**Methods**

## Field Collection and

*Solanum carolinense* plants were collected from two populations in Houston County, Minnesota and three populations in Collin County, Texas between October 2019 and August 2020. The Minnesota plants collectively will be referred to as the northern region and included 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. In Houston County, MN, the average monthly low temperature is -14°C (7°F) and the average monthly high is 29°C (85°F). The Texas plants together will be referred to as the southern region. All three TX populations were within a 1.5 Km radius near McKinney (Oil Patch: 33.173465 N, -96.615402 W; Reserve: 33.159962 N, -96.619011 W; and Cemetery: 33.173672, -96.615096 W). In Colin County TX, the average monthly low temperature is 18°C (65°F) and the average monthly high is 43°C (111°F).

*Solanum carolinense* is a perennial that reproduces by sexual reproduction and dispersal of seeds in a tomato-like fruit by frugivores and by asexual reproduction by the growth of ramets (genetically identical clones) from the rhizome. Genets (individual plants) were sampled by digging up plants with an inter-plant distance of 1 meter and cutting at least 10 cm of rhizome. The rhizomes were given unique ID numbers, placed in zip lock bags, and shipped to Fargo in a cooler with blue ice. The rhizomes were stored in a 4°C refrigerator until they were planted in one-gallon containers and grown through the summer of 2020. In October, the above ground material was cut and the rhizomes in pots were stored again at 4°C for a three-month period of dormancy. During the spring and summer of 2021, four ramets (A, B, C, and D) cut from the rhizome of each genet were grown and used in a previous study (methods described in Chapter 1). In October and November, the above ground material for all ramets of each genet was cut and the plants were returned to 4°C for a dormancy period.

## Growth Conditions and Experimental Design

On January 12, 2022, ramets A and B for all genets were placed in a randomized grid pattern in the Conviron XXX growth chamber. One ramet from each genet was randomly assigned to the heat and the other was assigned to the control treatment. For initial growth, all plants were placed in the same, “control” conditions. For the control growth conditions, the chamber was set at 25°C day/25°C night with fluorescent lights at setting 2 and incandescent lights at setting 1 for 14 hours per day. As plants grew to heights at which the incandescent bulbs damaged upper leaves on certain plants, the incandescent setting was reduced to 0. Plants were regularly fertilized with a high phosphorus fertilize to promote flower production.

Once a ramet flowered, all flowers and buds were removed before placing the plants in either the heat or control treatment chambers. The control treatment chamber (Conviron XXX) was set at the same conditions used for initial growth. The heat treatment chambers (Conviron XXX) were set at 32°C day/25°C night with the same light settings as the control. Plant in the treatment groups were watered daily. The date of first flowering and the date when a ramet flowered again in the treatment were recorded. The flower type, hermaphroditic or staminate, produced for the first flowering in the treatment was also recorded.

## Pre-pollination

Three hermaphroditic flowers that completely developed in the respective treatments were collected and used for flower morphology measurements, ovule counts, and pollen size measurements. The ovules were stained following a protocol adapted from Diaz and Macnair (1999). The flowers with petals removed were fixed in Eppendorf tubes with ethanol for 24 hours and washed with deionized water. The tubes were then filled with 1M NaOH and placed in a hot plate at 70°C for 2 minutes to soften the floral structures before a final wash in deionized water. The flowers were then stained in 0.1% aniline blue with 0.1M K3PO4 for 24 hours in darkness. The length of the style and stigma and one anther were measured under a dissecting scope. The ovary and one anther were sectioned and mounted on a microscope slide with 50% glycerol. Fluorescence microscopy was intended to be used for determining ovule viability based on the percentage of callose formation in ovules. The heat treatment did not incur visible callose formation for this species, thus only ovule number was recorded using microscopy. Pollen diameter was measured using Microscopy at 400x total magnification and the circle diameter tool on the Zen 3.1 software. Pictures of pollen grains from the slide of one flower per plant were taken and used for measurements. All pollen in a picture were measured and the number of pictures required to measure at least 100 pollen grains were used.

## Post-pollination

Pollen germination at 40°C was used as a male performance trait. In the previous study, there was variation in pollen germination at high temperatures. We used 40°C to determine how plants differ in germination at high temperatures and whether pollen development in long-term high heat affects pollen germination at high temperatures. One flower that completely developed in the treatment group was collected for pollen germination. Pollen from a mature flower, identified by petals in a open position perpendicular to the anthers and a fully developed stigma (if flower was hermaphroditic). Since horsenettle is naturally buzz pollinated, a device crafted from parts from robotic vacuum cleaner was used to vibrate stamen and release pollen. Pollen was dispersed evenly on a petri dish with a 3% Bacto-Agar based growth medium (sucrose, Ca(NO3)2, MgSO4, KNO3, H3BO3) and placed in a drying oven at 40°C for 16 hours. Three pictures of the pollen on the petri dish were taken using microscopy. Pollen germination was measured by counting the number of pollen grains that produced tubes of at least half the diameter of the pollen grain and dividing by the total number of pollen grains in a picture. All pollen grains in a picture were counted and the numbers of pictures required for total number of at least 100 pollen grains were use.

Female performance was determined by the number of ovules that were fertilized and developed into seeds. Once all flowers for morphological and male performance traits were collected, three flowers on one plant were pollinated with a mix of pollen from flowers in the control treatment. Horsenettle has a self incompatibility system, which prevents plants with the same S allele from fertilizing one another. The self incompatibility system is a measure to prevent inbreeding. Since we haven’t identified the S alleles for these plants, we mixed pollen from multiple populations from the north and south to ensure that there was the opportunity for fertilization between our crosses. Paternity was disregarded for these measurements. Since pollen developed in the control conditions, the only alternation is the treatment in which the ovules developed. The flowers were pollinated by applying mixed pollen on the stigma with a probe and labeling the flower with a jewelry tag. Once flowers were pollinated, the plant remained in the treatment for one week before being moved into a greenhouse for fruit to develop.

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

*Data Analysis*

All data analysis was conducted in R (version 4.1.2). Flower date was analyzed for regional differences using a linear mixed effects model (lmerTest; function lmer) with region as the fixed effect and genet nested in population as the random effect. Differences between flower type development were analyzed using a Cochran-Mantel-Haenszel chi-squared test (stats; function mantelhaen.test) with the variables: region, treatment, and flower type. Stamen length, style and stigma length, and ovule number were analyzed for regional differences in the control treatment using a linear mixed effects model (lmerTest; function lmer) with region and population as fixed effects and genet nested in population as the random effect. Treatment differences were only analyzed for northern plants because of low sample size in southern plants. A linear mixed effects model (lmerTest; function lmer) was used for treatment differences with treatment and population as the fixed effects and genet nested in population as the random effect. Mean pollen diameter was compared between regions using a linear mixed effects model (lmerTest; function lmer) with region as the fixed effect and population and the random effect. The treatment effect on mean diameter of pollen grains in the northern plants was analyzed using a linear mixed effects model (lmerTest; function lmer) with treatment as the fixed effect and population as the random effect.

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

Diaz, A., & Macnair, M. R. (1999). Pollen tube competition as a mechanism of prezygotic reproductive isolation between Mimulus nasutus and its presumed progenitor M. guttatus. *The New phytologist*, *144*(3), 471-478. <https://doi.org/10.1046/j.1469-8137.1999.00543.x>