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A common garden experiment suggests local adaptation in stomata size but not stomata density in Common Milkweed (Asclepias syriaca)

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ABSTRACT

Local adaptation is a common process that occurs in organisms with wide geographic ranges to maximize fitness in different environments. One specific species that has been shown to have certain locally adapted traits is Common Milkweed, *Asclepias syriaca*. A handful of these traits include height, stem count, number of leaves, and length and width of the leaves. The size and density of stomata, the pores underneath the leaf used for gas exchange and transpiration, may also be influenced by environmental conditions; however, this relationship has not been studied in common milkweed. In order to analyze population-level differences in the density and size of stomata in *A. syriaca*, a common garden experiment was used which allowed for multiple different genotypes from across the large geographic range to be planted in one specific region. The seeds were collected from 35 different locations ranging from northwest Minnesota to southern Maine and down to central Virginia. Representatives of ten different populations, from a total of 45 different mother plants with six replicates of each plant, were placed in a common garden experiment. After growth, a subset of leaves was collected to create leaf impressions which allowed analysis of stomata density and size. The underside of the leaves were coated in lacquer and cured, and then the impressions were slowly peeled off of the leaf. A microscope was used to count the number, and measure the size, of the stomata. The multiple regression test on stomata size showed evidence of latitudinal and longitudinal clines, while stomata density did not differ significantly among populations. This shows that stomata size shows genetic differences across the native common milkweed range, instead of being a plastic trait. Overall, the project can help to show that the focus of conservationists should be shifted toward using locally adapted seeds during restoration efforts.

INTRODUCTION

Individuals within a species vary in morphology, behavior, and physical characteristics (i.e., their phenotype) across populations based on both their genotype and multiple environmental variables. Ecologists are interested in disentangling genetic versus environmental effects on an individual's phenotype to better understand the selection factors acting on a population. A widely used method for doing this is called a common garden experiment. In a common garden experiment, individuals from disparate populations are reared together in a common environment, such as in a greenhouse or single field at one geographic location. By doing so, researchers are able to test the relationship between environmental factors such as soil type, pH, temperature, and precipitation against the fitness of individuals from these different populations (Pelini et al., 2012). If there are fitness differences among individuals from different populations that are grown in the same environment, then the differences can be tied to genetics (Pelini et al., 2012). On the other hand, if all the individuals from varying geographic regions grow the same under the same environmental conditions then the phenotypes of the individuals are assumed to be a plastic response to local environmental conditions (Pelini et al., 2012).

Local adaptation is a genetic change in a specific population of a species due to experiencing different environmental conditions over a given time. This is different from phenotypic plasticity which is when a given species exhibits different physical traits due to the environment but all maintain the same genetic information (Joshi et al., 2001). This act of locally adapting to the environment, and therefore changing the genetic makeup of the population, is common within plants. Plant traits, such as leaf length, leaf width, plant height, number of leaves, etc., are subject to selective pressures because of their influence on the growth and survival of the population. Certain morphological traits, such as leaf size, shape, and thickness,

can affect the photosynthetic rates of the plants (Carlson et al., 2016). Components of leaf size are known to be correlated specifically with latitude and elevation. Guerin et al. (2012) and Hill et al. (2014), for example, found that leaf width decreased with increased elevation and associated precipitation and temperature, while leaf length decreased with decreasing latitude. Leaf size changes with increased latitude, which increases the chance of reproductive success due to increased photosynthetic rates (Guerin et al., 2012). Therefore, by studying how leaf length and width are locally adapted within populations one can better explain their fitness in terms of growth, survival, and reproduction.

In turn, stomata size and density are important plant traits that have lacked in depth investigation in previous studies, thus leaving local adaptation unclear in regards to these traits. Stomata are the pores on the underside of the leaves which allow for transpiration and the exchange of gasses that drive photosynthesis and cellular respiration in plants. A pair of guard cells closes stomata under dry conditions to prevent water loss and open the stomata when moisture is abundant. By closing the stomata under low light conditions, the guard cells can also help to prevent water loss while photosynthetic production is already low (Mizutani & Kanaoka, 2018). Previous research on multiple plant species with large geographic ranges has shown that there tends to be an increase in stomatal density with an increase in mean temperature and increased drought intensity across populations (Carlson et al., 2016). The increase in stomatal density in hot, dry regions can increase reproduction because the plants produce more sugars and energy that can be allocated to reproduction instead of water conservation (Carlson et al., 2016). This allows the plants to maximize their photosynthesis abilities, as well as improve the transpiration cooling abilities and gas exchange rates (Carlson et al., 2016). Therefore, a positive relationship between stomata density and fecundity was found in hotter, drier regions and a

negative one in cooler, moist regions (Carlson et al., 2016). Research has also shown that there

is a negative correlation between stomata size and density based on water-use efficiency, but

water use efficiency increased with stomata size and density when considering each as

independent variables (Dittberner et al., 2018).

Because stomata are so fundamental to plant biology and fitness, it is reasonable to

expect stomata size and density to be under strong selection pressure which could lead to local

adaptation to different environments across a wide geographic range. Here, I present the results

of a common garden experiment to test the hypothesis that Common Milkweed, *Asclepias*

syriaca, shows local adaptation in stomata size and density. The common garden experiment

included multiple individuals from different populations of Common Milkweed from across a

large geographic area that were grown in one location simultaneously. The location is in the

southeast corner of the natural geographic range, where the growing season is longer and hotter

in respect to the more northern populations. If stomata size and density are locally adapted traits,

I predicted geographic clines in these traits associated with environmental variables such as

mean temperature, precipitation, and growing season length that vary clinally with latitude and

longitude. Mean annual temperature and growing season length are negatively correlated with

latitude, while precipitation is positively correlated with decreasing longitude from west to east

(Woods et al. 2012, Mohl et al., in review). Therefore, assuming stomata size and density are

negatively correlated, stomata density should increase from north to south and from east to west,

whereas stomata size should decrease from north to south and east to west.

METHODS

Propagating Common Milkweed Plants and Creating a Common Garden Experiment

Participants at over thirty institutions collected seed pods from local natural populations of Common Milkweed across its native range from Minnesota to New England to the border of Virginia and North Carolina. The seeds were sent to St. Olaf College (Northfield, MN), where they were organized by ecoregion (a major ecosystem characterized by specific geography and climate patterns), population (a collective group of individuals from a single location within an ecoregion), and genotype (one specific individual found within a population), and then redistributed to three institutions for use in a reciprocal transplant experiment (i.e., three identical common garden experiments established in three different locations at the same time). The three institutions involved in the reciprocal transplant experiment were separated widely in latitude and longitude: St. Olaf College (44.4621° N, 93.1840° W), Denison University (40.0734° N, 82.5229° W), and the University of Lynchburg (37.4006° N, 79.1832° W). The common garden experiment used in the study was conducted solely at the University of Lynchburg.

Each of the three reciprocal transplant experiments was set up using the same 180 genotypes with representatives from all sampled populations, resulting in 18 genotypes from each of 10 populations (Figure 1). Thirty to fifty seeds of each genotype were put into cold stratification in late 2019 to simulate winter. Milkweed seeds will not germinate if they are not stimulated through cold stratification. The reciprocal transplant experiments were supposed to be established in the spring of 2020 but the Covid-19 pandemic forced the delay until the spring of 2021. The seeds were removed from cold stratification in April 2021 and planted in a former dairy barn. Seventy-two trays, each filled with 12 cell-packs (6 cells per pack) were used to germinate seeds. Each cell within the trays was 4cm x 4cm, with a depth of 6.4cm. The cells

were filled with a growing medium which included perlite and other nutrients that would

encourage germination of the seeds. Each cell-pack was labeled by genotype, and the location of

the genotypes throughout all of the seventy-two trays was randomized. Thirty-six seeds were

planted from each genotype to ensure there was a surplus of seedlings available for transplanting

into the common garden. Some cells were planted with two seeds, one in each corner, and other

cells were planted with one seed, directly in the center. If two seedlings germinated, one was

removed after about two weeks.

After the seeds were planted, each tray was placed on shelves under banks of LED grow

lights. The grow lights could be raised or lowered using a rope and pulley system that allowed

the lights to be raised out of the way for watering and lowered back down directly above the

plants as they grew. The lights were scheduled on a 14-hour day, 10-hour night timer.

Germinating plants were watered liberally three times a week. The temperature in the growing

room was never allowed to fall below 70 F. Trays were moved to a different position on the

shelves every few days to minimize any effects of variation in light intensity.

The common garden experiment was established at the University of Lynchburg Claytor

Nature Center in a field mowed annually for hay. Native *A. syriaca* grows in fields adjacent to

this experiment location. The experimental field was organized into six blocks, each of which

contained a grid of 182 three-gallon pots sunk into the ground and filled with native soil. Pots

within blocks were separated by one meter and blocks were separated by at least two meters. A

chicken wire fence was placed along the perimeter of the common garden to ensure that small

mammals, such as rabbits, and other potential hazards to the milkweeds would be kept out of the

plots. After six weeks, the seeds had germinated and the seedlings were ready to be transplanted

into the field. The week prior to transplanting, the seedlings were hardened off by bringing each

tray into the sunlight for four to six hours for five consecutive days. The seedlings were

transplanted from 1 June to 3 June, 2021. The arrangement of the genotypes within each

experimental block was randomized to minimize effects of variation in soil. When transplanting,

the seedlings of each genotype were chosen based on their stature (how sturdy and upright the

stems were) and vigor (how green their leaves were). After transplanting, the individually

labeled seedlings were watered until the water was tipping over the brim of their pots. From then

on, the plants were watered three times a week for a month, continuing to fill to the brim, unless

the field site received substantial rainfall. By July, the plants were no longer watered and

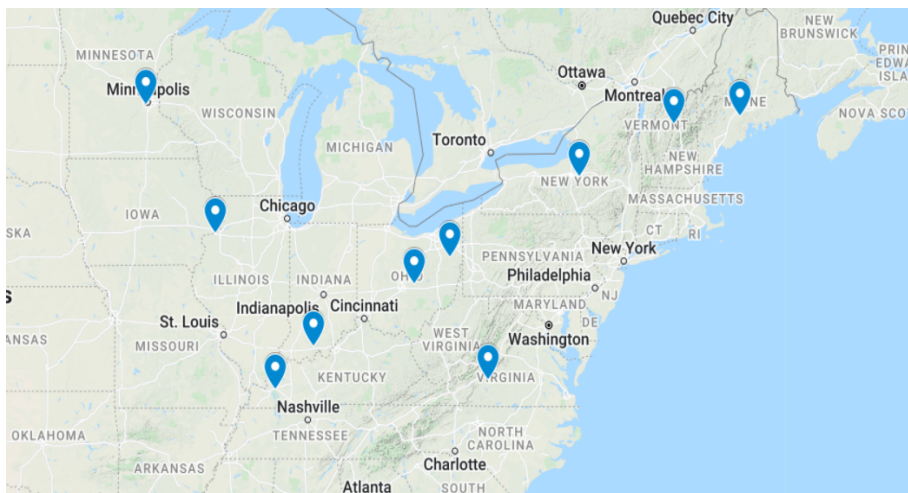thereafter received only natural precipitation.

Figure 1. Locations of the ten source populations for plants used in the common garden
experiment at the UL Claytor Nature Center in Bedford County, Virginia.

Collecting and Analyzing Leaves for Stomata Density and Size

At the end of July 2021, I collected leaves from four to five genotypes from ten different

populations. Forty-five genotypes in total were sampled from each of the six replicate blocks

(Table 1). The genotypes collected were chosen at random with the exception of the Interior

River Bottoms, Highlands, and Plateau (IRHP) populations, where only one genotype was

selected due to the lack of availability of healthy plants to sample. A single leaf from the third

node down the stem from the apex was collected from each sampled plant and these were placed

within labeled bags in a cooler to preserve them. The collection of the leaves occurred over a two

week period in July, and three more populations were sampled over one weekend in the

beginning of August 2021.

Table 1. A summary of the different populations, each with six replicates, of *A. syriaca* used in the stomata size and density analysis.

Ecoregion*	Population	Genotype	Latitude	Longitude	Avg. Precip. Inches
MWP	1	9	41.497	-90.5605	37
MWP	1	7	41.497	-90.5605	37
MWP	1	3	41.497	-90.5605	37
MWP	1	4	41.497	-90.5605	37
MWP	1	6	41.497	-90.5605	37
NCW-NF	2	86	44.924	-93.3976	32.1
NCW-NF	2	83	44.924	-93.3976	32.1
NCW-NF	2	89	44.924	-93.3976	32.1
NCW-NF	2	84	44.924	-93.3976	32.1
NCW-NF	2	85	44.924	-93.3976	32.1
CP	3	48	40.084	-82.5168	38
CP	3	49	40.084	-82.5168	38
CP	3	43	40.084	-82.5168	38
CP	3	46	40.084	-82.5168	38
CP	3	42	40.084	-82.5168	38
SA	4	33	37.392	-79.5376	20.1
SA	4	31	37.392	-79.5376	20.1
SA	4	36	37.392	-79.5376	20.1
SA	4	34	37.392	-79.5376	20.1
SA	4	37	37.392	-79.5376	20.1
AH	5	54	44.434	-71.9818	38.6
AH	5	56	44.434	-71.9818	38.6

AH	5	60	44.434	-71.9818	38.6
AH	5	58	44.434	-71.9818	38.6
AH	5	51	44.434	-71.9818	38.6
NEW	6	154	40.825	-81.0942	35
NEW	6	153	40.825	-81.0942	35
NEW	6	152	40.825	-81.0942	35
NEW	6	159	40.825	-81.0942	35
NEW	7	166	44.608	-69.3306	45
NEW	7	167	44.608	-69.3306	45
NEW	7	168	44.608	-69.3306	45
NEW	7	169	44.608	-69.3306	45
NEW	8	93	42.982	-75.817	40.1
NEW	8	94	42.982	-75.817	40.1
NEW	8	96	42.982	-75.817	40.1
NEW	8	98	42.982	-75.817	40.1
NEW	8	99	42.982	-75.817	40.1
IRHP	9	202	38.335	-86.6232	48.4
IRHP	9	203	38.335	-86.6232	48.4
IRHP	9	196	38.335	-86.6232	48.4
IRHP	10	172	40.086	-88.2394	40.7
IRHP	10	173	40.086	-88.2394	40.7
IRHP	10	180	40.086	-88.2394	40.7
IRHP	10	181	40.086	-88.2394	40.7

*Ecoregion codes: MWP - Midwest Prairie; NCW-NF - North Central Woods; CP - Central Plains; SA - Southern Appalachian; AH - Atlantic Highlands; NEW - North East Woods; IRHP - Interior River Bottoms, Highlands, and Plateau

To determine the stomata density and size, I used a clear lacquer (Vishine® Top Coat) to make leaf impressions on the same day the leaves were collected in the field. One half of the bottom side of the leaves was brushed with the lacquer back and forth three times and then placed under a UV light for two minutes to cure the lacquer. After the lacquer cured, I gently peeled the impression off the leaf with forceps. In some cases, removing the impression also peeled off leaf epidermal tissue. I attempted to remove this tissue and gently wiped it with a non-abrasive paper towel to ensure that no damage occurred to the impression. I stored leaf impressions on labeled microscope slides until I could count and measure stomata size and density.

I estimated stomatal density using a reticle in the eyepiece of a compound microscope to form a transect. The transect was a long, thin rectangle defined by the length of the reticle and the width of the cross-hatches at both ends. Any stomata at least partially within this rectangle were counted at a 100x magnification. The area of the reticle was 250 micrometers^2 which allowed for the stomatal density to be calculated. The slide was randomly placed on the stage and moved haphazardly until there were stomata discernable. I quantified stomatal density in five separate transects per impression. These were averaged to provide an estimate of stomatal density for a single plant. Mean stomatal densities from each plant were then averaged to provide a mean stomatal density for each genotype. After stomatal density was sampled, magnification was increased to 400x to acquire digital photographs of stomata from which I could measure their size. I measured five individual stoma from each leaf photograph (i.e., from each plant) using ImageJ image analysis software. After setting the measurement scale on ImageJ, it allowed me to measure the length and width of each stomata, then a formula of an ellipse was used to provide an estimate of stoma area. Individual stomatal areas were averaged for each plant and

then for each genotype.

Statistical Analysis

I tested for local adaptation in two ways using SAS statistical software (SAS Institute, Inc. 2022). A one-way ANOVA was used to test for differences in mean stomatal density and size among populations without respect to latitude and longitude. Block was included as a fixed effect. Data did not need to be transformed to meet assumptions of normality and homoscedasticity in the residuals. Multiple regressions of latitude and longitude of each population on mean stomatal density and size were used to test for geographic clines. A term for the interaction of latitude and longitude effects was included in the regressions initially, but removed if non-significant. Data were square-root transformed to improve normality and heteroscedasticity of the residuals. P-values ≤ 0.05 were considered significant in all tests.

RESULTS

Mean stomatal density (+/- 1 SE) ranged from 12.96 (+/-0.74) to 20.14 (+/-0.74) (#/250 square nanometers) among populations. Although there was a trend towards population-level differences in mean stomatal density (population: $F_{9,198} = 1.77$, p = 0.076) (Fig. 2a), the overall ANOVA model was not significant (model: $F_{14,198} = 1.37$, p = 0.17). In contrast, mean stomata size varied more dramatically among populations, ranging from 354.36 (+/-75.02) to 1150.43 (+/-75.02) (square nanometers). The results of an ANOVA showed that this population-level effect was significant (population: $F_{9,176} = 3.74$, p = 0.0002) (Fig. 2b).
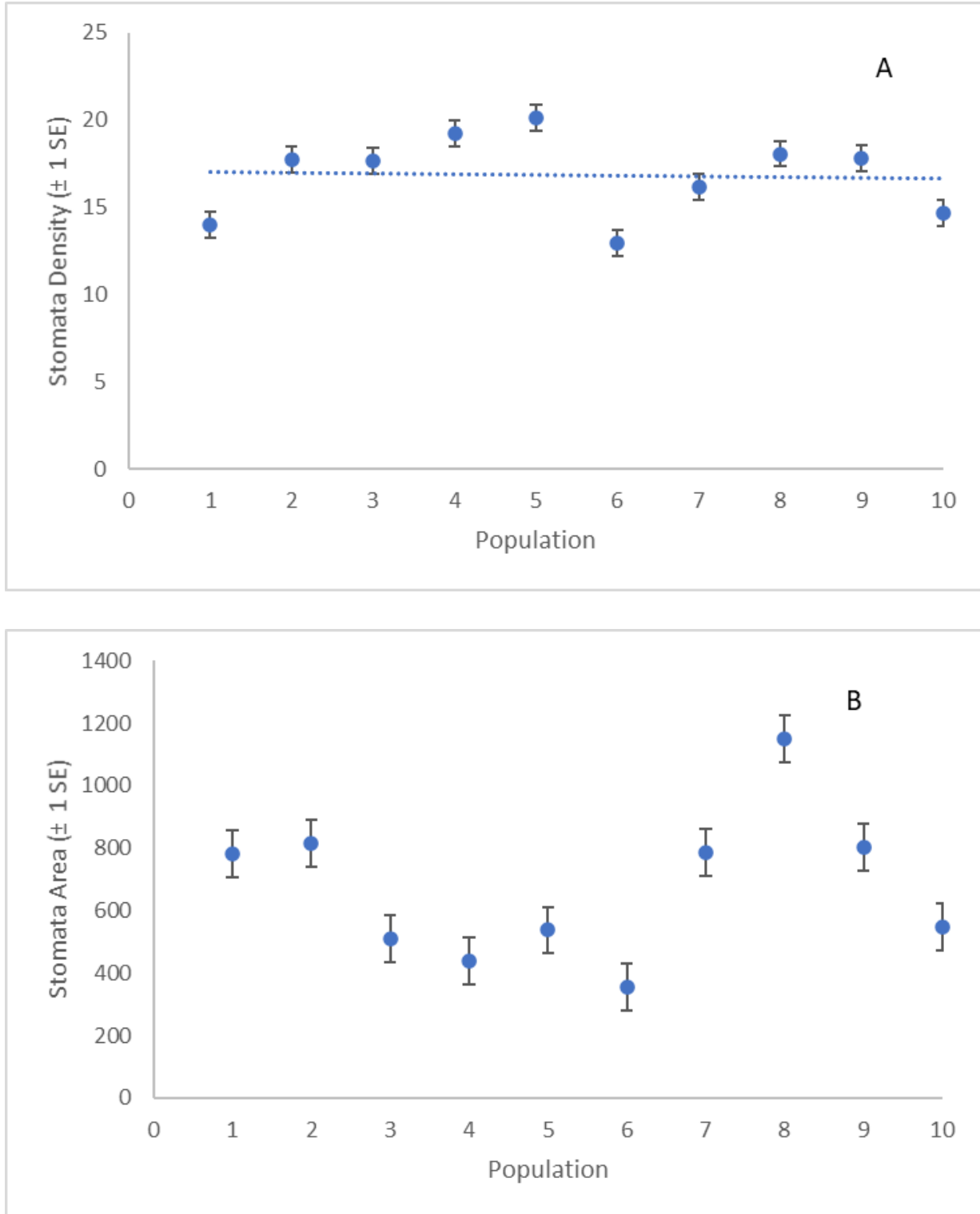
Figure 2. A) Mean stomatal density +/- 1 SE (#/250 square nanometers) and B) mean stomatal size +/- 1 SE (square nanometers) in Common Milkweed (*A. syriaca*) plants from 10 populations (see Table 1) from across the species' range grown in a common garden in central Virginia in 2021.

Results of multiple regressions showed no significant effect of either latitude ($F_{1,57}$ =

0.01, p = 0.93) or longitude ($F_{1,57}$ = 0.43, p = 0.52) on mean stomatal density (Figs. 3a and 3b,

respectively). Mean stomata size showed a trend towards an increase in size with an increase in

latitude (from south to north), but the effect of latitude was not significant ($F_{1,61}$ = 3.16, p = 0.08)

(Fig. 4a). In contrast, mean stomata size increased significantly with increasing longitude (from

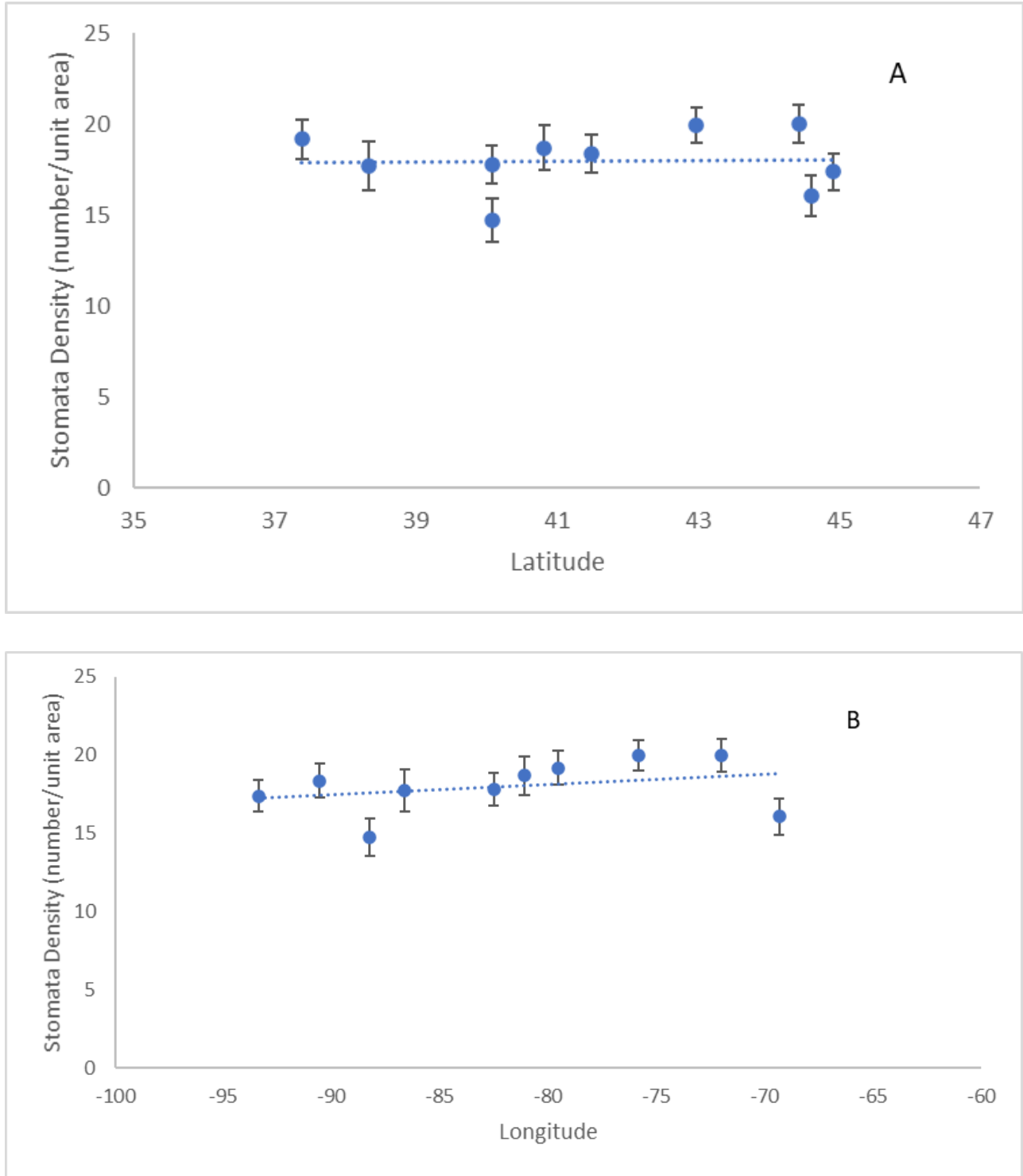east to west), thereby providing evidence of a geographic cline ($F_{1,61}$ = 5.85, p = 0.02) (Fig. 4b).

Figure 3. The effect of A) latitude and B) longitude on mean (+/- 1 SE) stomatal density (#/250 square nanometers) in Common Milkweed (*A. syriaca*) plants from 10 populations (see Table 1) from across the species' range grown in a common garden in central Virginia in 2021.

Figure 4. The effect of A) latitude and B) longitude on mean (+/- 1 SE) stomatal size (square nanometers) in Common Milkweed (*A. syriaca*) plants from 10 populations (see Table 1) from across the species' range grown in a common garden in central Virginia in 2021.
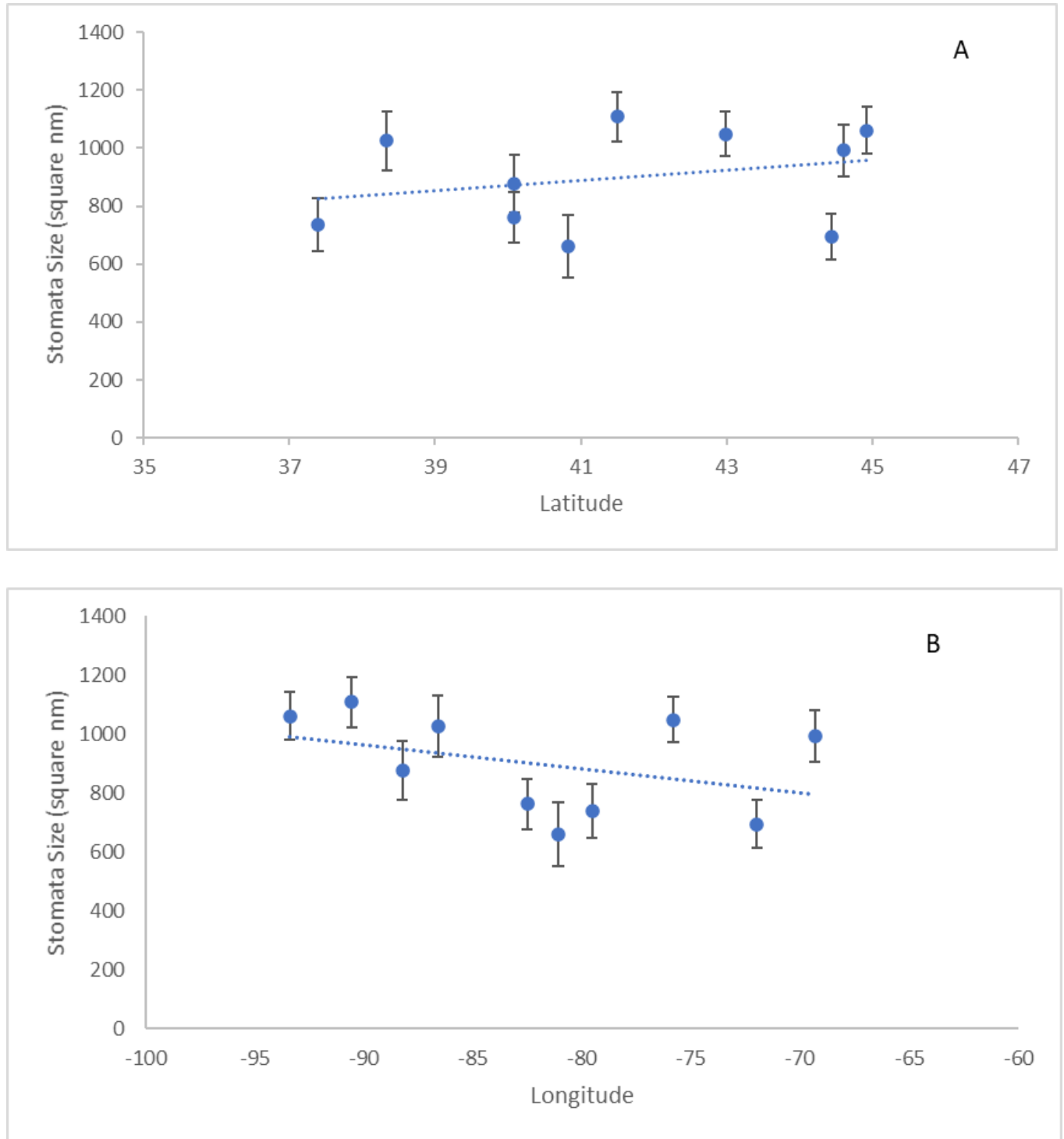
DISCUSSION

I established a common garden experiment in which I transplanted common milkweed seedlings from across the species' native range to test the hypothesis that common milkweed is locally adapted with respect to stomatal density and size. Leaf impressions from representative plants of 45 genotypes from 10 populations were collected to determine mean stomatal density and size by population. Stomatal density did not vary significantly with either longitude or latitude. In contrast, stomatal size did decrease significantly from south to north. Stomatal size tended to increase from west to east, but the cline was not quite significant. Therefore, my results partially support the hypothesis that stomata size and density are locally adapted and vary clinally.

The fact that stomatal area varied clinally with latitude but stomatal density did not is confusing because stomata area and density are typically negatively correlated with one another. As stated, Dittberner et al. (2018) found that there is a negative correlation between stomata size and density based on water-use efficiency. Similarly, Carlson et al. (2016) found that there is a positive relationship between stomata density and fecundity, but a negative relationship between stomata density and the stomata area. However, another study also found clinal variation in stomata size but not area. Franks, Drake, and Beerling (2009) compared field-grown *Eucalyptus globulus* at two plantations and took measurements from both the high and low rainfall site to compare the stomatal morphology. The researchers explanation for differences in clinal variation for stomata size but not area is competition between stomatal and non-stomatal cells on the leaf surface including trichomes, hair-like follicles on the underside of the leaf surface, and other glands.

Woods et al. (2012) conducted a study on local adaptation in Common Milkweed to investigate effects of latitude and longitude on fitness-related plant characteristics. They found that aboveground biomass was negatively correlated with latitude, meaning that plants farther south produced more above-ground biomass than did plants farther north. Additionally, latex production was positively correlated with latitude and precipitation levels. Latex is a sticky white sap that serves as a defensive trait because it gums up insect mouthparts and contains toxic cardenolides that kill insects by stopping their hearts. Finally, early-season height of milkweed plants, which is used as an estimate of phenology, was positively correlated with both precipitation and latitude in Common Milkweed (Woods et al., 2012). Many fitness-related traits seem to be locally adapted in common milkweed, but local adaptation in stomata size and density has not been studied previously.

Local adaptation has been studied in many other plant species as well. For example, Clay and Quinn (1987) experimented with another plant, *Danthonia sericea*, that had been experiencing local adaptation and determined that plants in drier regions had greater stomatal density and less stomatal size than wetter regions, following the trend that stomata size and density are negatively correlated. The results from Clay and Quinn (1987) are inconsistent with my work in the idea that the stomata area was significantly larger the closer to more wet conditions. Therefore, while Clay and Quinn (1987) saw significant results for stomatal density and area, the results from this experiment show only stomatal area was significantly affected by latitude, and not stomata density, meaning possible limitations could have affected the overall results.

One of the biggest factors that could be affecting the results is that stomatal density could be a plastic trait instead of genetically adapted. This means that the stomata size and density may

vary from environment to environment, but all common milkweed would genetically be the same when it comes to stomata production and quantity. However, some possible limitations that could have occurred include only sampling one leaf per plant. If more leaves were sampled, there could have been a difference in the stomata density based on the developmental stage of the leaf. When leaves are smaller and expanding, all the stomata are packed into a smaller area, meaning higher density. When the leaves are fully expanded, the stomata are more dispersed, leading to a lower density. Therefore, sampling leaves of different ages could have masked any population-level efforts. By consistently sampling a leaf the third node down from the top, the efforts tried to avoid this probability, but sometimes those leaves were missing or were just replaced by the plant if they had been damaged.

Importance of Studying Local Adaptation and Common Milkweed

In recent years, the eastern Monarch butterfly population has been declining (Thogmartin et al., 2017). This is possibly due to the declines in Common Milkweed populations caused by agricultural practices such as tilling soil for crop growth and removal of milkweed which is seen as a 'weed' species. The most significant reduction of Common Milkweed plants is caused by the use of herbicides such as glyphosate to kill weeds in agricultural fields (Pitman et al., 2018). In fact, there has been a 58% decline in Common Milkweeds in the Midwest and 81% decline in the monarch butterfly population in the Midwest from 1999-2010 (Pleasants et al., 2013). This correlated decline is likely one of causation because, 92% of Mexican and 84% of southern United States Monarchs were associated with *A. syriaca*, which is easily recognized by the concentrated cardenolides in the gut of Monarch butterflies (Malcolm et al., 1993).

Investigations of local adaptation in Common Milkweed can be used to inform ongoing conservation efforts of both Common Milkweed and the Monarch butterfly. If Common Milkweed is locally adapted into regional ecotypes, the seeds used for restoring Common Milkweed populations should be locally sourced to ensure the plants have a fitness advantage. By analyzing the local adaptation and different ecotypes associated with *A. syriaca*, Monarch preservation has a promising future if researchers and conservationists understand Monarch oviposition preference (Pitman et al., 2018). Evidence shows that Monarch oviposition is influenced by the size and density of a milkweed patch, as well as landscape, in that a small and low density patch in agricultural settings has the highest egg density (Pitman et al., 2018). Therefore, certain ideal areas for Monarch restoration areas could be within the edges of crop fields, the edges of fields, or other land that is close to agricultural land that may support Monarch oviposition (Pitman et al., 2018). Similarly, conservationists and researchers should consider planting native ecotypes of *A. syriaca* in their natural area. More specifically areas where machinery, livestock, herbicides, or other industrial agricultural techniques are used and would hinder the Monarch breeding seasons (Pitman et al., 2018). Therefore, understanding the Monarch preservation techniques associated with *A. syriaca* and local adaptation allows researchers to delve deeper into looking at how stomata size and density within *A. syriaca* may be an additional feature necessary for determining the best ecotype of *A. syriaca* that would ensure the highest success rate and the greatest chance of Monarch butterflies preservation.

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