then cut, and the pots were again stored in a 4°C

131 refrigerator to induce a period of dormancy.

132   After the dormancy period (3 months), equal sections of rhizome (at least 2 cm for thick

133 rhizomes and increased lengths for thinner rhizomes) were cut into four equal-sized pieces.

134 These were ultimately used to grow genetically identical plants (ramets) from each genet at

135 different times (temporal blocks A, B, C and D) because of a lack of sufficient space to grow

136 them all at once. The rhizome pieces were placed in 3.8 cm diameter cone-shaped containers in

137 the greenhouse.

138   Of the ramets planted each week, half were from the southern region and half were from

139 the northern region. Since we had a total of 26 genets from the south, we randomly selected 26 of

140 the 42 genets from the northern populations using a random number generator. All ramets in

141 block A were planted over the course of five weeks prior to the planting of the ramets in block B

142 and so on. Each northern plant was paired with a southern plant spatially on the greenhouse

143 benches. The plants were fertilized every other week with 10-10-10 fertilizer and transplanted to

144 larger, 4.5 L containers when they outgrew the small cone-shaped containers. These conditions

145 were established for Experiment 2, which occurred prior to Experiment 1. We present the

146 experiments out of order for the sake of clarity.

147 **Experiment 1: The effect of long-term moderate heat on reproductive traits**

148   Just prior to Experiment one, we removed the aboveground portions of each plant and

149 stored them at 4° C for a final dormancy period of 3-9 months. Two ramets of all 26 genets from

150 the north and south were placed in a randomized grid pattern in a growth chamber (Conviron

151 PGC-FLEX). Due to space constraints in the environmental chambers, only A and B ramets were

152 grown initially. Ramets C and D were placed in the chambers six months later. For initial

153 growth, all plants were exposed to "control" conditions (25°C day/25°C night; fluorescent and

154 incandescent lighting for 14 hours per day. Plants were fertilized once every two weeks with a

155 high phosphorus fertilizer (12-55-6) to promote flower production (Super Bloom, Scotts).

156   Upon flowering, two ramets per genet were randomly assigned to the control conditions

157 (25°C day/25°C night; 14hr/10hr) and the other two to the heat treatment conditions (32°C

158 day/25°C night; 14hr/10hr). By necessity, these were in different chambers (control: Conviron

159   PGC-FLEX; heat treatment: Conviron E7/2). Plants were watered daily. Subsequent flowers and

160   fruits developed at either elevated temperatures (32°C) or control temperatures (25°C).

161   Pre-Pollination Phase Variables

162   The first three hermaphroditic flowers (*Solanum carolinense* is andromonecious) per

163   plant that developed in the respective treatments were collected in ethanol and used for flower

164   morphology measurements, ovule counts, and pollen size measurements. Floral morphology

165   traits (length of the style, stigma, and one anther) were measured under a dissecting scope. The

166   number of ovules in each ovary was counted following a modified staining protocol adapted

167   from Diaz and Macnair (1999). Pollen diameters of approximately 100 grains were measured

168   with the use of a microscope (Axio Scope A.1 Carl Zeiss, Germany) at 400x total magnification

169   and the circle diameter measurement tool on the Zen 3.1 software.

170   Post-Pollination Phase Variables

171   We pollinated three additional flowers and collected the pollen of one more flower to

172   measure subsequent female and male reproductive traits. Mature flowers were pollinated with a

173   mix of pollen from 2 to 5 flowers, represented by northern and southern plants, that developed in

174   the control conditions. Pollinations were accomplished by applying a mixture of pollen on the

175   stigma with a dissection probe. Each pollinated flower was labelled with a jewelry tag. Once

176   flowers were pollinated, the plant remained in their respective treatments for one week before we

177   moved them into a greenhouse for the remainder of fruit maturation (Average Daily

178   Temperatures 25.1°C day / 21.3°C night). Once fruits were at least one month old, they were

179   harvested. We measured fruit set (number of fruits produced / three flowers pollinated) and the

180   seed set (number of viable seeds, aborted seeds, and unfertilized ovules per fruit). The number

181   of viable seeds, aborted seeds, and unfertilized ovules were counted under a dissecting scope.

182   In-vitro pollen germination at 40°C was used as a proxy for male reproductive success in

183   high temperatures. Pollen germination at extreme temperatures was measured following a

184   protocol from Reddy and Kakani (2007). Pollen from each plant was dispersed over a petri dish

185   containing 3% Bacto-Agar based growth medium (sucrose, $Ca(NO_3)_2$, $MgSO_4$, KNO_3, H_3BO_3)

186   and incubated at 40°C for 16 hours. Four pictures of each plate were taken using a compound

187   microscope (Leica DM500 microscope, Leica ICC50 HD camera) and the LAS EZ 2.1.0

188   software. We measured pollen germination (Germ) for each plate by counting the number of

189   pollen grains per image that had produced pollen tubes and dividing that by the total number of

190   pollen grains observed.

191   **Experiment 2: Life-stage specific responses to extreme temperature**

192   <u>Temperature tolerance variables</u>

193   To assess the impact of extreme heat on plants, we measured three vegetative variables

194   (cell membrane stability (CMS), chlorophyll content (CHPL), and net photosynthetic rate (PS).

195   and two reproductive variables (the propensity for pollen grains to germinate (GERM) and the

196   growth rate of pollen tubes (PTGR). We measured each variable on each plant in three

197   temperature treatments, hot treatment (acronym preceded by "H"), an extreme cold treatment

198   (acronym preceded by "C") and control conditions.

199   CMS was calculated according to the protocol from Gajanayake et al. (2011) and Fang

200   and To (2016). Ion leakage from leaf material exposed to either heat (HCMS: 55°C water bath

201   for ten minutes) or cold (CCMS: -18°C) was measured using an electrical conductivity probe and

202   compared to the conductivity of leaf material in control (27°C) and maximum damage (98°C)

203   treatments. Difference in chlorophyll content (CHPL) of leaves was estimated, as in Gitelson et

204   al., (1998) for material exposed to either a hot temperature treatment (HCHPL: 60°C for 1 hr) or

205   a cold temperature treatment (CCHPL: 4°C for 1 hr followed by -18°C for 1 hr) using a

206   chlorophyll meter (Opti-Sciences CCM-300). The chlorophyll meter measures the fluorescence

207   emitted at 735nm/700nm for a constant leaf area. Chlorophyll content before and after treatments

208   was used to estimate the difference in chlorophyll content in mg/m^2. PS was a measure of the

209   effects of temperature treatments on the photosynthetic capabilities of leaves. PS was estimated

210   as the ratio of net photosynthetic rates before and after a temperature treatment (HPS: 33°C,

211   CPS: 10°C for 48 hrs) based on leaf measurements made with a Licor. More detailed methods

212   are available in the Supporting Information.

213   We focused on two pollen traits for estimates of male thermotolerance during the

214   reproductive stage: 1) the propensity for pollen grains to germinate (GERM) and 2) the growth

215   rate of pollen tubes while exposed to a range of temperatures (PTGR). We paired measurements

216   of pollen traits from plants in the north and south by sampling mature anthers of plants flowering

217   simultaneously. Pollen from each flower in a pair was dispersed over five petri dishes containing

218   the mixture described in experiment 1 (3% Bacto-Agar based growth medium (sucrose,

219     $Ca(NO_3)_2$, $MgSO_4$, KNO_3, H_3BO_3). The dusted plates were each placed at one of the five

220 temperature treatments (10°C, 20°C, 25°C, 30°C, 40°C) for 16 hours. Four pictures of each plate

221 were taken using a compound microscope (Leica DM500 microscope, Leica ICC50 HD camera)

222 and the LAS EZ 2.1.0 software. We measured pollen germination (Germ) for each plate by

223 counting the number of pollen grains per image that had produced pollen tubes and dividing that

224 count by the total number of pollen grains observed. Pollen tube growth rate (PTGR) was

225 calculated by dividing the length of the 20 longest pollen tubes measured using ImageJ

226 (Schneider et al. 2012) by the time allowed for growth (16 hours). Detailed methods provided in

227 the Supporting Information. Each experimental plant was cut back to soil level and rhizomes

228 were stored at 4°C for 3-9 months.

229 **Data Analysis**

230 <u>The effect of long-term moderate heat on reproductive traits</u>

231 Flower date was analyzed for regional differences using a linear mixed effects model in

232 the *lme4* package (Bates et al. 2015) with region as the fixed effect and genet as the random

233 effect. Differences in flower type development between the treatments were analyzed using a

234 chi-squared test in the *stats* package (R Core Team 2020). All pre and post pollination traits were

235 analyzed with different versions of mixed effects models depending on the data type. The

236 general structure for the model was region, treatment, and the interaction of region and treatment

237 as fixed effects and genet as the random intercept. We used general linear mixed effects models

238 (*lme4*; function lmer) for anther and style plus stigma length. To avoid overfitting the model for

239 pollen diameter, we omitted genet as a random effect and used a general linear model (*stats*;

240 function lm). We used generalized mixed effects models (*lme4*; function glmer) with a Poisson

241 distribution for all count data, which included counts of ovules, viable seeds, unfertilized ovules,

242 and aborted seeds. Since pollen germination at 40°C was a proportion, we used a generalized

243 mixed effects model (*lme4*; function glmer) with a binomial distribution for analysis. We

244 conducted correlation analysis for mean anther and mean style plus stigma lengths (*stats*;

245 function cor.test). Fruit set was analyzed using a chi-squared test (*stats*; function chisq.test).

246 <u>Life-stage specific responses to extreme temperature</u>

247 To analyze differences in vegetative traits between plant origins and among genets, we fit

248 linear mixed effects models using the lmer function from the *lme4* package (Kuznetsova et al.

249 2017). Region (north vs. south) was considered the fixed effect and block (A, B, C, D) and genet

250 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 (*stats*; 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 (*stats*; function aov) to determine if the response curves differed between regions. One outlier was identified using the Grubbs' test for outliers, grubbs.test function in the *outliers* package (Komsta 2011), and subsequently dropped from the analysis.

We used Pearson's method for correlation analysis (*stats*; function cor) to identify associations between vegetative and reproductive variables. The Holm-Bonferroni method (*stats*; 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).

Results

Experiment 1: The effect of long-term moderate heat on reproductive traits

<u>Pre-pollination</u>

We found that long-term moderate heat negatively impacted style plus stigma length, anther length, pollen grain diameter, and ovule number (Table 1, Figure 4). However, flowering time and first flower type (hermaphrodite and male) did not differ between the treatments or region of origin. On average, flowers that developed in the heat treatment had smaller floral structures. Style plus stigma length decreased by 14% (X^2=240, $p<0.001$) and anther length decreased by 11% (X^2=183, $p<0.001$) in long term moderate heat conditions relative to the control. Style plus stigma length also differed by region of origin. Plants from Texas on average had 5% longer style plus stigma than plants from Minnesota (X^2=11, $p=0.001$). The relationship between anther and style plus stigma length changed with development in heat. Mean anther length and style plus stigma length were correlated in the control treatment (r=0.55, t_{52}=4.81, $p<0.001$), but not in the heat treatment (r=0.21, t_{40}=1.35, $p=0.184$; Figure 5). Development in heat increased the average number of ovules by approximately 1 ovule (X^2=11, $p=0.001$) and reduced pollen size by 10% ($F_{1,100}$=82, $p<0.001$). Neither trait differed by region. We found

11

282 significant interactions between treatment and region in style plus stigma length ($X^2=6$,

283 p=0.014), anther length ($X^2=9$, p=0.002), and ovule number ($X^2=53$, p<0.001; Figure 4).

284 <u>Post-pollination</u>

285   Pollen development in long-term moderate heat did not affect germination at high

286 temperatures and germination did not differ between regions (Table 1). Fruit set was also not

287 affected by the heat treatment. The number of viable seeds was affected by heat ($X^2=100$,

288 p<0.001) and on average heat decreased seed set by 16 seeds. The number of unfertilized ovules

289 increased by six in the heat treatment compared to the control ($X^2=11$, p<0.001) and the number

290 of aborted seeds increased by about 1.64 seeds on average ($X^2=42$, p<0.001). We note here that

291 the average number of aborted seeds in the control group was relatively low with an average

292 number of 0.63 seeds. The number of unfertilized ovules did differ by region ($X^2=6$, p=0.011).

293 There was a significant interaction between the treatment and region for the number of

294 unfertilized ovules ($X^2=64$, p<0.001) and aborted seeds ($X^2=12$, p<0.001; Figure 4).

295 Table 1. ANOVA results with the fixed effects temperature treatment (control and heat), region

296 of origin (north and south), and the interaction between treatment and region. Genet was

297 included as a random effect (excluded in pollen grain size due to overfitting the model).

Variable		Treatment		Region		Treatment:Region	
	dF	X^2	p	X^2	p	X^2	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**

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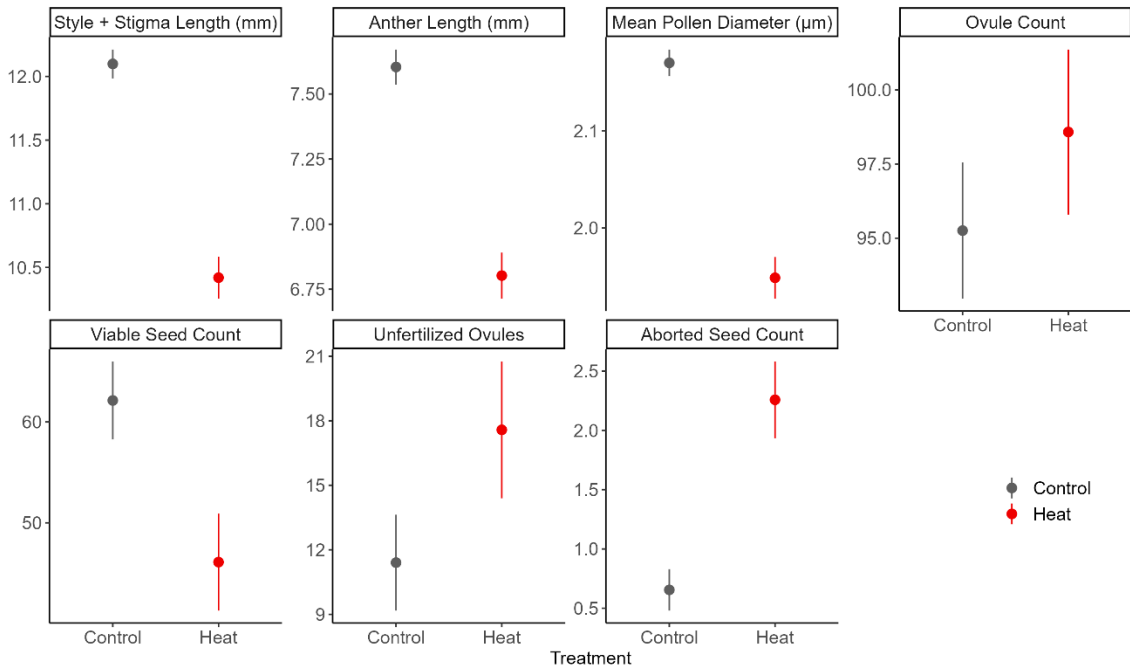
299 significant (α=0.05).

300

301 Figure 3. The effects of long-term moderate heat on morphological traits and seed set. Plant

302 development in heat reduced the size of the stigma + style, anther, and pollen grains. The number

13

303   of ovules increased. Development and fertilization in heat reduced the number of viable seeds

304   per fruit. The number of unfertilized ovules and aborted seeds increased.
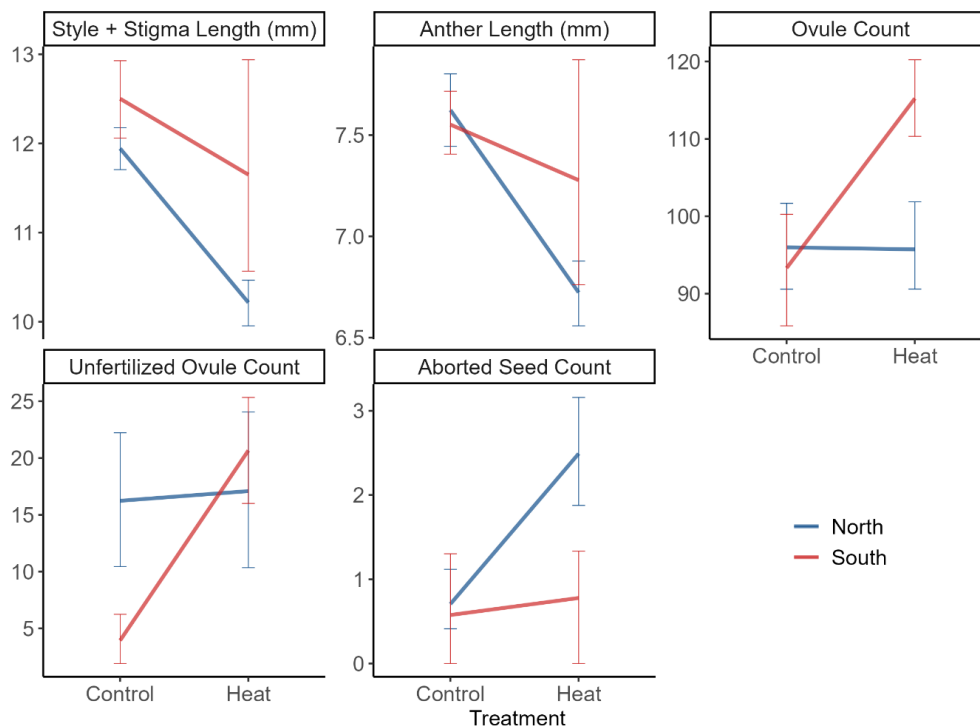

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307   Figure 4. Statistically significant interactions in Experiment 1 between heat treatment and region.
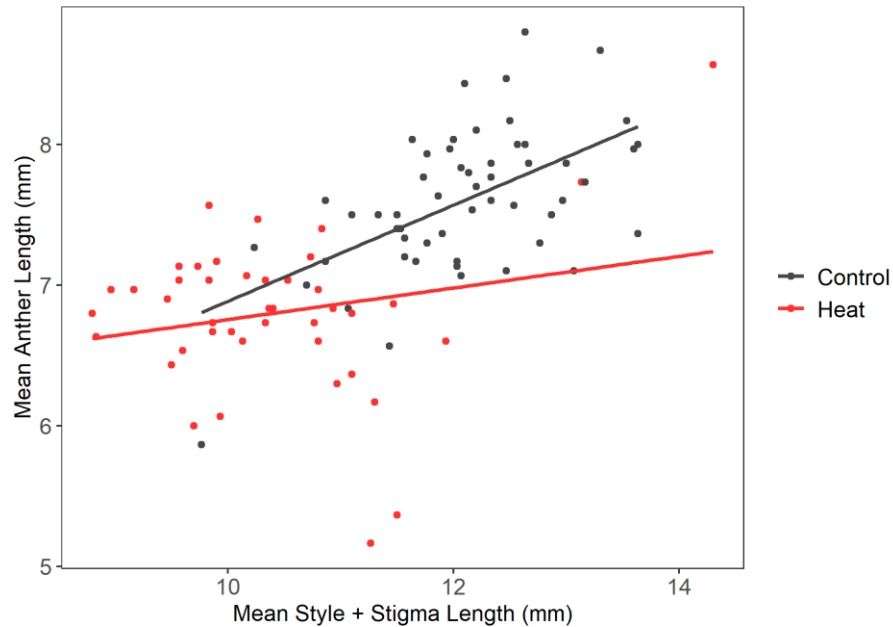
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309

310 Figure 5. Correlation of anther and style plus stigma length averaged across genets in

311 Experiment 1. Control shown in dark grey and heat treatment in red. Correlation between

312 morphological traits (r=0.55, t_{52}=4.81, p<0.001) deteriorated in the heat treatment (r=0.21,

313 t_{40}=1.35, p=0.184).

314 **Experiment 2: Life-stage specific responses to extreme temperature**

315 <u>Vegetative traits</u>

316   Of the six vegetative traits measured in this experiment, three differed between regions.

317 In extreme heat (HCHPL: $F_{1,51}$=4.418, p =0.041) and cold (CCHPL: $F_{1,50}$=66.369, p <0.001),

318 northern plants retained chlorophyll content more effectively than southern plants (Table 2). The

319 chlorophyll content of northern plants was 8% and 19% higher than southern plants for the heat

320 and cold treatments respectively. In contrast, southern plants had a 5% higher cell membrane

321 stability in the extreme cold treatment than did northern plants (CCMS: $F_{1,191}$=66.369, p <0.001;

322 Table 2).

323   There was no overall significant difference between regions for HCMS, but heat

324 tolerance was higher for northern than for southern plants in block A (Supplementary

325 Information). Temperatures in the greenhouse progressively rose throughout the spring and

326 summer leading to a block effect in both the hot and cold treatments of CMS (Supporting

327 Information Fig. S2). The block effect on CMS may be due to the capacity of *S. carolinense* to

15

328   induce temperature tolerance and acclimate to environmental conditions (Clarke et al. 2004). In

329   block A, northern plants had a higher HCMS, but this difference degraded in the later blocks

330   during the times when greenhouse temperatures were higher during plant development

331   (Supporting Information Fig. S3). We considered block A values the baseline HCMS and

332   determined that northern plant have higher baseline heat tolerance. Finally, there were no

333   regional effects on photosynthetic rate in response to either cold or heat.

334

335   Table 2. Vegetative and reproductive temperature tolerance results from mixed effects linear

336   models with the fixed effect region (north vs south) and the random effects genet and block

337   (omitted for reproductive). Due to overfitting the model genet was omitted from CCMS, HPS,

338   and Tmin PTGR. Block was not included in the analysis for reproductive traits and CPS.

339   Random effect statistical values reported in the Supporting Information (Table S2), as well as

340   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

341   * Outlier removed. Bolded values: statistically significant (α=0.05).
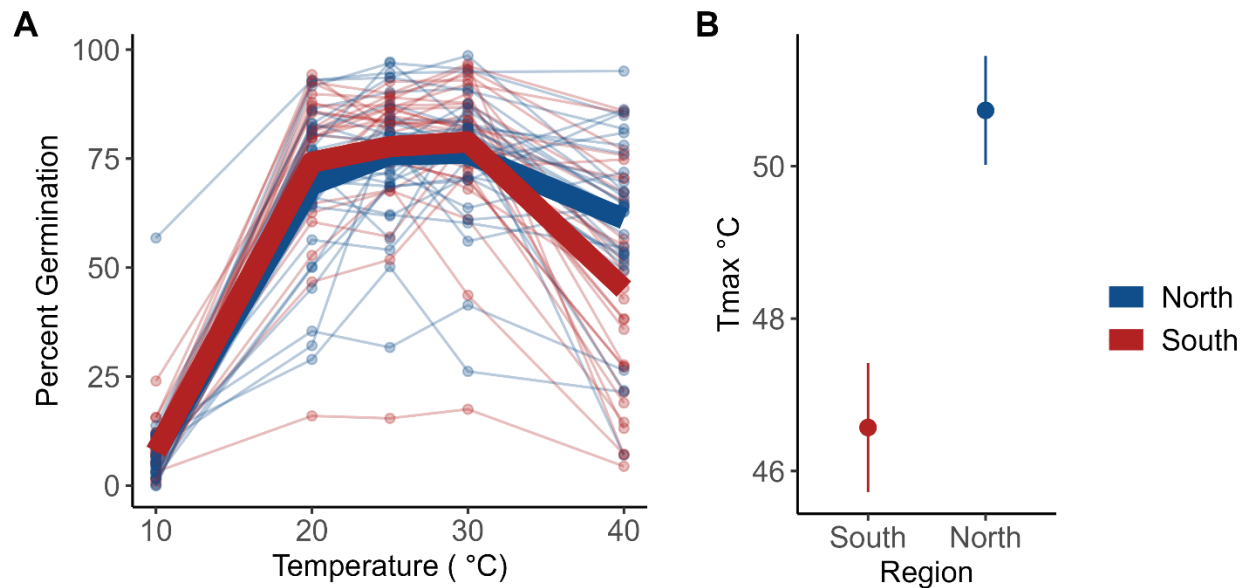
Figure 6. Results from Experiment 2 measures of pollen germination. (A) Percent pollen germination per genet (points) and regional mean (bolded lines: blue=northern, red=southern) . (B) Mean (±se) Tmax for northern (blue) and southern (red) genets. Tmax is the upper x-intercept of the quadratic fit for each individual (i.e., the highest temperature predicted with pollen germinatipn). Pollen from the northern region germinates at higher temperatures compared to pollen from the south (Tmax: $F_{1,26}=12$, p =0.002).

Reproductive traits

There was a significant difference between regions for Tmax (Figure 6, Table 2) and Topt (Table 2). Pollen from plants from the north germinated more readily at high temperatures (Tmax: $F_{1,26}=12$, p =0.002) and had higher thermal optima (Topt: $F_{1,24}=11$, 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.

362   Tmax and Tmin of pollen tube growth rates were positively correlated (r = 0.46). There was also

363   a significant correlation between Tmax for pollen tube growth rate and for pollen germination (r

364   = 0.3).

365   **Discussion**

366   The results of the combined experiments we present here indicate that not only do high

367   temperatures affect viable seed production and morphological traits but, the responses to heat

368   differ between plants from northern and southern populations in both moderate and extreme

369   temperature conditions. As in other studies (Muller et al. 2016, Fahad et al. 2017), we found that

370   exposure to higher temperatures during plant and floral growth led to negative effects on traits

371   tied to successful reproduction. In experiment one, where plants were exposed to moderate heat

372   (32 °C) and control conditions (25 °C) during floral development, there was a significant

373   treatment effect on 7 of the 8 characteristics we measured (Table 1, Figure 3) including floral

374   morphology measurements, pollen size and ovule fate (viable, aborted, unfertilized). Regardless

375   of where they were from, flowers were smaller and the number of viable seeds decreased in hot

376   conditions.

377   Several other studies have found that heat affects the floral structures in other taxa, but

378   not necessarily in the same way (Lyrene 1994). Muller et al. (2016) found anther deformations

379   when tomato flowers developed in mild heat (32°C/26°C). Charles and Harris (1972) found that

380   as temperatures increased, the distance between the antheridial cone and the stigma (herkogamy)

381   in tomatoes decreased (longer pistil or shorter stamen). We didn't specifically look at

382   herkogamy, but the differing style lengths we observed could have implications for pollen

383   competition (Ramesha et al. 2011) and the position of anthers relative to the stigma could affect

384   the receipt of outcross versus self pollen from pollinators. We did find that the correlation

385   between the length of male and female reproductive structures breaks down in heat (Figure 5)

386   suggesting that the fundamental proportions of floral structures are disrupted. Further

387   investigations would be useful to determine if observed changes to positions of integral

388   reproductive structures in heat affect rates of self-pollination and inbreeding in *Solanum*

389   *carolinense*.

390   The effect of heat on viable seed production and pollen size in our study represent

391   important responses to temperature stress that could have fitness consequences. Pollen diameter

392   dropped significantly in Experiment 1, when flowers developed in hotter conditions (Figure 3).

393 McCallum and Chang (2016) found evidence of pollen size influencing siring success; larger

394 pollen grains were more competitive (sired more seeds) than smaller pollen grains in common

395 morning glory. Our result of reduced viable seed counts in fruits developed in moderate heat has

396 mixed support in the literature for a close relative, tomatoes. Xu et al. (2017) found that heat had

397 little influence on seed number compared to other reproductive traits, but Din et al. (2015) found

398 that seed set was reduced in heat, especially in more temperature sensitive accessions. They

399 attributed this difference to heat reducing pollen viability, or pollen tube growth in the style. In

400 sum, these results suggest that the stress of warmer temperatures during floral development can

401 have important negative effects with potential evolutionary consequences.

402 Regional differences

403 Despite the fact that all of the plants used in our experiments were the same species and

404 were grown in approximately the same conditions, there were significant differences between the

405 responses to hot temperatures between plants from northern versus southern latitudes (Figure 4).

406 These differences are consistent with long-term local adaptation of plants to the thermal patterns

407 and environments in the two different places (MN and TX). The floral morphology reductions in

408 response to heat described previously, were not consistent between northern and southern plants.

409 Reductions in the length of female and male floral structures were significantly more dramatic in

410 plants from northern populations relative to southern populations (Table 1 – treatment x region

411 effects, Figure 4). These patterns and the increase in ovule counts for southern plants suggest that

412 they will maintain allocations of energy to floral structures despite the heat stress. In contrast,

413 northern plants may reduce their energy allocation to floral structures as an alternative strategy for

414 tolerating heat stress.

415 Another key difference between northern and southern plants was how heat, during

416 pollination and fruit development, influenced the number of aborted and unfertilized ovules. In

417 southern plants, heat led to increases in the number of ovules that were unfertilized, while in plants

418 from the north, heat led to increases in the number of aborted ovules. In control conditions,

419 southern plants had a much lower percentage of ovules that were unfertilized than northern plants.

420 In heat, the percentage was about the same for the two regions. The reduced efficiency of

421 fertilization in southern plants when exposed to heat may be the result of two separate phenomena.

422 First, in heat, southern plants produced more ovules and generally had longer styles than northern

423 plants. The increased number of unfertilized ovules may be a result of southern plants producing

424   a larger number of ovules during development and the inability of a full pollen load to fertilize the
425   excess ovules. Second, prolonged heat exposure and termination of pollen tube growth in the
426   longer styles of flowers on southern plants could also contribute to the number of ovules that went
427   unfertilized in heat. Both of the above phenomena and the generally low seed abortion rate suggest
428   that pollen is the limiting factor.

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

436   A second set of evidence that regional differences in *Solanum carolinense* have evolved
437   over time comes from the divergent patterns of tolerance to extreme temperatures between
438   northern and southern plants in experiment two (Table 2). Contrary to our expectations, we found
439   that in multiple life stages, northern plants were more tolerant of extreme heat than are southern
440   plants and southern plants were more tolerant of cold in terms of membrane stability. Typically,
441   heat tolerance is measured by exposing plant material to heat and quantifying cell membrane
442   stability and chloroplast integrity under high relative to control levels of heat (Murty and
443   Majumder 1962; Martineau et al. 1979; Gajanayake et al. 2011; Fang and To 2016). In Experiment
444   2, we found northern plants had higher chlorophyll content (HCHPL) and baseline cell membrane
445   stability (HCMS) under hot conditions, as well as lower membrane stability under cold conditions
446   in comparison to southern plants (Table 2, Figure 3).

447   Plants from the north had more stable chlorophyll content in both the hot (HCHPL) and
448   cold treatments (CCHPL; Table 2). The capacity of chloroplasts in northern plants to outperform
449   southern plants in both extreme cold and heat might be due to northern plants experiencing a larger
450   range of temperatures and broad adaptation to stress in general. Between 2018 and 2021,
451   temperatures during the growing season (March to September) in Houston County, MN ranged
452   from -28°C to 34°C (62°C difference), while in Collin County, TX they ranged from -7°C to 42°C
453   (49°C difference). If the evolution of tolerance to extreme cold in the north, where wintering
454   rhizomes remain in frozen ground for months, yields general physiological tolerance to any

20

455   temperature extremes, then northern plants should be more tolerant than southern. Instead, we

456   found that chloroplasts seem to be more tolerant in the north but not cell membranes. Heat shock

457   proteins, that play an important role in maintaining tolerance to heat in plant cells (Feder and

458   Hofmann 1999; Frank et al. 2009; Heckathorn et al. 1998), can also confer tolerance to cold by

459   stabilizing protein configurations and functions in cells at stressful temperatures (Neta-Sharir et

460   al. 2005). Therefore, selection for extreme temperature tolerance may be more common in northern

461   latitudes. Northern plants will suffer severe fitness consequences if they do not maximize growth

462   and reproduction during the relatively short growing season (Fig.2), whereas southern plants can

463   avoid growing and reproducing during the hottest portions of summer and still have months of

464   mild temperatures available. Our results suggest that evolved thermotolerance applies to

465   chloroplasts in the north but not to cell membranes.

466   The results from reproductive trait comparisons also countered our expectations for the

467   direction of temperature tolerance. Pollen from the north had a higher propensity to produce pollen

468   tubes (Germ) at high temperatures than their southern counterparts (Table 2, Figure 3). One

469   possible explanation for these results is that there is an avoidance strategy in southern populations

470   where maximum summer temperatures can reach over 38°C consistently. Under these conditions,

471   there could well be a selective advantage to pollen remaining dormant rather than germinating at

472   high temperatures. In contrast, there may be no selection for dormancy at relatively high

473   temperatures in the north. This explanation is supported by a theory regarding pollen dormancy

474   developed in Rutley et al. (2022). They proposed the "two baskets model" categorizing pollen and

475   stating that there are active (high-ROS) and backup (low-ROS) subpopulations of pollen within

476   anthers of flowering species. Active pollen readily germinate and have fast metabolisms,

477   increasing pollen tube growth rates, and typically outcompete the smaller, partially dehydrated

478   backup pollen with low metabolisms. The two subpopulations of pollen are adaptive under

479   different conditions. In stressful environments, such as extreme heat or drought, asynchrony in

480   pollen germination permits some pollen to remain dormant and grow pollen tubes later in more

481   favorable conditions. In favorable conditions, active pollen tubes grow faster and are more likely

482   to fertilize ovules than backup pollen. While the two-pollen system has not been established in

483   *Solanum carolinense*, there have been studies demonstrating these two pollen types in *Solanum*

484   *lycopersicum*, tomato (Jegadeesan et al. 2018; Keller and Simm 2018; Luria et al. 2019).

485   Predictions about how species will be affected by climate change can be improved with a

486   better understanding of how different populations of the same species differ in their responses to

487   heat now but at different latitudes with different thermal patterns. The future climate experienced

488   by plants in Minnesota is predicted to more closely resemble that experienced by plants in Texas

489   now and in the past including both higher average temperatures in summer months and higher

490   maximum daily temperatures (IPCC 2014). Growing seasons are already getting longer in

491   northern populations (Badh et al. 2009; Dunnell and Travers 2011). Given the responses to heat

492   by plants in our experiments in the form of relatively moderate heat (32°C) during floral

493   development, pollen tube growth and fruit maturation and extreme heat (40-60°C) in acute doses,

494   we suggest that plants in the two regions we studied have evolved some differences that

495   represent differing strategies for surviving thermal stress. There is little evidence that southern

496   plants have evolved greater cellular tolerances to extreme heat despite growing in an

497   environment that can have daily maximum temperatures above 40.5° C. The stability of

498   chloroplasts and pollen germination capabilities were reduced after exposure to extreme heat in

499   southern relative to northern plants (Table 2, Figure 3). If plants in the south have shifted to an

500   avoidance strategy where the temperature extremes of summer months are avoided by dormancy

501   of pollen or flowering patterns shifted earlier or later, then selection for tolerance of high heat

502   may not occur. In contrast, northern plants that experience relatively short seasons when growth

503   and flowering are possible will need to flower and develop fruit during the hottest times of the

504   year to produce viable seed. However, as climate change leads to longer, hotter growing seasons,

505   our expectation is that an upper limit to tolerance of heat will ultimately lead to different

506   phenological patterns and perhaps dormancy. Thus, the evolutionary consequences of climate

507   change for flowering plants is likely to be a complex shift in phenological and physiological

508   patterns.

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