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Barlow R. Wagner
Denison University

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Barlow Wagner

Dr. Andrew McCall

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Department of Biology

Denison University

Abstract

Common milkweed (*Asclepias syriaca*) plants are currently being planted as a food source for the declining Eastern monarch butterfly (*Danaus plexippus*). It is hypothesized that common milkweed populations from lower latitudes will have higher levels of defensive traits in comparison to their northern counterparts. This latitudinal hypothesis is important for restorative purposes because researchers might have to use different seeds at different sites when planting milkweeds. This hypothesis is founded on the idea that plants closer to the equator tend to experience higher rates of herbivory. In general, the purpose of this experiment was to determine whether a latitudinal cline in defensive traits exists between 19 milkweed populations sourced across North America and transplanted into a common garden in Granville, Ohio. The 19 populations were sampled for their mean latex exudation weights, specific leaf areas, and trichome densities. Statistical significance was determined using a mixed model ANOVA. There were no significant differences between the populations regarding their latex exudation weights and specific leaf areas. However, trichome densities were significantly different between populations. A significant negative linear relationship between latitude and trichome density was found. The differences between the populations regarding their trichome density data supports the idea that lower latitude populations are more defended against herbivores. This suggests that trichome density is a trait under selection across these populations, and likely indicative of local adaptation in *Asclepias syriaca*.

Introduction

Eastern monarch butterfly populations have declined by roughly 80% over the last two decades (Semmens et al. 2016). To help prevent monarch butterflies from becoming extinct, individuals planting common milkweed should plant populations of seeds that are able to survive and reproduce well in an environment that is suitable for monarchs (Thogmartin et al. 2017). *A. syriaca* local adaptation plays an important role in monarch conservation strategy as in addition to helping scientists understand how a changing climate can affect a population's distribution of phenotypes within their tolerable niche breadth (Woods et al. 2012; Thogmartin et al. 2017). Reciprocal transplant experiments are used to study how a plant species' niche distribution could be locally adapted to abiotic and biotic conditions in different areas (Woods et al. 2012; DeLaMater et al 2021; Zhou et al. 2021). Local adaptation occurs when a population is more fit in its local environment in comparison to a different population, which is less fit in that same location (Woods et al. 2012). In this experiment, plants would be locally adapted if they are more fit in one environmental location in comparison to another.

In reciprocal transplant, multiple genotypes grown in multiple common gardens are analyzed to determine whether phenotypic variation exists among the genotypes, and if such phenotypic variation could be explained through local adaptation (Woods et al. 2012; DeLaMater et al. 2021). Importantly, the effect that seed source location has on phenotypic traits helps scientists understand the implications that abiotic conditions in those different environments could have on a species, such as common milkweed and monarch butterflies (Woods et al. 2012; Zhou et al. 2021). Precipitation, nitrogen availability, and temperature are known to be changing ecosystems across the world differently (Parmesan 2006; Zvereva & Kozlov 2006; Pelini et al. 2009; Cornelissen 2011; Jamieson et al. 2012; DeLaMater et al 2021).

If milkweed populations can't adapt to these changing environmental conditions, then monarch populations could become extinct (Semmens et al. 2016; Outhwaite et al. 2022).

For common milkweed, latitudinal clines in defensive traits could be explained by a difference in herbivory pressure between the populations (Woods et al. 2012; Abdala-Roberts et al. 2016; DeLaMater et al 2021). For instance, a past study that performed a reciprocal transplant experiment on common milkweed found that higher-latitude populations exuded more latex than their southern counterparts (Woods et al. 2012). In another common garden experiment studying plant defensive traits, researchers noted specific leaf area showed plasticity along a geographical cline (Kooyers et al. 2014). Researcher have also noted that in *Encella farinosa,* trichome densities displayed an intraspecific cline across a moisture gradient (Sandquist & Ehleringer 1998, 2003ab). These studies show that herbivory pressure or latitudinal clines in precipitation could be responsible for driving local adaptation (Woods et al. 2012; Finch et al. 2018; DeLaMater et al. 2021; Zhou 2021).

Woods et al. (2012) found a negative linear relationship between seed source latitudes and stem biomass. Additionally, theses researchers also found a negative linear relationship between seed source latitude and aboveground plant biomass (Woods et al. 2012). In their New Brunswick, New York, and North Carolina common gardens, seeds sourced from more southern populations showed higher aboveground and stem biomass in comparison to their northern counterparts (Woods et al. 2012). They also found that populations from higher latitudes (where precipitation was higher) were positively correlated to the amount of latex the plants exuded (Woods et al. 2012). The Woods et al. 2012 model shows that that latitude was positively correlated to seed mass, precipitation, and latex exudation, but negatively correlated to overall aboveground plant growth (Woods et al. 2012). Their seed source latitudes were negatively

correlated to precipitation, so relationships could be inversely related in common gardens where seed source precipitation is not correlated to seed source latitude in the same way as in their experiment (Woods et al. 2012). That is to say, precipitation gradients may not always follow clines across latitude in the same way across the planet (Moreira et al. 2018).

Interestingly enough, herbivore pressure along latitudinal gradients is known to drive investments in defensive and growth traits for species across the globe (López-Goldar & Agrawal 2021). However, plants often face a tradeoff in resource allocation to growth and defense (Coley et al. 1985; López-Goldar & Agrawal 2021). For this reason, measuring both constitutive and inducible defenses is necessary in getting a wholistic picture of how the plants may be locally adapting to herbivory (Coley et al. 1985; López-Goldar & Agrawal 2021). In this experiment, it is hypothesized that populations growing closer to the equator will possess a higher level of defensive traits in comparison to their more northern counterparts (Woods et al. 2012; Kooyers et al. 2014; Abdala-Roberts et al. 2016; DeLaMater et al. 2021). This gradient in trait differentiation has been observed previously and could driven by herbivory (Woods et al. 2012; Kooyers et al. 2014; Abdala-Roberts et al. 2016; DeLaMater et al. 2021).

Although, it is very possible that in a changing environment the niche breadth of common milkweed could shift, and thus shift clines in defensive traits as well (DeLaMater et al. 2021; Outhwaite et al. 2022). In environments that are warming, conservationists should plant milkweeds that would retain water and limit expensive investments in defense (Cipollini et al. 2018). For this reason, understanding how milkweed are potentially adapting to and will adapt to a shifting environment will be critically important in replenishing populations of milkweed (Semmens et al. 2016; Outhwaite et al. 2022). Currently, because the plants are locally adapted to shorter growing season in the north and a higher rate of herbivory in the south, the plants

could shift their allocation of resources to best conserve water in environments that are warming (Agrawal et al. 2005; Woods et al. 2012; Cipollini et al. 2018; DeLaMater et al. 2021). It is likely that milkweeds are going to invest more resources into constitutive defenses when resources are limited, and when resources are plentiful, they are more likely to invest more resources into inducible defenses (Agrawal et al. 2005; Bingham and Agrawal 2010; Rasmann and Agrawal 2011). Although all three defensive traits measured in this experiment can be induced, some are less inducible than others. For instance, latex exudation mass is known to be more inducible than trichome densities and specific leaf areas (Agrawal et al. 2005; Bingham and Agrawal 2010; Rasmann and Agrawal 2011). This is because constitutive defenses cost less resources in comparison to inducible defenses (Agrawal et al. 2005; Bingham and Agrawal 2010; Rasmann and Agrawal 2011). Several other studies show that because inducible defenses are more expensive to produce, a cline is likely to be observed that shows a positive linear relationship between the amount of precipitation the plants populations receive in their surrounding environments and the amount of latex that is exuded (Agrawal et al. 2005; Bingham and Agrawal 2010; Rasmann and Agrawal 2011). Areas where there is more water likely correlates to higher inducible defenses and areas with less water likely correlates to higher constitutive defenses (Agrawal et al. 2005; Bingham and Agrawal 2010; Rasmann and Agrawal 2011; Woods et al. 2012; Cipollini et al. 2018).

In this experiment, it is possible that these traits are either dependent or independent from herbivory (Kooyers et al. 2014; Abdala-Roberts et al. 2016). Research in the future will focus on discerning whether such trait differentiation in this experiment is due to herbivory. For instance, trichome density decreases in density down a latitudinal gradient in the species of perennial herb, *Ruellia Nudiflora,* and researchers were able to correlate the results to higher rates of herbivory

in the south (Abdala-Roberts et al. 2016). These experiments studying defensive traits are showing that plant herbivore interactions show clines across the world differently and that the same trend in trait differentiation is not observed interspecifically (Kooyers et al. 2014).

In this experiment, we question whether there are differences in the levels of defensive traits between the populations, as well as if there are clines in such defensive traits along a latitudinal gradient. We hypothesized that the populations would show differences in their levels of defensive traits. Additionally, we also hypothesized that populations closer to the equator would have higher levels of defensive traits in comparison to their more northern counterparts (Woods et al. 2012; Kooyers et al. 2014; Abdala-Roberts et al. 2016; DeLaMater et al. 2021). If the results of this experiment show any significance, a model for how milkweed populations may adapt to a changing environment in the future could be made (Woods et al. 2012).

Methods

Milkweed seeds were sourced from 20 populations and 180 separate genotypes across the Midwest and East Coast ranging from latitudes 36° to 47° and longitudes -95° to -70°. The seeds were planted and germinated in the Denison greenhouse during the week of April 9, 2021. Plants were transplanted into the Denison University Biology Reserve during the week of 5/7/21. Plant genotypes were randomized and placed in six plots partitioned into 50m by 50m. The same genotypes were represented in each plot. Plants were watered with backpack sprayers and plot location and genotypes were identified with pin flags. During peak summer growth, the plants were sampled for latex exudation and trichome density from 7/6/21-7/17/21. A pair of leaves two nodes below the top-most leaves, or the third pair of nodes from the top was selected. If no leaf was present in that location, then a leaf with the least amount of damage was selected for

sampling. A filter paper, produced by a half circle whole punch was placed against the stem of the plant after the leaf was removed until all latex was exuded. Leaves were collected and placed into a Ziploc bag and returned to the lab. The leaves were hole punched into disks of an area of 19.63 square millimeters, so that the edges were cut without getting the leaf sample stuck in the collector of the hole puncher, that is between the main vein and the edge of the leaf. A 1 millimeter-by-1millimeter grid was placed on the disks for counting the density of the trichome for each sample. The image analysis and counting software ImageJ was used to determine the amount of trichomes in a millimeter radius of a leaf disk. Calculations were performed on each measurable sample in the dataset. For samples with greater than 250 trichomes, subsections of the frame were counted and then multiplied by the number of subsections to get the total number of trichomes in the sample area. If densities were too dense to count, the value was recorded as too many to count (TMTC) or not applicable (NA) if the picture was not clear. These values were not included in the statistical analysis.

Latex samples were placed in a microcentrifuge tube and later weighed using a Mettler Toledo XA105DU scale after the latex was dried to the filter paper. The mass of the filter paper with no latex for each sample was subtracted from the mass of the filter paper with the dried latex. Latex masses were then analyzed using JMP. Leaf disks were also dried for determining the specific leaf area. This was determined by dividing the area of the leaf disk by the mass of the leaf disk. Samples were dried in microcentrifuge tubes and later massed using a Mettler Toledo XA105DU. The dry mass of the leaf disks was determined by subtracting the pre-weight of the microcentrifuge tube from the dry weight of the disk and the weight of the tube.

Statistical Data Analysis

Data was analyzed on February 9, 2022 (n=987). Common milkweed's defensive traits of trichome density, specific leaf area, and latex exudation were analyzed on JMP, using a mixed model ANOVA. Latex exudation weights, SLA, and trichome densities were normalized using a logarithmic transformation on JMP. For the mixed model ANOVA, genotype and plot were assigned as random effects, while population was assigned as a fixed effect. Regressions of defensive traits against their seed source latitudes were analyzed on JMP. Additionally, due to the potential confounding effects of insecticide application in plot 7, sample data (n=229) was excluded in this analysis.

Results

A significant difference was found between populations and mean trichome densities using a mixed model ANOVA (Fig. 1; p<0.0001; F=5.1980). No significant difference was found between populations and their mean SLA (p=0.4821; F=0.5166; DF=18). No significant difference was found between populations and their mean latex weights (p=0.2255; F=1.5821; DF=18). Populations from southern populations had higher trichome densities than northern populations (Fig. 2; p=0.0009; F=15.992; DF=18). No linear relationship was found between population latitude and SLA (p=0.4821; F=0.5166; DF=18). No linear relationship was found between population latitude and latex exudation weights (p=0.2255; F=1.5821; DF=18).
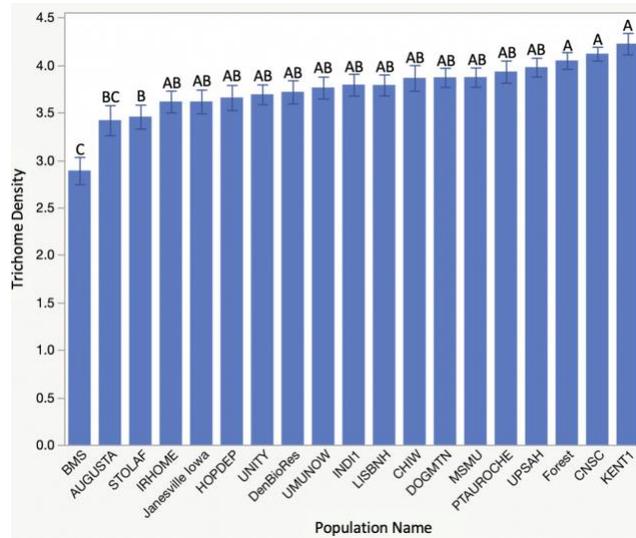
Figures

Figure 1. Bar graph of trichome densities broken down by population. Bars are standard error. Bars differing in letters shared are statistically different.
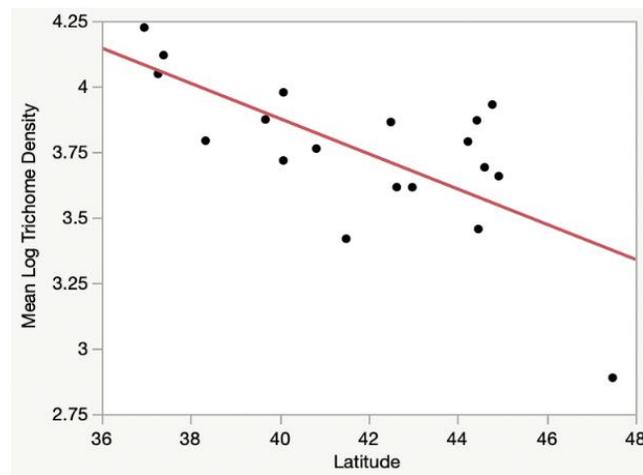

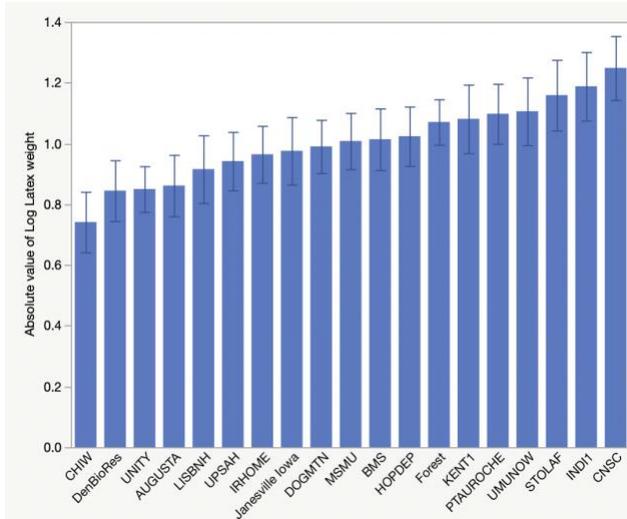Figure 2. Linear regression of trichome densities vs. population latitude.

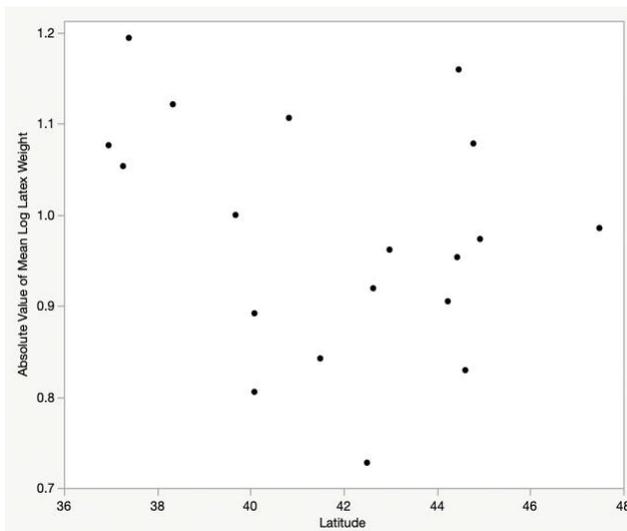Figure 3. Bar Graph of latex exudation weights broken down by population. Bars are standard error.

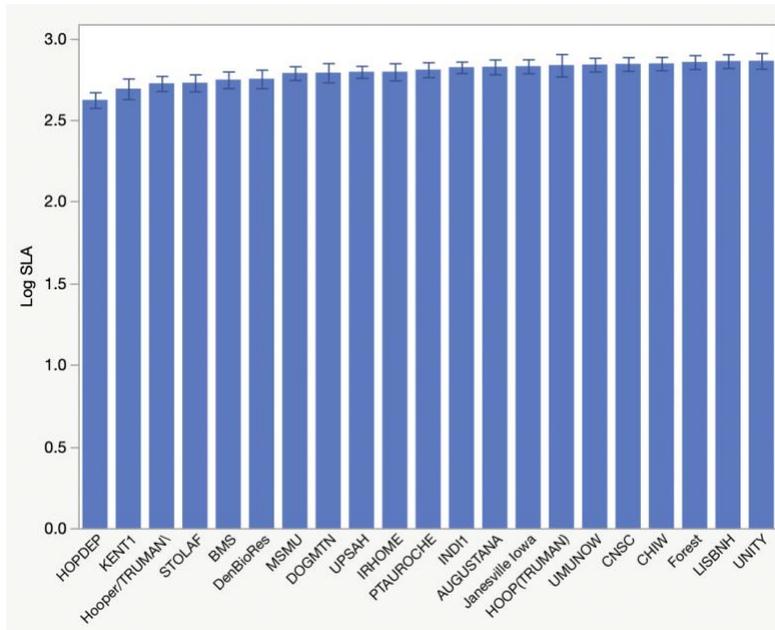Figure 4. Linear regression of latex exudation weights vs. population latitude.

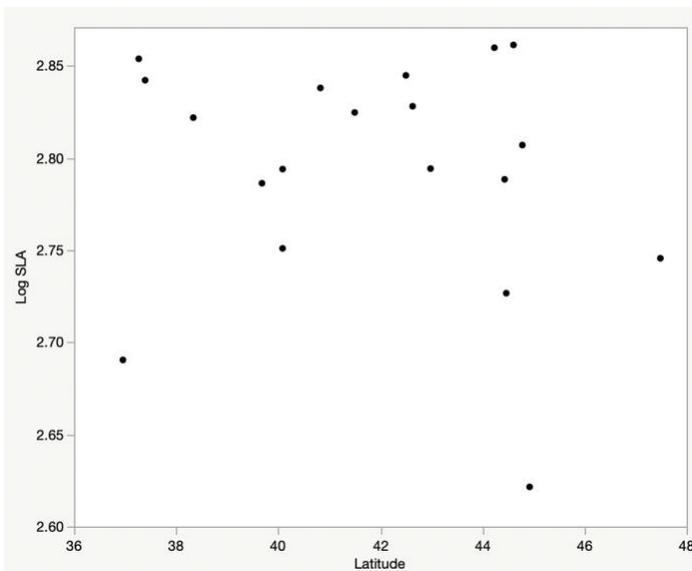Figure 5. Bar Graph of specific leaf areas broken down by population. Bars are standard error.

Figure 6. Linear regression of specific leaf areas vs. population latitude.
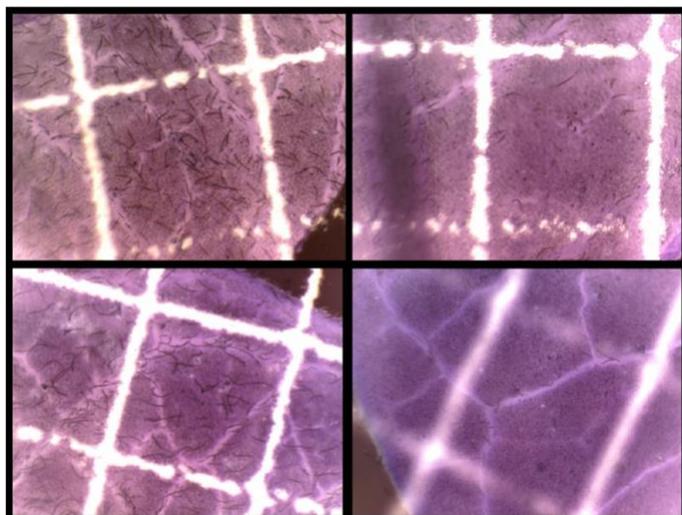
Figure 7. Inverted colorized microscope photographs of 1-millimeter square grid over abaxial side of four different common milkweed genotypes.

Discussion

The trend in our data supports the hypothesis that populations of common milkweed closer to the equator would show higher levels of defensive traits in comparison to their northern plant counterparts and that such populations are locally adapted to higher rates of herbivory in their surrounding environments (Woods et al. 2012; Kooyers et al. 2014; Abdala-Roberts et al. 2016; DeLaMater et al. 2021). This was true regarding the trichome density data, where we observed a significant difference between the 19 populations. An increase in trichome density in lower latitude plant populations has been found previously in several other species, including *Ruellia nudiflora* and *Oenothera biennis* (Kooyers et al. 2014; Anstett et al. 2015; Abdala-Roberts et al. 2016). Although we found no statistical difference between the plant population's mean SLA and latex exudation masses, the results from this study were not surprising, for past research has also found a mix-matching of results on correlations between latitude and defensive traits (Kooyers et al. 2014). Though, trichome densities still differed along a latitudinal gradient, suggesting that the agents of selection across milkweed's midwestern and eastern niche breadth

are environmental factors that differ in distribution and drive investments in defensive traits (Kooyers et al. 2014; DeLaMater et al. 2021).

Difficulty finding latitudinal clines in defensive traits suggests that there are more environmental variables to account for (Gao et al. 2019). Experiments that seek to investigate the latitudinal herbivory hypothesis (LHH), where plant-herbivore interactions decrease as latitude increases not only in the field, but in reciprocal transplant studies as well should certainly focus on interactions of environmental variables at higher trophic levels and how populations of herbivores are affected by the species distributions of their predators (Hairston et al. 1960; Lawton & Strong, 1981; Strong et al. 1984, Agrawal et al. 2005; Gao et al. 2019). For instance, the distribution of higher trophic levels and species distributions of herbivore predators is known to drive herbivory pressures worldwide (Hairston et al. 1960; Agrawal et al. 2005). Because this experiment did not find any differences between the populations and their mean SLA and latex exudation weights, future experiments should focus on determining why that was the case, potentially surveying animal distributions at higher trophic levels (Hairston et al. 1960). It could be that distributions of higher trophic levels within milkweeds' native niche breadth also varied significantly and could help explain defensive traits along a latitudinal gradient (Hairston et al. 1960; Agrawal et al. 2005). A lack of differences between populations and the mass of latex that the plants exuded could have been due to herbivory shortly before sampling latex as well; if a plant experienced herbivory shortly before we sampled its latex, it could affect the mass of latex that was exuded from the sample (Agrawal et al. 2005; Rasmann et al. 2009). Latex is an expensive defense to produce and a temporal delay in replenishment could throw off the distribution of mass data if one population was fed on more than another (Agrawal et al. 2005;

Rasmann et al. 2009). More research in the future should also help to explain why we observed a latitudinal cline in only 33% of the measured defensive traits, while also focusing on how a changing climate and landscape is going to affect the phenotypic and genotypic expression of these traits (Kooyers et al. 2014; Semmens et al. 2016; Zhou et al. 2021; Outhwaite et al. 2022).

A multitude of studies have shown that there are latitudinal clines in plant defensive traits, but such clines are not always consistent for results in how defenses are expressed (Andrew and Hughes 2005, Adams and Zhang 2009, Pennings et al. 2009). For instance, this experiment did not find a cline in the mean mass of latex that was exuded in milkweed populations, while another experiment did (Woods et al. 2012). In another study, differences in plant trichome densities were found between populations, but such differences could not be explained through a latitudinal gradient (Loughan and Williams 2018). There was also controversy as to whether any intraspecific patterns of plant defenses locally adapting to environmental herbivory even exists (Levin 1976, Coley and Aide 1991, Bolser and Hay 1996, Coley and Barone 1996, Schemske et al. 2009, Moles et al. 2011, Rasmann and Agrawal 2011). Therefore, there is still more evidence needed to support the latitudinal herbivory hypothesis in common milkweed. If all three measured defensive traits showed significant differences and clines across latitude, then supporting the LHH would be easier. However, because the results from this study do not completely align with past research on how common milkweed defenses are locally adapting to its environment, support for the LHH in the context of this experiment is made more difficult (Moles et al. 2011; Woods et al. 2012; Kooyers et al. 2014; DeLaMater et al. 2021).

The allocation of plant resources to trichomes, specific leaf area, and latex production

and exudation also differs from plant species to species (Woods et al. 2012; Kooyers et al. 2014;

Abdala-Roberts et al. 2016; DeLaMater et al. 2021). Regarding common milkweed, the

investments of resources into constitutive and inducible defenses also changes based on which

herbivores are present or absent (Woods et al. 2012; Kooyers et al. 2014; Abdala-Roberts et al.

2016; DeLaMater et al. 2021). In the past, when common milkweed was translocated across the

Atlantic Ocean, populations that were transposed showed substantially reduced latex exudation

in response to herbivory, showing that continental trait differentiation can significantly reduce

defenses in plants (Agrawal et al. 2015). These differing levels of inducible latex could be due to

differing levels of herbivores and distributions of higher trophic level predators (Hairston et al.

1960; Agrawal et al. 2005; Agrawal et al. 2015). Woods et al 2012 also found that in their

reciprocal transplant experiment that populations in higher latitudes exuded more latex than their

southern counterparts. The researchers also showed that in their reciprocal transplant experiment,

the latex exudation mass was positively correlated with the precipitation at each population site

(Woods et al 2012). Populations that experienced more precipitation also experienced more latex

exudation (Woods et al 2012). Interestingly, milkweed plants can differentiate between

herbivores as well (Bingham and Agrawal 2010). In the past researchers have found that

milkweed plants exuded five-fold variation in the amount of latex that was exuded among

genetic families (Bingham and Agrawal 2010). Such a result suggests that species specific

herbivory cues play a role in how much latex is exuded from the plant and that sampling

techniques for latex exudation studies should aim to standardize the sampling methodology to

best compare latex exudation results to each other (Agrawal and Hastings 2019).

In addition, after performing a reciprocal transplant experiment in New York, researchers

showed that the specific leaf areas of the common milkweed plants did not show a latitudinal cline in SLA, despite population differences (Woods et al. 2012). This is interesting, because although they showed differences among populations, no latitudinal cline was observed (Woods et al. 2012). Plants with a high SLA often are more susceptible to herbivory and differing levels of SLA across a population would likely show differing levels of herbivory, but in this experiment and in Woods et al. 2012, that was not found to be the case (Da Silva and Batalha 2011). Plants with greater cell wall toughness also tend to have more leaf mass per area, and therefore lower specific leaf areas, suggesting that we would find clines across common milkweed's latitude due to differing herbivory pressure across the populations (Da Silva and Batalha 2011). Plants with higher internal water levels tend to have lower specific leaf areas and tougher leaves (Da Silva and Batalha 2011). One would expect that because there are differences in precipitation across common milkweed's native niche breadth, that we would expect to find clines in SLA, but a lack of SLA significance in our experiment suggests that the populations are not under selection for this trait, nor are they locally adapted to their environments. In the past, experiments studying defensive traits across a latitudinal gradient have found both a lack of differences and clines in SLA (Woods et al. 2012; Loughan and Williams 2018) It could be that in the future, after the plants are more developed and more resources are invested into cellular development that a difference between populations or even a latitudinal cline is found.

Even though we found no differences between populations regarding their mean latex exudation weights and their mean specific leaf areas, we still found differences between the population's trichome densities as well as a latitudinal cline in such densities. This result is supportive of the LHH and that the plants closer to the equator have higher levels of defenses to protect themselves from herbivory in their environments (Woods et al. 2012; Kooyers et al.

2014; Abdala-Roberts et al. 2016; DeLaMater et al. 2021). However, past research shows that in common milkweed there were no previous clines in trichome densities while looking across a latitudinal gradient (Woods et al. 2012). Additionally, past research found that trichomes serve a purpose beyond defensive traits, preventing water loss and evapotranspiration (Bickford 2016). Therefore, one would expect that in locations with longer growing seasons, that the trichome densities would also be greater than those in the north with shorter growing seasons (Woods et al. 2012). In this experiment, we found that more southern populations had higher densities of trichomes in comparison to their northern counterparts. However, it is difficult to discern whether such a result is due to the plants locally adapting to herbivory or locally adapting to the climate in their surrounding environment (Bickford 2016). This discernment of trait by environment interactions is concerning to those that seek to better understand how milkweeds are locally adapted to different regions in the United States (DeLaMater et al. 2021). For this experiment, disentangling whether trichomes densities increase closer to the equator because of herbivore pressure or for water retention purposes should be considered.

The fact that milkweed populations in this experiment showed differences in their mean trichome densities in addition to displaying a positive linear relationship between the densities of those populations and a decreasing latitude suggests that the plants could be experiencing a difference in selective pressures across their native niche breadth (Kooyers et al. 2014; DeLaMater et al. 2021). This result could support the hypothesis that plants closer to the equator are locally adapted to higher rates of herbivory in those locations (Woods et al. 2012). Importantly, whether herbivory is the driver of such selection is up to debate, for the increasing trichome density could also be beneficial to the plants in southern locations to maximize water retention (Bickford 2016).

A changing environment will have an impact the restoration efforts of common milkweed and monarch populations (Outhwaite et al. 2022). After performing this experiment, we found that trichomes in common milkweed show a latitudinal cline in density, which differed from what Woods et al. found 2012. In their experiment, they found no differences among the populations regarding their trichome densities and no clines. However, in 2022, we did find a difference in trichome densities with the populations that were sampled. Future research should certainly aim to investigate the driver of trichome densities, and whether the driver of selection is responsible for such a difference in results between the years 2012 and 2022 (Loughnan and Williams 2018). Answering this question would help restoration efforts that aim to plant the most fit milkweeds across the Unites States (Mondoni et al. 2012). In a changing environment, planting a maladapted plant could hamper the reproductive growth of the plant, if a seed that is locally adapted to a shorter growing season is transplanted into a region where the growing season is longer, the seeds that are locally adapted to sprout at a certain time could never produce seeds of their own (Mondoni et al. 2012). Such a scenario could be detrimental to common milkweed and would likely have lasting consequences on populations of monarch butterflies across the nation (Mondoni et al. 2012; Semmens et al. 2016; Zhou et al. 2021; Outhwaite et al. 2022). During a time when the landscape and climate is changing rapidly, the need to understand how milkweed is locally adapted to different regions in the United States is now more important than ever so that future generations can continue to appreciate the beauty of the monarch butterfly (Outhwaite et al. 2022). Additionally, the need to find the underlying drivers of selection in defensive traits for plant species across Earth could help scientists understand how a changing climate is making real time impacts on plant and insect species globally.

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