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Ecology and Evolution of Common Milkweed

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Ecology and Evolution of Common Milkweed

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ABSTRACT

All organisms must interact with and adapt to their surrounding environment. There are myriad ways in which species accomplish this; ultimately resulting in the vast diversity of life on earth today. Changes in the environment can have profound impacts on an organisms' ability to compete and utilize their surroundings. Plants are particularly impacted by local environmental differences because of the fact that they are immobile. This environmental variation exists at both large and small spatial scales. For example, on larger scales, forces such as fire and grazers can remove dominant plant competitors. On smaller scales, variation in resource availability (e.g. light, nutrients, water) may benefit more phenotypically plastic species. To better understand how changes in the environment, on both large and small spatial scales, I established a two part study using milkweed (*Asclepias spp.*) as a model system. In the first chapter, I ask how fire, large grazers, and nutrients have affected milkweed abundance over relatively long time and large spatial scales. Here I found that most milkweed species increase in abundance with burning alone but expressed species-specific responses to other treatment combinations. This indicates that milkweed species have likely experienced unique fluctuations in abundance as fire and large herbivores moved across the landscape. The second aspect of this research focuses in on a single year and relatively small spatial scales. Here, using common milkweed (*A. syriaca*), I ask how environmental variation shapes spatial structuring of phenotypes within fine-scale physical distance and how genotypes impact phenotypes. I found that environment, not genotype, had a relatively larger role on fine-scale phenotypic variation. Combined, these results have implications for understanding the role of large and small scale environmental variations in plant phenotypes and plant abundance.

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CHAPTER 1. LONG AND SHORT-TERM RESPONSES OF *ASCLEPIAS* SPECIES DIFFER IN RESPECT TO FIRE, GRAZING AND NUTRIENT ADDITION

INTRODUCTION

Over the last few decades, milkweed numbers have declined at an estimated rate of 2 million stems per year (J. Pleasants 2016). Common milkweed has a wide natural range that includes the tallgrass prairie. The tallgrass prairie ecosystem once covered an estimated 162 million hectares (Samson and Knopf 1994) of the central United States. Nearly all of this system has since been converted to row crop agriculture: in many central states only 0.1% of the original tallgrass prairie remains (Samson and Knopf 1994). Disturbance and nutrient load regimes differ between agricultural use and that of the native prairie; for example, agricultural settings have higher levels of nutrients and frequent disturbance that includes soil turnover from tilling, removal of grasses or other forbs, and differences in light regimes. Plants within tallgrass prairie also experience disturbance and nutrient fluxes, albeit, under relatively different processes and timescales -- with the two principle disturbances being fire and grazing (Knapp et al. 1999). Fire removes detritus, surrounding plant tissue, and canopy cover; large grazers also remove competitors, “till” the soil, and redistribute nitrogen back into the system. Some plants native to the tallgrass prairie may do especially well within agricultural settings. Common milkweed (*Asclepias syriaca)* is one such species and is known for its ability to quickly colonize highly disturbed, nutrient rich agricultural areas.

The Flint Hills region of central Kansas, where the shallow soils and limestone rock prevented plowing (Hickey and Webb, 1987), provides an ideal place to study the effects of fire and grazing disturbance regimes on plant composition. This region contains the largest tract of remaining tallgrass prairie covering 1.6 million hectares (Knapp 1998). Much of the area is currently used for ranching and is annually burned. Konza Prairie Biological Station (KPBS) is a tallgrass prairie LTER station that studies multiple drivers of grassland communities, such as fire and grazing. For over three decades (1980) researchers at this 3,487-hectare preserve have examined the influence of fire and grazing regimes on tallgrass prairie ecosystems (Collins et al., 1998). Nutrient addition, another important driver to plant composition, has also been examined at KPBS since 1986 (Collins et al., 1998). Like other grasslands worldwide, KPBS has three main ecological drivers including fire (Knapp et al. 1999; Collins et al. 1998), grazing (Knapp et al. 1999), and climate variability (Knapp 1998). Both fire and grazing have large effects on plant species composition by altering the competitive interactions between grasses and forbs for light and belowground nutrients such as nitrogen and phosphorus (Collins et al. 1998). Inter-annual climate variability is extreme creating years with productivity similar to desert grasslands and other years with productivity as high as deciduous forest (Knapp and Seastedt 1996).

Historically, fire in tallgrass prairie was frequent, with the majority of fires occurring in late summer and less frequently in early spring (Collins and Wallace 1990). Fire alters several aspects of the competitive environment for plants including removal of detritus and an opening of the canopy (Knapp et. al, 1999; S. L. Collins et al. 1998). This increases soil temperature in the spring and thus promotes early green up (Certini 2005; Wang et al. 2015; Winter et al. 2015). Fire also alters nitrogen availability within grasslands in two ways: first, nitrogen is volatilized during the combustion of detritus and plant tissues, which is a major cause behind nitrogen losses in this system (Blair 1997). Secondly, overall plant productivity is reduced with the *lack* of fire, as energy demands become higher due to either shading effects of accumulated detritus, increased demands from higher overall plant cover, or both (Blair 1997). In general, grasses benefit more from frequent burning, but this can depend upon the season of burn (Blair 1997; Knapp 1998; Towne and Kemp, 2003; Towne and Kemp, 2008).

The cumulative effects of fire (i.e. increased plant biomass and productivity; reduction in plant species richness) have also shown to be largely mediated by grazing of bison, especially when fire is infrequent (Knapp 1998). This effect is driven by the preferential consumption of dominant grasses by bison which increase the abundance of forbs by reducing the cover of competitive grass species (T. M. Abel, 2012; Knapp et al. 1999). Grazers also affect nutrient cycling and availability by redistributing nitrogen through dung and urine (Johnson and Matchett 2001). Redistribution of nutrients by these large herbivores can strongly influence plant responses, although this response can differ among grassland species (Leriche et al. 2003). A limiting factor within tallgrass prairie is the availability of soil nitrogen. Generally, excess nutrients will increase overall productivity of grassland communities, but will lower species diversity thereby altering overall composition (Avolio et al., 2014). Two main macronutrients that have been heavily explored are nitrogen and phosphorous; however, individual effects of phosphorous alone are relatively understudied (Avolio et al., 2014).

In addition to management practices such as fire, grazing, and nutrient addition, species composition in tallgrass prairie can be altered through fragmentation (Cully, Cully, and Hiebert 2003) and land-use change (Weiner et al. 2011); both of which have been highly influential within the tallgrass prairie ecosystem. Currently, the greatest threat to grasslands is the conversion to agricultural cropland (Stephens et al., 2008), and is considered to be “one of the most important land cover/land use change(s) in US history” (Wright and Wimberly, 2013). By 1990 nearly half (49%) of grassland habitat worldwide had been lost, with a subsequent five to six-fold increase in cropland and pastures (from 265 to 3451 million hectares and 524 to 3451 million hectares for cropland and pasture habitat, respectively; Goldewijk, KK, 2001). For tallgrass natives, then, species responses to losses of historical drivers (burning), introduction of large grazers in pastured area, and nutrient runoff from agricultural cropland can have large implications for future populations.

Asclepias *species in this study*

As many species in the genus *Asclepias* are native to grasslands, it is unsurprising that fragmentation and land-use change are considered to be major drivers behind milkweed declines (Flockhart et al. 2014). *Asclepias* species have come to prominence recently because of their connection to the iconic monarch butterfly, which has suffered alarming declines over the past 20 years (Flockhart et al. 2017; Flockhart et al. 2014; Semmens et al. 2016). Monarch butterflies (*Danaus plexippus*) utilize a variety of milkweed species as obligate host plants (Mattila and Otis 2003), but primarily use common milkweed, *Asclepias syriaca;* (Malcolm and Brower 1989). Monarch’s frequent use of common milkweed may not be because monarchs prefer common milkweed, rather, it may be due to the fact common milkweed is more abundant. An estimated 92% of overwintering monarchs have been shown to have fed on common milkweed at some point in their lifetimes (Malcolm and Brower 1989; Malcolm and Zalucki 1993). A growing number of studies have been aimed at quantifying the relationship between milkweed abundance and monarch declines, with mixed results. Inamine and colleagues (Inamine et al. 2016) report that, regardless of relative milkweed losses, monarch butterflies were able to rebound in the northern U.S. even after apparent population bottlenecks of monarchs were observed. In contrast, Pleasants and Oberhauser (Pleasants and Oberhauser 2012), state that known milkweed declines "will set a new, lower ceiling" for migratory monarch population estimates. Regardless of the eventual outcome of these studies, it is clear that monarchs need milkweed.

Currently, common milkweed may be more important for the monarch butterfly, than other *Asclepias* species (Malcolm and Zalucki 1993, Flockhart et al. 2013), due to its high availability and intermediate toxicity (Fink & Brower, 1981; Alonso-Mejia & Brower, 1994). However, this preference can shift based on generation and latitude. For example, when first generation monarchs enter the southern United States, *A. viridis* appears to be an important hostplant with an estimated 84% of larvae having fed on this species (Malcom and Brower, 1989; Baum and Mueller, 2015); perhaps due to its earlier emergence (Malcom et al., 1992). Further evidence indicates that larvae receive increased protection from highly toxic milkweeds such as *A. viridis* over less toxic milkweed such as *A. incarnata* (Roode et al., 2008; Roode et al., 2011). This can be especially important for first generation migrants as many overwintering adults have low levels of cardenolides (Nishida, Ritsuo, 2002), the toxic defensive compounds that milkweed specialists are capable of sequestering. Further, monarchs have been shown to use high cardenolide composition as a means to "self-medicate" from parasites prevalent in nature (*i.e. Ophryocystis elektroscirrha*; Roode et al., 2011). Milkweed with low levels of cardenolides, such as *A. tuberosa* (Malcom and Brower, 1986), can significantly reduce the life span of adults with parasites (Lefevre et al., 2012). Oviposition preference on this milkweed species also been shown to be significantly lower in relation to intermediate or even highly toxic milkweed (Haribal and Renwick, 1998), though monarchs will generally oviposit on milkweed with intermediate cardenolide levels (Oyeyele and Zalucki, 1990; Van Hook and Zalucki, 1991). As milkweed species inherently possess differential levels of toxicity, shifting abundances of hostplants could have pronounced effects on monarch populations. Monarch oviposition decisions do not seem to depend on the local density of nectar sources, as monarchs egg densities on Asclepias species found in monoculture row crop agriculture are just as high as egg densities on milkweeds in non-agriculture settings (Oberhauser et al., 2001).

Milkweed species have been shown to significantly increase overall cover with burning, especially when burning is applied during spring months (Towne and Kemp, 2008); including *A. stenophylla*, *A. syriaca*, and *A. verticillata*. Not all milkweed respond to fire similarly; for example, abundance of *A. meadii* decreased in response to annual burning, but remaining individuals had 68% more flowering ramets than in plots that were not burned (Bowles et al., 1998). If fire promotes flowering in other milkweed, then one might expect to see higher seed set in sexually reproducing species, which may lead to higher cover of these species over relatively long time scales. Regardless of life history, milkweed species have been shown to increase overall cover with burning, especially when burning is applied during spring months (Towne and Kemp, 2008).

For this study, we focused on tallgrass prairie natives that, due to their shared evolutionary history, one might expect relatively similar responses to major ecological drivers such as grazing. Due to their toxicity to mammals, grazing on milkweeds, even in small quantities (1% of total body mass), can detrimentally affect large herbivores (Kingsbury, J.M.; 1964; Panter et al. 2011). Therefore, cattle and bison should avoid grazing on them, especially in the presence of more beneficial vegetation (Fleming et al. 1920; Kingsbury 1964; DiTomaso & Healy 2007). Interactions between grazing and milkweed, however, are still largely unexplored. Towne and colleagues (Towne et al., 2005) found similar responses of forbs overall to grazing in KPBS from 1995 to 2004; yet a single milkweed included in that study (*A. verticillata*) had higher cover in plots that were *not* grazed.

Both fire and grazing have the potential to greatly influence *Asclepias* species, but we lack an understanding of how these drivers shape the abundance of milkweed and how this may differ across species. Here, we sought to determine how milkweed species respond to herbivory, fire, and nutrient addition. We specifically sought to answer three questions: (1) What is the long-term consequence of fire and grazing on milkweed abundance across large spatial scales? (2) What is the long-term consequence of fire and nutrient application in a field experiment? And (3) What is the effect of nutrient application to *A. syriaca* growth on in the absence of fire and biotic interactions in a greenhouse experiment? To address the first two questions, we utilize long-term datasets from KPBS that allow us to examine the effect of fire, grazing, and nutrient addition on milkweed species under field conditions. Next, using a greenhouse nutrient addition experiment in the absence of abundant biotic interactions and fire, we study how nitrogen and phosphorus impact growth on one of monarchs' most important host plants, *A. syriaca*. In light of the limited previous literature, we expected that both fire and grazing should increase the abundance of milkweed species. *Asclepias syriaca* in particular should increase in response to nutrient addition due to its ability to thrive in nutrient-rich agricultural fields.

MATERIALS and METHODS

Konza Prairie Long Term Ecological Research (LTER) site

Konza Prairie Biological Station (KPBS), located in the Flint Hills region of northeastern Kansas, maintains large watershed level plots in addition to relatively smaller plots that examine the additive effects of fire on species composition when nutrients are added (Collins et al., 1998). These “nutrient addition” plots were established in 1986, but milkweed responses within these plots were not available until 1989. We therefore used data for watershed and nutrient plots from 1989 onward. Nutrient inputs within smaller plot-level experiments was also explored. Using data collected from this site we sought to answer questions related to the impact of herbivory, fire, and nutrient addition on relative abundances of *Asclepias* species.

What is the long-term consequence of fire and grazing on milkweed abundance across large spatial scales?

From 1983 to 2016, plant species identity and cover and were recorded in long term fire and grazing watersheds at Konza prairie (Collins, 2016). Watersheds were sampled three times a year until 1991, when the sampling frequency was changed to twice a year (Collins 2016). In each watershed, four 50-meter-long transects were established with five evenly spaced, permanently marked, 10 m^2 sampling plots (Collins 2016). Species composition was recorded as a cover class value on a modified Daubenmire cover scale (Blair 2016). A value of 1 denotes present, but less than 1% cover, 2 denotes 1 – 5% cover, 3 denotes 5 – 25% cover, 4 denotes 25 – 50% cover, 5 denotes 50 – 75% cover, 6 denotes 75 – 95% cover, and 7 denotes 95 – 100% cover (Blair 2016). We only analyzed watersheds that were burned in intervals of 1, 2, 4, or 20 years. Burning of these watersheds occurred in early spring.

What is the long-term consequence of fire and nutrient application in natural experimental treatment within nutrient application plots?

Nutrient addition plots at Konza Prairie were established in 1986 as a full factorial design in which nutrient, burning, and mowing treatments were assigned to 64, 10 m x 10 m plots (Blair 2016). The plots were either burned annually or not at all (Blair 2016). The plots were also given one of four annual nutrient treatments: the addition of nitrogen (10 g/m^2), the addition of phosphorus (1 g/m^2), the addition of nitrogen and phosphorus (10 g N/m^2 and 1 g P/m^2), or a control in which no additional nutrients were added (Blair 2016). Mowing was found to have no appreciable effect on *Asclepias* cover and was excluded from all analyses.

In 1989 and 1994, plant species composition data was collected from one 10 m^2 circular subplot per plot (John Blair, personal communication). In 1999, two permanent plant composition subplots were established in each of the 64 plots, and cover data was recorded in these 5 m^2 circular plots (Blair 2016). Species composition was recorded following the same methods in the watershed level plots. Starting in 1989, plots were sampled at approximately 5-year intervals in June and August (Blair 2016). After 2005 sampling was reduced to once in late July (Blair 2016). Starting in 2015, cover was measured annually (Blair 2016). Since annual sampling did not start until 2015, we averaged data from 2015 and 2016 over all samples and used this as the last time point.

Statistical analysis for long-term consequences of fire, grazing, and nutrient addition

Linear regressions were used to determine how the change in mean *Asclepias* cover over time differed among burning and grazing regimes. One-way ANOVA was used to evaluate differences in cover class between nutrient treatments in those plots. Although our data did not follow a normal distribution, ANOVA tests are relatively robust to deviations from normality, especially when samples sizes are large, as in our case (nutrient addition and watershed-level data sets contained 4,716 and 696,080 observations, respectively; Blanca et al., 2017). Post hoc multiple comparisons were made using the Tukey-Kramer method to quantify significantly different responses to burning, grazing, and/or nutrient addition. Any instance of wildfire in a treatment plot was considered as burned, regardless if it was a prescribed or non-prescribed (wildfire) burn. All statistical analyses were performed using R version 3.4.1. (R Core Team, 2017).

What is the effect of nutrient application to milkweed growth in the absence of fire and biotic interactions?

Asclepias syriaca seeds were taken from three different sources; Blandy Experimental Farms (39°03’45.4”N, 78°03’46.9’W), Sky Meadows State Park (38°59’11.1”N 77°57’41.6”W), and from the North Carolina Botanic Garden collections. To control for variation due to shared ancestry, we sampled 24 individual pods over the three sources (Table 1) so that plants were half siblings at most. Seeds were scarified and then planted in trays on May 15th, 2017. Approximately ten days after germination, 600 healthy seedlings were transplanted into pots (8” round x 5” deep) filled with Fafard 52 soil mix. Fafard 52 soil mix consists of peat moss, bark, perlite, vermiculite, dolomitic limestone, a wetting agent, and RESiLIENCE ® (a Fafard registered silicone soil additive; Sungrow Horticulture Growing Products). Original content of carbon and nitrogen within this soil were determined using an elemental analyzer (Perkin-Elmer 2400 Series). Results of three samples were averaged for percent weight (%wgt) of the solute in solution (carbon 43.88 %wgt; nitrogen 1.54 %wgt).

Table 1. Sample sizes for greenhouse nutrient experiment. Sample sizes of experimental plants. Number of individuals is the total number of plants from a given population. Initial harvest refers to the 99 plants that were sampled at time=0 to allow for calculation of allometric relationships. All populations experienced 25 unique treatment combinations.

Population	Maternal lines	Individuals	Initial Harvest	Replicates per treatment
BLANDY	5	150	24	2 to 9
NC	11	330	55	7 to 15
SKY	4	120	20	1 to 8
Totals:	20	600	99	

Transplanted individuals were given ten days to acclimate before initiation of the experiment. Five individuals from each seed pod were randomly selected for an initial destructive harvest, leaving 25 individuals for the remainder of the experiment. One of the 25 unique treatment combinations (below) was then randomly assigned to each plant. Any fallen leaves were removed daily to avoid build-up of detritus within pots.

We used a fully factorial design in which five levels for both nitrogen (ammonium nitrate: $(NH_4)(NO_3)$); FisherScientific # S25172) and phosphorus (calcium dihydrogen phosphate: $Ca(H_2PO_4)_2$; SCBT #Sc-252539A) were combined to create 25 unique treatment applications. To ensure that we did not measure the effect of potassium limitation, potassium (potash) was kept constant for all solutions. Fertilizer was applied twice over the course of the 78-day experiment (Supplemental Table 1). Our first application occurred on day 22, one day after the initial harvest on June 5th. The second application occurred on day 67 (July 27th).

Nutrient Addition: Concentration of nutrients were estimated from agricultural recommendations; the recommended nitrogen application is 190kg/hectare (7th Edition MAFF RB209, 2000). Assuming penetration depth of 0.1 meter, this equates to 0.19 g of nitrogen per liter of soil. We established five levels of nitrogen with final concentrations of 0, 0.18, 0.35, 0.70, and 1.40 g/L soil added after two treatments. Phosphorus levels were 60% of that of nitrogen, with final concentrations of 0, 0.10, 0.21, 0.42, and 0.84 g/L soil added after two treatments. Potassium was kept constant at a final concentration of 0.30 g/L soil.

Plant phenotyping: Plant height was measured in centimeters from the soil surface to the top of the shoot apical meristem. Longest leaf length was measured in centimeters from the tip of the leaf to the top of the petiole. Leaf number was counted and leaves shorter than 1 cm were disregarded. Stem diameter was measured with a digital caliper where the stem met the soil. The age of plants at each measurement date is shown in Supplemental Table 1.

Determining allometric relationships: To determine allometric relationships between above and below ground biomass, as well as linear measurements (i.e. height, leaf length, leaf number and stem diameter) and biomass (Supplemental Table 2), five plants per pod (n=99; one individual was lost) were harvested on June 6th (22 days old). For each individual, we measured both the above and below ground biomass as well as height (cm), total number of leaves greater than 1 cm, average length of the four largest leaves (cm), and the stem diameter (mm). Plants were cut were the stem met the soil to separate above and below ground biomass. Roots were washed and all material dried in ovens at 60 degrees Celsius for 48-72 hours. Above ground tissue was also dried in the same conditions before final dry mass was recorded.

Statistical analysis for short-term consequences of nutrient addition on *A. syriaca*

To predict the initial biomass and other allometric relationships (leaf length, leaf number, stem diameter, and plant height) of all 500 plants, we created a linear model of above and below ground biomass from the 99 plants that were initially harvested. Explanatory variables were included in these regressions if they increased the explanatory power of the model. Variables were evaluated for multicollinearity using variation inflation factors (VIF; Supplemental Table 2). VIF values provide an index of how much the variance of an estimated regression coefficient is increased due to collinearity. The models that explained the most variation in above and belowground biomass of harvested plants were then used to predict the initial biomass of the remaining 500 plants.

Nitrogen and phosphorus additions were treated as continuous variables and their impact on the final change in milkweed growth was evaluated. Measurer, site, and seedpod were treated as random effects and linear mixed effect models were created for biomass, height, stem diameter, leaf length, and leaf number by nitrogen and/or phosphorus levels. Stepwise regression was used to produce the minimal adequate model then compared each model to a null model with no predictors using a likelihood ratio test. A summary of the final, fitted models for each response variable can be found in Table 3.

Table 2. Summary of linear mixed effect models for the final change in all response variables. Backward stepwise selection from the full model - which included nitrogen (N), phosphorous (P) and the interaction of N and P - was performed to determine the most parsimonious model that described the final change in all response variables by treatment. Population and measurer were included as random effects (except in the cases of belowground biomass and leaf number, where only population was a random effect because it resulted in a lower AIC). To compare between slopes (β) across different phenotypic measurements, we standardized β for nitrogen treatments by dividing by the mean trait value (β_s). Final change in a response variable (Δ) was calculated as the final value (on August 1st) minus the initial value (on June 8th). ΔABOVE refers to change in aboveground biomass, and ΔBELOW to belowground biomass.

	Δ Height	**Δ Stem Diameter**	**Δ Leaf Length**	**Δ Number of Leaves**	**Δ ABOVE**	**ΔBELOW**
N	$\beta = 9.8 \pm 1.5$	$\beta = 1.2 \pm 0.10$	$\beta = 1.6 \pm 0.21$	$\beta = 3.2 \pm 0.81$	$\beta = 4.1 \pm 0.32$	not included
	$\beta_s = 0.17$	$\beta_s = 0.20$	$\beta_s = 0.14$	$\beta_s = 0.16$	$\beta_s = 0.38$	
	t = 6.4	t = 11	t = 7.6	t = 3.9	t = 13	
	$P < 0.001$	**$P < 0.001$**	**$P < 0.001$**	**$P < 0.001$**	**$P < 0.001$**	
P	$\beta = 2.2 \pm 2.6$	not included	not included	$\beta = -0.7 \pm 1.4$	not included	$\beta = -1 \pm 0.57$
	t = 0.87			t = -0.51		t = -1.7
	$P = 0.390$			$P = 0.610$		$P = 0.082$
N*P	$\beta = 2.5 \pm 3.5$	not included	not included	$\beta = 4.2 \pm 1.9$	not included	not included
	t = 0.72			t = 2.2		
	$P = 0.470$			**$P = 0.027$**		

RESULTS

What is the long-term consequence of fire and grazing on milkweed abundance across large spatial scales?

Across watersheds at KPBS, eight different milkweed species were observed over the 27-year study period (Figure 1). In general, *Asclepias* cover increased over time in watersheds that were burned but not grazed (average $\beta = 2.546e^{-05}$, $p < 0.001$), except for *A. viridis* ($\boldsymbol{\beta} = -1.157e^{-04}$, $p < 0.001$). Burning in the absence of grazing did not affect abundance for several species, including *A. tuberosa* ($p = 0.347$), *A. stenophylla* ($p = 0.271$), and *A. viridiflora* ($p = 0.320$). In watersheds that were both burned and grazed, seven of the eight species decreased in cover. When grazers were present in watersheds that were not burned, cover increased for all species except for *A. verticillata* ($\boldsymbol{\beta} = -1.621e^{-04}$, $p < 0.001$) and *A. syriaca* ($\boldsymbol{\beta} = -4.218e^{-05}$, $p < 0.001$). In the absence of both burning and grazing, only *A. viridis* ($\boldsymbol{\beta} = 0.445$, $p < 0.001$) significantly increased in cover. Collectively, then, *Asclepias* species share broad patterns in response to burning without grazing but exhibit more species-specific patterns with other combinations (Figure 1).

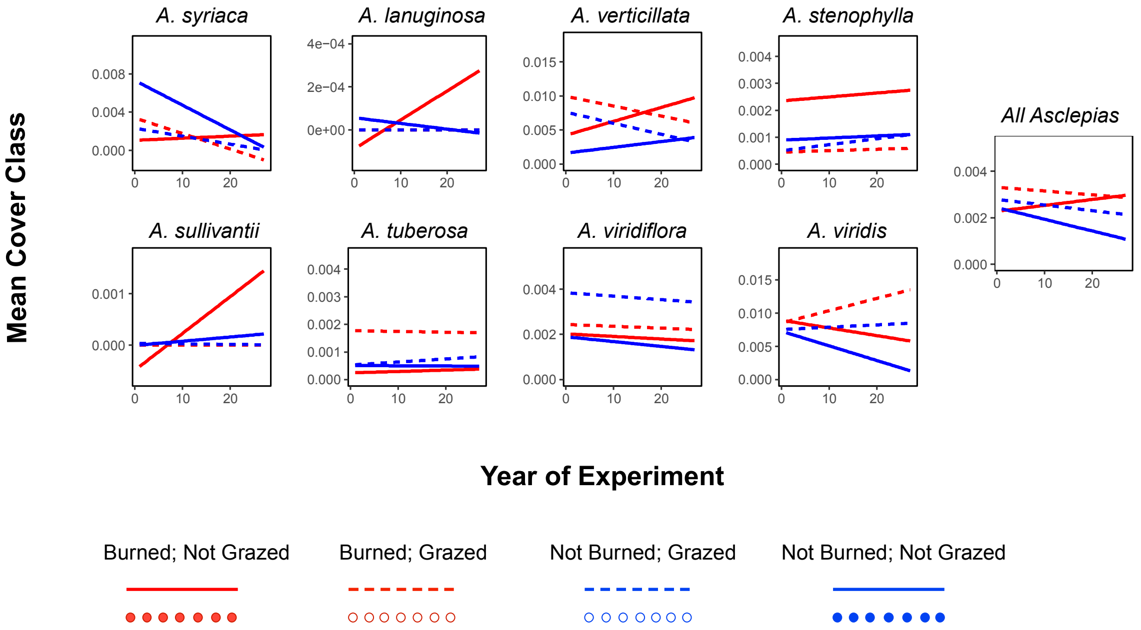

Figure 1. Long-term effects of burning and grazing for eight *Asclepias* species at Konza Prairie watershed plots. Results of mean cover class for experimental plants without nutrient addition. Lines depict the means, and grey bars represent 95% confidence intervals. Cover class could not be calculated for *A. lanuginosa* in either of the grazed watersheds, as this species was not found at all in these plots. Intercepts reflect differences in starting cover class at time zero; these may not reflect actual responses to treatments. Burning without grazing increases mean cover class over time for all species except *A. viridis* and *A. viridiflora*. For plots that were not burned or grazed, all but *A. verticillata* and *A. stenophylla* experience a decrease in mean cover class over time. *A. tuberosa* was the only species to consistently have a relatively high coverage when both burned and grazed (red dotted line).

Specifically, grazing appeared to have the most pronounced effect on *A. verticillata*, such that cover switched from a positive trend to a negative trend once grazing was introduced. The opposite relationship was seen in *A. viridis*, with grazed watersheds increasing in cover, and watersheds that were not grazed decreasing in cover. *A viridiflora* exhibited a slight negative trend across all treatments; however, this species was also the only species to have the highest cover in grazed but not burned watersheds. Finally, while *A. tuberosa* had the highest cover in watersheds that were burned and grazed, the opposite effect was found in *A. stenophylla*. In fact, these two species mirror each other in respect to their incremental decreases in cover across experimental watersheds.

In burned watersheds, cover of *A. syriaca* decreased over time when watersheds were grazed (**β** = -1.621e^{-04}, $p < 0.001$) but increased slightly, though not significantly, when watersheds were not grazed (**β** = 2.180e^{-05}, $p = 0.07$). Watersheds that were not burned showed a decrease in cover over time in both grazed (**β** = -8.333e^{05}, $p < 0.001$) and not grazed watersheds (**β** = -2.582e^{-04}, $p < 0.001$) for *A. syriaca*. This negative relationship between grazing (without burning) and mean cover did not hold for *A. stenophylla*, *A. tuberosa*, and *A. sullivantii*. It is important to note that *A. syriaca* and *A. sullivantii* look strikingly similar and have been shown to hybridize (Klips and Culley, 2004), indicating that there is some potential of mis-identification on either of these species. Observer codes were not available for these data, so we were unable to examine any significant effects of individual observers.

Several species, including *A. viridis* and *A. viridiflora*, did not show significant differences between burning treatments when plots were not grazed (Figure 1). When plots were grazed, however, burning treatment had opposite effects on these species. While *A. viridis* showed a positive trend when burned and grazed, *A. viridiflora* did not. Four of the eight *Asclepias* had the highest initial cover in burned and grazed plots, including *A. viridis*. However, this species was the only species to maintain this pattern at the end of the 27-year experiment (Figure 1).

What is the long-term consequence of fire and nutrient application in natural experimental treatment within nutrient application plots?

Within the nutrient addition plots at KPBS, there were six *Asclepias* species observed from 1988 to 2016 (Figure 2). For four of these six species, cover class declined over time in both burned and not burned plots. Exceptions to this are *A. syriaca* and *A. sullivantii*. For *A. syriaca*, cover decreased over time in plots that were not burned (p = $2.859e^{-06}$) but did not significantly increase or decrease in burned plots (p =0.1226). The only significant increase in *A. syriaca* cover, then, was in plots that experienced nitrogen addition (Figure 3A; Tukey Kramer Test: F(3,778) = 4.43, p = 0.005). Post-hoc multiple comparisons showed that the only significant difference in cover was between addition of nitrogen and the control plots in the presence of burning (p = 0.042). There was no significant difference between any nutrient treatments when the plots were not burned.

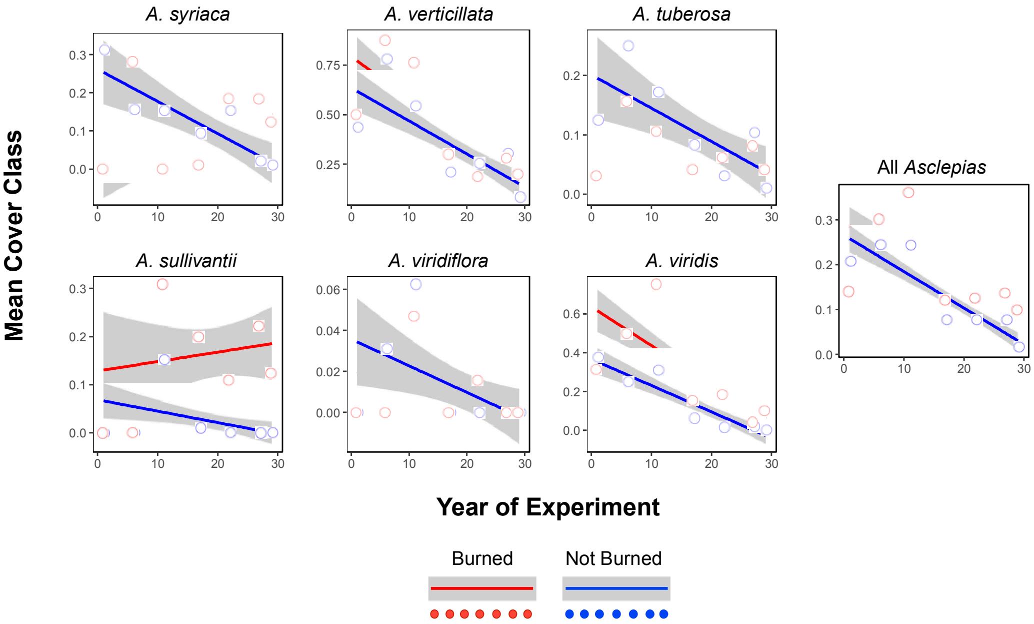

Figure 2. Mean cover class over time for six *Asclepias* species in nutrient addition plots at Konza Prairie in response to burning. All plots include the addition of both nitrogen and phosphorous. Lines indicate the mean cover class and grey bars depict 95% confidence intervals *A. syriaca* cover increases with burning but decreases in the absence of fire. All other species show decreases in cover in both conditions. Intercepts reflect differences in starting cover class at time zero; these may not reflect actual responses to treatments.

What is the effect of nutrient application to milkweed growth in the absence of fire and biotic interactions?

Nitrogen showed a significant positive effect on the growth in all phenotypic variables except belowground biomass (Table 3). As a proportion of mean trait value, nitrogen had the largest effect on aboveground biomass. Belowground biomass appeared to increase linearly with added nitrogen until nitrogen levels exceed 0.69 g/L soil (Supplemental Figure 1, panel F), which could explain why it had no significant effect in the minimal adequate model. When the highest nitrogen level was excluded from the data and model selection was repeated, nitrogen, phosphorus, and their interaction all entered the minimal adequate model (cover = 9.2 + 0.99N - 1.3P + 2.0N*P), although none of these effects were significant.

Nitrogen appeared to be the main driver of plant growth; none of the variables were significantly affected by phosphorus. Overall, plants with higher levels of nitrogen grew taller, developed thicker stems, and produced more leaves that were longer than those in lower treatment levels (Supplemental Figure 2), while phosphorus had no impact on plant growth (Supplemental Figure 1). Finally, increased nitrogen availability allowed for longer duration of plant growth; however, the rate of this growth did not appear to be affected by nitrogen treatment (Supplemental Figure 3).

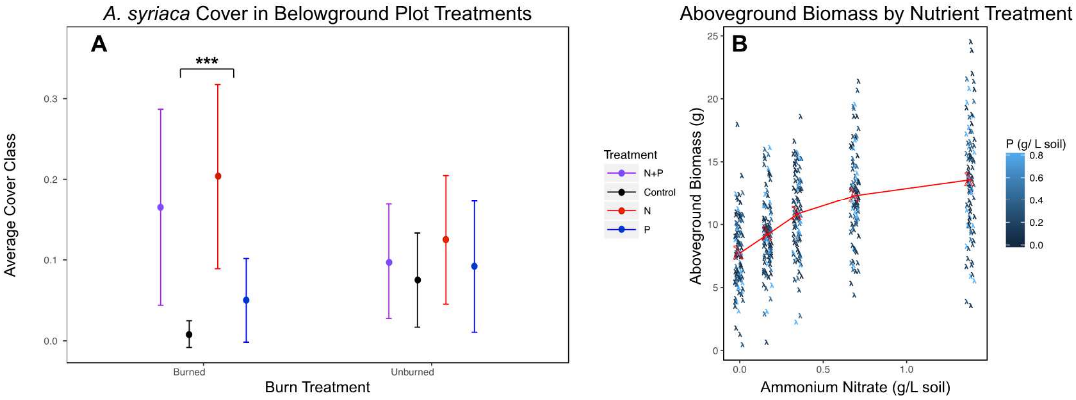

Figure 3. *Asclepias syriaca* responses to burning, nitrogen (NH_4NO_3), and phosphorous [$Ca(H_2PO_4)_2$)] treatments. (A) *Asclepias syriaca* cover at Konza Prairie in nutrient and burning treatment plots. Cover was averaged across the 27-year study. Asterisks indicate significant differences among treatments ($p < 0.001$, “***”). Nutrient applications without burning do not change the average cover class of *A. syriaca.* In the presence of burning, the addition of nitrogen alone increases *A. syriaca* cover. (B) In the greenhouse, above ground plant biomass of *A. syriaca* is positively related to amount of nitrogen added. The amount of phosphorus added (blue) did not influence aboveground biomass (Table 3).

DISCUSSION

Conversion of the native tallgrass prairie to row-crop agriculture has had a massive impact on tallgrass prairie species, including milkweed. However, we have little information of how milkweed respond to fire, grazing, and nutrient application within this native ecosystem. To address the effects of these forces on milkweed abundance we utilized two long-term datasets and a controlled greenhouse experiment.

At KPBS we found that burning increases *Asclepias* abundance overall (Figure 1); especially on *A. syriaca* when nutrients are added (Figure 2). Watershed data further indicate that two of the four non-clonal milkweeds included in this study (*A. viridiflora*, and *A. viridis*) decrease in abundance when burning without grazing was applied (Figure 1); although *A. tuberosa* and *A. stenophylla* increased at relatively low rates compared to other milkweed studied (approximately 0.05% change in cover class over 27 years). Clonal milkweed studied (*A. syriaca*, *A. languinosa* and *A. verticillata*) all dramatically increased in abundance in burned plots that were not grazed, while *A. verticillata* also increased at a similar rate in plots that were not burned (Figure 1). This pattern may nod at the idea of clonal milkweed having a higher advantage to burning regimes than non-clonal milkweed but is hardly conclusive without further study. However, this response could be especially important for milkweed with characteristically low seed set, such as *A. languinosa*, *A. stenophylla*, and *A. verticillata* (Betz and Lamp, 1992) and therefore warrants further investigation. Additionally, summer burning has been shown to provide pre-migratory monarchs fresh leaves oviposit on which otherwise would have senesced by late summer (Baum and Sharber, 2012), providing additional corridors for later generation monarchs (Baum and Sharber, 2012). Overall, burning can be an important driver to milkweed abundance over large timescales, but we found that this response can vary in the presence of large grazers for multiple milkweed species.

Grazers were found to have a negative effect on abundance for most milkweed (Figures 1 and 2). This result was surprising, as grazing has previously been shown to reduce cover of other grasses, thereby reducing competition, presumably creating a better environment for milkweed (Elson and Harnett, 2017; Knapp et al. 1999). In our case, the only species found on grazed plots that did not decrease in cover were *A. stenophylla* and *A. viridis*; the latter of which is considered to be one of the more toxic milkweed species (Malcom and Brower, 1986). A similar response was seen in *A. tuberosa*, although this species only increased in grazed plots that were also burned. The decline in *A. syriaca* abundance in the presence of grazing and burning was stronger than declining patterns observed in other *Asclepias* species studied. Overall, *Asclepias* abundance did not uniformly increase with the presence of grazers, as other forbs have been shown to do. Historically, this suggests that milkweed species, especially *A. syriaca*, may have experienced fluctuations in abundance as bison and fire moved throughout the tallgrass prairie.

In our greenhouse experiment, which did not include competition or herbivory, we predicted both nitrogen and phosphorus would have a significant effect on growth as they are both key macronutrients. Previous research has also shown that nitrogen and phosphorous-together-will increase *A. syriaca* growth ring patterns, which can be linked to above ground growth (Dee and Palmer, 2016). Here we discover that phosphorus had no effect on either above or belowground biomass. Previous research has shown (Hartnett and Wilson 1999) that colonization of arbuscular mycorrhizal fungi on *Asclepias* species increase the plants' ability to intake both nitrogen and, particularly, phosphorus. It is possible that in the absence of applied phosphorous, *A. syriaca* is able to outcompete other plants due its mycorrhizal interactions.

CONCLUSION

Historically, the tallgrass prairie was shaped by stochastic processes such as fire and roaming ungulates. These dynamic processes open up canopy cover, remove competitors, and alter levels of soil nutrients. Vast amounts of tallgrass prairie have been converted to agricultural settings, thereby changing species composition and abundance on a large scale. Croplands in particular may mimic these processes as they contain open canopy cover, low levels of competitors, and high levels of nutrients from fertilizer runoff. We found that *Asclepias* species overall increase in the presence of burning, but found alternate, species-specific responses when grazing or nutrients were applied. This implies that milkweed will respond differently to land conversion of their natural habitat. While species such as *A. viridis* may be more suited to pasture settings, others such as *A. syriaca*, *A. languinosa*, and *A. stenophylla* may do best surrounding cropland.

CHAPTER 2. FINE-SCALE SPATIAL STRUCTURE OF GENETIC, FUNCTIONAL, AND DEMOGRAPHIC TRAITS IN *ASCLEPIAS SYRIACA*

INTRODUCTION

Phenotypes are the product of genetic and environmental interactions. While the contribution of environment to phenotypic variation has been well studied in the field of quantitative genetics (Frank et al., 2017; Manel and Holderegger, 2013; Stöcklin et al., 2009), we have limited understanding of how much within population phenotypic variation is due to fine-scale environmental differences. Within a population of non-motile organisms, fine-scale environmental differences may contribute significantly to phenotypic variation. For instance, consider a population of predominately selfing wildflowers existing on a hillside. Despite the fact that this population may largely consist of a single genotype due to inbreeding, small-scale variations in water availability, sunlight duration, and nutrients may result in significant phenotypic differences due to selection pressures for structures that take advantage of higher resources (Moraes et al., 2016; Sultan, Sonia; 2000). The contribution of fine-scale environmental variation to phenotypes may be detected by quantifying their spatial structure. For example, when looking at plant height, if fine-scale environmental differences are present, tall plants might not be uniformly distributed across the population but may be clustered in localized regions. Localized (structured) phenotypes can be driven by an organisms' competitive, dispersal, and adaptive capabilities (Benot et al., 2013; Husband and Barret, 1998; Manel et al., 2003; Soininen, Janne; 2016) and therefore is a key aspect behind understanding an organisms' ability to function and adapt in the environment (Sultan, Sonia 2000).

Plants provide an excellent model to study spatial structuring due to their high degree of plasticity (Bradshaw, Amy, 1965; Sultan and Bazzaz 1993a, b) within multiple traits that are relatively easy to quantify. Broadly, phenotypic plasticity refers to the range of a phenotypic response induced by differential environmental factors (Huber et al., 1999). Populations with high plasticity are capable of exploiting a wider range of differential environmental conditions and exhibit larger variation in phenotypes within a given genotype. On larger spatial scales, this can increase the formation of specialized ecotypes, while on relatively smaller spatial scales this may result in divergence within a population (Sultan and Spencer, 2002).

Clonal plants provide a unique opportunity to examine these traits within genetically identical individuals (ramets) in relatively close proximity of each other. Use of clonal ramets creates the possibility to “remove” the influence of genetic variation in plant traits and therefore assess the relative contribution of environmental variation on phenotypic plasticity within fine-scale physical distance. If clonal individuals are not functionally similar, it stands to reason that environmental factors may play a relatively larger role in phenotypic variation. On the flip side, functional similarity in clones could imply that genotypes are relatively important. However, within fine-scale physical distance, functional similarity between clonal ramets could also be due to a lack of environmental variation. For plants that can switch between sexual and asexual reproduction, a lack of phenotypic similarity between clones may describe an ideal situation in which populations *i.* avoid the cost of sexual reproduction, *ii.* preserve some level of phenotypic variation for natural selection to act upon, and *iii.* maintain the ability to increase genetic diversity through sexual reproduction in areas of higher resources.

Although the vast majority of angiosperms can switch between sexual and asexual reproduction (Van Groenedael, J. A. N., 1997), we are still unclear on the relative role of clonal genotypes in the spatial structuring and variation of plant traits. Although there are many studies that examine population structure within the context of clonal genotypes (Cheliak and Dancik, 1982; Maddox et al., 1989; Furman et al., 2015), there are relatively few spatial structure studies that combine this genotypic data with ecological plant trait data. Those that do largely ignore intraspecific patterns (Manel et al., 2003) or patterns within fine-scale physical distance. The former is especially problematic as biological interactions are likely to occur within fine-scale physical distance (Benot et al., 2013).

Here we use common milkweed, *Asclepias syriaca* (*A. syriaca*), as a model to explore the relative role of clonal genotypes in fine-scale spatial structuring of plant phenotypes. The genus *Asclepias* consists of ~130 species that vary widely in chemical and physical defensive traits (Agarwal et al, 2015; Fishbein et al., 2018), leaf characteristics (Fishbein et al., 2018), and habitat preference (Betz and Lamp, 1992). *Asclepias* species exhibit mixed reproductive strategies with some entirely reliant on sexual reproduction and others that accommodate sexual and asexual reproduction simultaneously (Pellissier et al., 2016). *Asclepias syriaca* can both sexually and asexually reproduce. More specifically, *A. syriaca* adventitiously clones, thereby producing multiple ramets within close physical distance of each other. This establishes an excellent framework to examine how environmental and genotypic factors ultimately drive natural variation within fine-scale physical distance. Here, we use demographic, functional, spatial, and genotypic data from three *A. syriaca* populations in Virginia to ask: (1) What is the extent of clonality between and within populations? (2) How genetically structured are these populations? (3) Do plant traits exhibit spatial structuring? And (4) Are genetically identical individuals more functionally similar than unrelated individuals?

METHODS

Sample Collection and Phenotypic Measurements

In 2017, phenotypic data was collected on 565 individual stems from 12 transects across three sites in Virginia, USA -- Blandy Experimental Farms (BLD) (39°03'45.4"N 78°03'46.9"W), Presquile National Wildlife Reserve (PWR) (37°21'01.8"N 77°16'01.6"W), and Sky Meadows Park (SKY) (38°59'11.1"N 77°57'41.6"W; Figure 4). Transects were one meter wide with variable lengths, ranging from five to 36 meters (Table 3). Plants were assigned a unique identifier and individual locations of stems were recorded using a PosTex positioning system (Lindstrom, Patrick 2015). This system allowed recording of stem location to within a few centimeters relative to each other.

Figure 4. Demographic, functional, and spatial data on 565 stems from three sites in eastern Virginia. Plant tissue samples for genetic analyses were collected from a random sample of 195 of these stems. Phenotypic trait data was collected from six transects at Blandy Experimental Farms (insert), two transects at Sky Meadows Park, and four transects at Presquile National Wildlife Reserve. Tissue samples for microsatellite analysis were collected from all transects at Blandy, and a single representative transect from Sky Meadows (transect 73) and Presquile (transect 83).

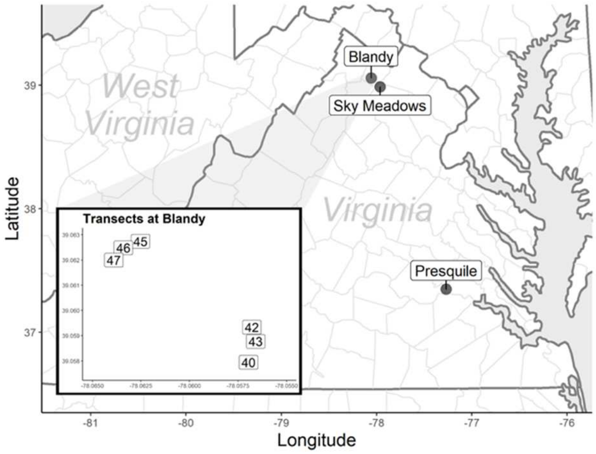

Table 3. Milkweed sampling. Transect lengths were variable between sites and were rounded to the nearest half meter for simplicity. Total stems represents sample size per transect. All transects at Blandy were used in genetic analyses (Genotyped Stems) and a single transect at Presquile and Sky were used as representative transects for genetic analyses. Site-wide sums are provided in bold next to the corresponding site name.

Population	Transect Length (m)	Date Collected	Total Stems	Genotyped Stems
Blandy	**22.5**		**249**	**135**
40	36	May 11, 2017	33	25
42	27	May 11-May13, 2017	71	29
43	19	May 11-May13, 2017	75	30
45	30	May 11, 2017	48	30
46	4	May 11, 2017	8	7
47	18	May 12, 2017	14	14
Presquile	**15**		**223**	**30**
80	9.5	May 8, 2017	33	0
81	10	May 8-May 9, 2017	33	0
82	22	May 8-May 9, 2017	45	0
83	18	May 8, 2017	112	30
Sky	**10**		**93**	**30**
72	10	May 12, 2017	38	0
73	10.5	May 12, 2017	55	30

In addition to recording physical location, demographic traits recorded in the field include apical height (cm), number of leaves, leaf width (cm), leaf length (cm), stem width (mm), and number of inflorescences. Average herbivory was measured in two steps; first, the average herbivory per leaf was recorded based on a Daubenmire scale. This score was then averaged across all leaves to produce the average herbivory score per individual stem. Additionally, toxicity of plants was quantified by measuring cardenolide composition -toxic specialized plant metabolites - using a spectroradiometer (Malcom, Stephen 1994). This instrument records the spectra of multiple compounds from leaf tissue simultaneously which can then calibrated by comparing their individual absorbance spectra with known chemical measurements (Couture et al., 2013). Validation of this initial calibration model was done by comparing observed and predicted responses. Once validated, this model can then be used to predict concentrations of plant compounds based on their unique spectral patterns (Couture et al., 2013). Percent nitrogen, carbon, chlorophyll, lignin and the carbon to nitrogen ratio were also quantified using a spectroradiometer. All spectroradiometry data were sent to Dr. John Couture (Purdue University) for final quantification.

Fine-scale phenotypic structure

Physical distances from the PosTex positioning system were then compared to functional distance using mantel correlations. Before calculating functional distance, we first identified collinear traits using a PCA (Figure 6). Highly correlated traits (number of leaves, stem width, cellulose, fiber, percent carbon, percent nitrogen, carbon to nitrogen ratio) were removed from all analyses. Further, any traits that were considered to be highly correlated with environmental conditions (Normalized Difference Water Index) were also removed. All remaining traits, except for average herbivory and number of inflorescences, were then z-transformed and combined into a composite score that described either vegetative (apical height; Leaf Mass Area; chlorophyll) or defensive (cardenolide composition; percent lignin) investment. Here we use “vegetative” traits to characterize direct vegetative growth (apical height; leaf density) but also use percent chlorophyll as a proxy for the ability of a plant to maintain this growth via photosynthesis. For defensive traits, chemical defensive capability (toxicity) was combined with a physical defense in the form of percent lignin. Plants with higher lignin content are less digestible which may act as a deterrent to herbivores (Benot, M-L., 2000; Cornelissen et al., 2004) and is considered to be a "first line of defense" against invasion from microorganisms (Moura et al., 2010). We were unable to collect spectroradiometry data from two transects at Blandy (Transect 40 and 42) as well as a single transect from Presquile (Transect 80). Additionally, we were unable to collect spatial data for Transects 72 (Sky), 81 and 82 (Presquile). Subsequently, these transects were not included in any normalized responses. Data from a total of six transects and 95 stems were then normalized. Linear regressions were used to determine potential tradeoffs between defensive investment, vegetative investment, and average herbivory. Additionally, investment in sexually reproductive structures (number of inflorescences) was also examined as a binary response where plants without sexual reproductive structures were scored as zero and plants with at least a single inflorescence scored as one. These responses were then log transformed and compared against normalized traits and average herbivory scores to determine if sexual reproductive investment correlated with other plant traits across all sites.

Microsatelite analysis

From the 565 stems where phenotypic data leaf tissue was collected 195 of these individuals were used for genotypic analyses. Tissue samples were stored with silica beads in coolers upon collection and placed in -80° Celsius within 12 hours thereafter. DNA extractions were performed following the MyTaq™ Plant-PCR Kit (BIOLINE: catalogue number BIO-25056), which combines DNA extraction and amplification into a single-step process. In short, we used 1mm disks of plant tissue, submerged disks in liquid nitrogen for 30 seconds, and disrupted using a mini pestle and centrifuge tube. After disruption, SDS buffer was added into each tube and incubated at 95°C for 5 minutes. Individuals were amplified using seven unique primers designed by Kabat et. al (2010; Supplemental Table 1). PCR protocol included 32 to 35 cycles of: denaturing at 95° C for three minutes, primer specific annealing (Supplementary Table 1) for one minute, and a 45 second elongation (68° C) followed by a five-minute (68° C) extension step.

Assigning multi-locus genotypes

Fragment analysis was performed at Cornell Institute of Biotechnology using a Liz 500 internal size standard (ThermoFisher Scientific: catalogue number 4322682). Genotypes were called using the program Geneious (Drummond et al., 2011). Once fragments sizes were called, these raw genotypes were then standardized such that each allele (per locus) was divisible by three as all primers were trinucleotide repeats. In short, “true” allele sizes were initially established as those that were only found in multiples of three from each other (*e.g.* 120 and 123) and also at high frequency. Here, “high frequency” calls refers to allele sizes that scored at least twice as much as any other potential call. These calls where then used to round the remaining allele sizes such that they were in accordance with a “true” fragment size. This was done by rounding each call by +/- 1.5 base pairs from the lowest “true” allele to the highest. For example, in the instance that two alleles (at a single locus) had been called as 121.3 and 122.4, and the “true” size had been determined as 120, allele 121.3 was rounded down to 120 and 122.4 was rounded up to 123.

Multilocus genotypes were assigned using the allelematch package in R Studio (Galpern et al., 2012; Team R Studio version 3.5.1). To avoid over or under estimating the total number of genotypes present, this package first estimates the optimal number of genotyping errors that should be allowed (Galpern et al., 2012). This involves a multi-step process: first, allelematch computes a similarity matrix that is initially based on pairwise comparisons of standardized genotypes. When these genotypes are exactly matched (no missing or mismatched alleles at any locus) these pairs are given a score of 1. If missing data is present, these individuals are then penalized by 1/2N for every mismatch (where N equals the total number of alleles). Further, when alleles do not match, this package further penalizes these scores by 1/N. Therefore, if any two individuals have a single allele that does not match, this score would be reduced to 1-1/N. If these same individuals have a single missing allele and also have missing data at a single locus, their score would be [1 – 1/N – 1/2N].

Once the similarity matrix is calculated, the next step in assigning individuals to genotypes is to determine the optimum number of genotyping errors to allow. This is done through the "amUniqueProfile" call which initially calculates the total number of unique genotypes under the assumption that there are no genotyping errors. However, if genotyping errors were present, this would greatly overestimate the true number of genotypes in the population. Therefore, successive steps in this process are necessary to estimate the "true" number of unique genotypes. To find this optimum number, the "amUniqueProfile" call will iteratively allow for n + 1 number of genotyping errors (with n equal to the number of mismatches allowed), up to N -1 loci (where N is equal to the total number of loci used). For example, when running this function on our data, we had 175 unique genotypes when no mismatches were allowed, 170 when one mismatch was allowed, and 140 unique genotypes when two mismatches were allowed (Supplemental

Figure 1). However, by allowing individuals to mismatch, even at a single allele, this creates the possibility of an individual “matching” across multiple genotypes. These individuals are flagged as “multiple matches” by the final output. The optimal number of genotyping errors to allow, then, is found where the number of multiple matches equals - or approaches- zero. Based on this estimation, we allowed for no more than two genotyping errors when assigning individuals (Supplemental Figure 4). Finally, 19 individuals that were classified as multiple matches were removed from analyses as any individual cannot in reality possess two different genotypes.

Once the optimal number of genotyping errors was determined individuals were assigned to genotypes using the maximum linkage hierarchical clustering method as implemented by allelematch (Galpern et al., 2012). In short, this method first combines individuals that have the highest similarity score into a single cluster. The (genetic) distance between this clustered pair and the remaining (unclustered) individuals is then used to recalculate a secondary matrix by finding the maximum distance between the clustered pair and all remaining individuals. This secondary matrix is then filled with a new set of scores that are based on the maximum distance between the originally clustered individuals (with the highest similarity scores described previously) and the remaining unclustered individuals. The process is then repeated, with a new cluster created between the most similar individuals from the secondary matrix, and a tertiary matrix created based on the maximum distance between the secondary cluster and the remaining (unclustered) individuals. This process continues until no new clusters can be formed. Finally, to ensure that we used enough microsatellite loci to obtain the maximum number of genotypes present, we generated rarefaction curves using GenClone (Arnaud-Haond et al., 2007; Supplemental Figure 5).

Fine-scale genetic structure

Mantel correlations were used to examined the relationship between genotypes and fine-scale physical distance. Physical distance matrices were created using Euclidean distance between ramets from the PosTex positioning information. Genotypic distance matrices were computed using the allelematch package (above; "Assigning Multi-Locus Genotypes") which accounted for both missing data and genotyping error. Mantel correlations were calculated using these distances matrices to determine if genotypes were correlated with physical distance (genetic structure).

Determining functional similarity between individuals

In order to determine if clones were more functionally similar than unique individuals, we preformed pairwise comparisons between "unique" individuals and clonal individuals. Here, "unique" individuals were comprised of any two randomly chosen individuals that were not assigned to the same genotype, while clonal individuals were any two randomly chosen individuals that were assigned to the same genotype. To account for disproportionate sample size the mean of an initial subsample of 56 non-clonal responses (equal to clone sample size) was calculated and compared to the mean of clonal individuals using an ANOVA. This process was repeated 100 times to create a posterior distribution of the significance values between ANOVA's. The mean of this posterior distribution was reported for each trait.

RESULTS

How clonal are populations of common milkweed?

First, to determine the extent of intrapopulation clonality within common milkweed, individual ramets were assigned to genets before spatial positing data was used to find the probability of two stems being a clonal pair as a function of physical distance (Figure 5). The proportion of clonality was calculated as the number of clones (shared genotype) divided by the total number of stems within incremental two-meter bins. This physical distance ranged from less than two meters to nearly 30 meters long. Overall, this probability was relatively low (less than 8% on average), even within relatively small distances (two meters or less). That said, there was still variation between sites. For example, some transects had only two clones (Transects 46 and 83), while others had ten or more (Transect 45 and 73; Table 4). The majority of clones were found at least three meters apart, but this intraclonal distance was highly variable and ranged from just over one meter to nearly 15 meters. The average intraclonal distance of Blandy was approximately eight meters; nearly eight times the distance of clones within Presquile and almost twice that of Sky Meadows. Despite relatively low interclonal distance, Sky Meadows had the highest proportion of clones overall (61%; Table 4).

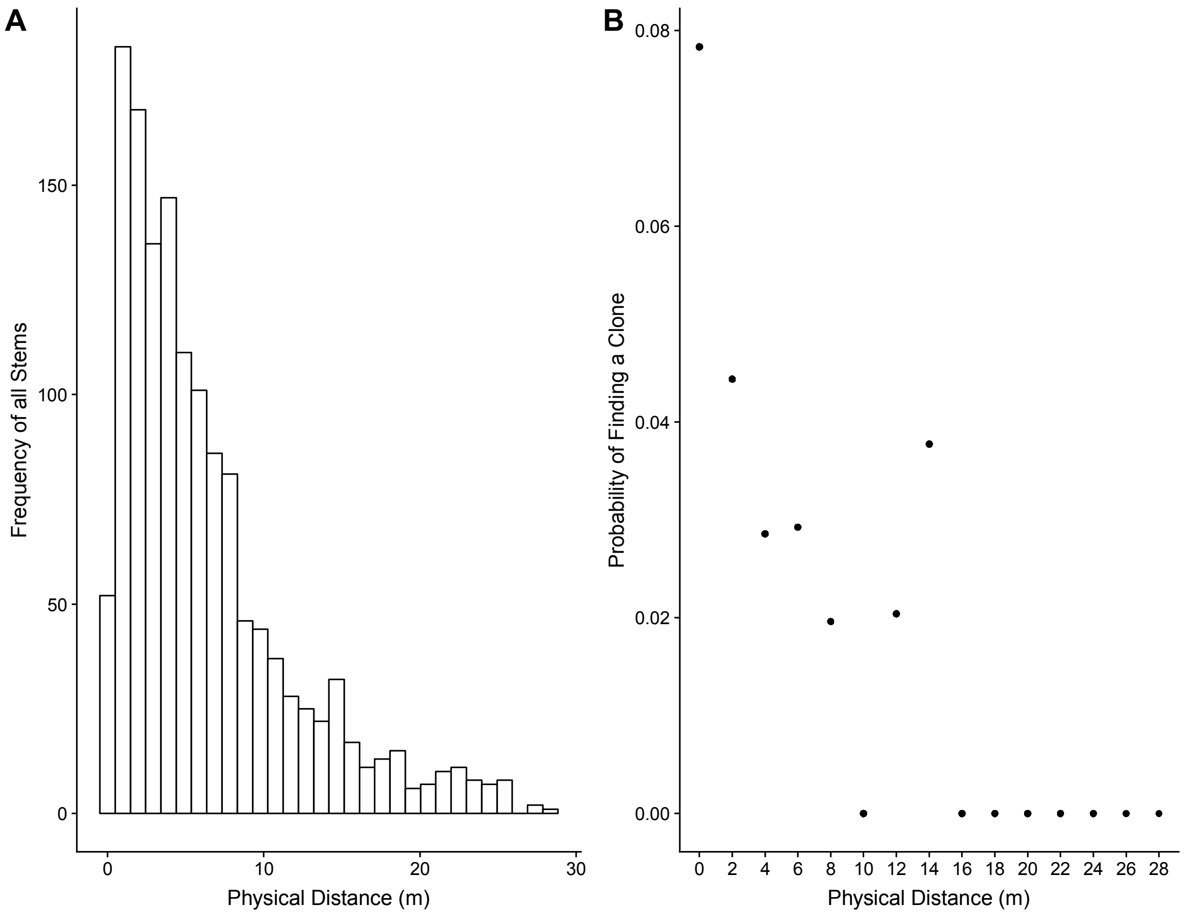

Figure 5. Sampling scheme and probability of finding a clone within fine-scale physical distance. Frequency of all stems throughout all three sites shows that the majority of samples were collected within 10 meters or less of each other (A). The probability of finding a clone versus physical distance was surprisingly low even when ramets were sampled from zero to two meters apart from each other (B). Clones were not found with more than 16 meters of physical space between them. However, there was still a relatively low probability of finding clones with 12 to 14 meters of space between them (2 – 4% respectively).

Table 4. Summary of clonality per transect. All transects exhibited significant genetic structure (significant M_R correlations in bold) regardless of the proportion of clonality (Prop. Clones). The probability of finding a clone within a given transect was determined by dividing the number of shared genotypes (#Clonal Ramets) by the total number of stems (#Total Stems) per transect. All remaining samples were assigned to unique MLG's (#Unique Stems). Transects with relatively high proportions of clones did not necessarily have shorter or larger distances between clonal ramets. Instead, the maximum distance (Max Length) and standard deviation of the distance (SD Length) between clonal ramets was highly variable.

Population	Mantel (M_R)	#Total Stems	#Unique Stems	#Clonal Ramets	Prop. Clones	Max Length	SD Length
Blandy		18.3	13.5	4.8	0.326	8.236	2.895
40	**0.252**	21	17	4	0.190	7.304	2.543
42	**0.243**	22	17	5	0.227	7.753	2.768
43	**0.213**	27	23	4	0.148	8.200	0.196
45	**0.355**	24	14	10	0.417	8.068	2.195
46	**0.411**	5	3	2	0.400	3.151	NA
47	**0.298**	11	7	4	0.364	14.939	6.773
Sky							
73	**0.245**	23	9	14	0.609	3.647	0.849
Presquile							
83	**0.244**	8	6	2	0.250	1.160	NA

How structured are genotypes within populations?

Although clonal genotypes were not always found in close physical proximity to each other (two meters or less; Table 4), there were still strong spatial and genotypic correlations ($p < 0.001$ to 0.026). The strength of this correlation was largely similar between transects (M_R ~0.25), with Transects 45 and 46 as potential exceptions to this. Regardless, higher mantel correlations did not result in noticeable marked differences between interclonal length as might have been expected.

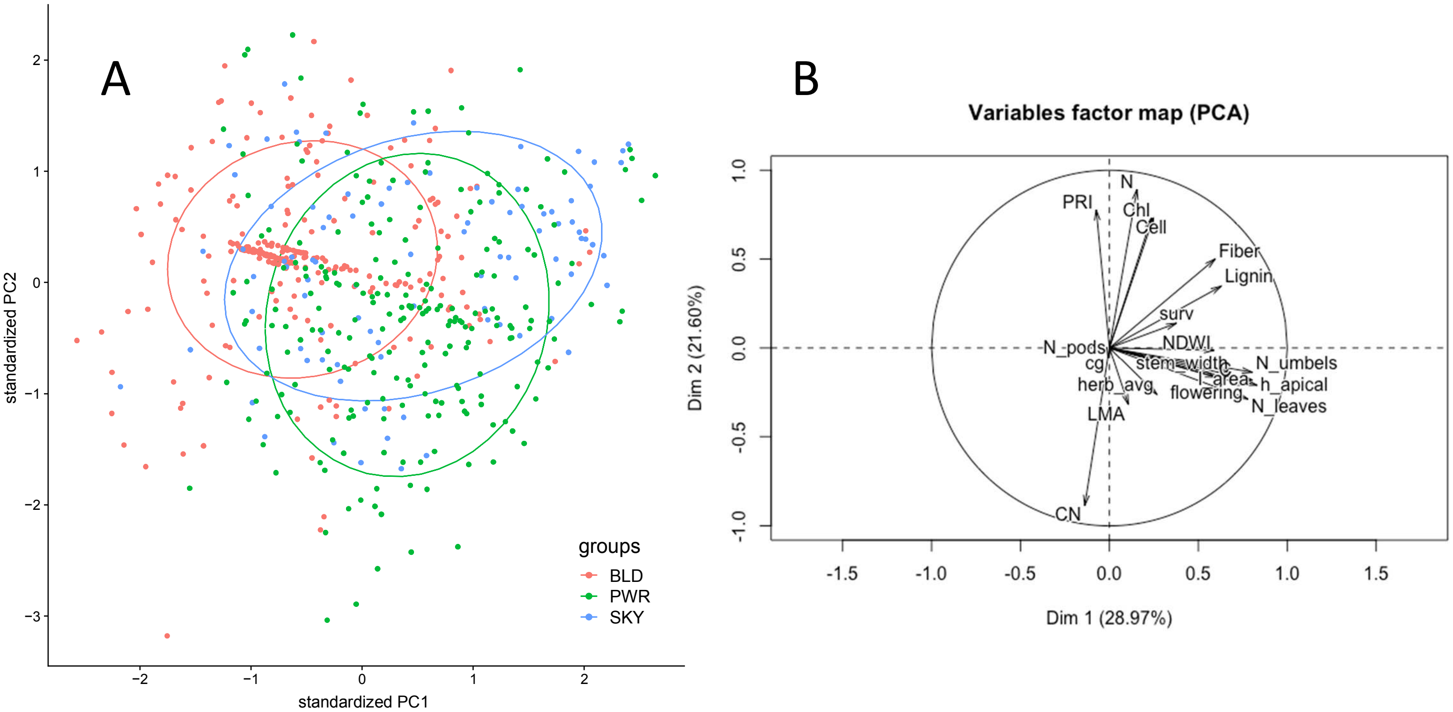

Figure 6. PCA were used to identify highly collinear traits. Overall, (A) there was not a strong signal of differentiation between sites when all traits were considered. (B) The number of leaves, stem width, cellulose; fiber, percent carbon and percent nitrogen were all highly correlated and were therefore removed from future analyses.

Are plants from different sites phenotypically similar?

A Principal Coordinate Analysis was used to examine plant traits between all three populations of *A. syriaca.* When including all traits, no strong signal of population differentiation in plant traits between sites was evident (Figure 6). Post hoc Tukey-Kramer tests of composite scores revealed that plants from Blandy did not invest as much in defensive, vegetative, or sexually reproductive traits as plants from Presquile or Sky Meadows (Figure 7; Table 3). Individuals from Sky had the highest investment in sexual reproduction but did not differ from Presquile in either defensive or vegetative traits. Out of all sites plants from Presquile experienced the most herbivory, yet this did not seem to affect their defensive or vegetative investments.

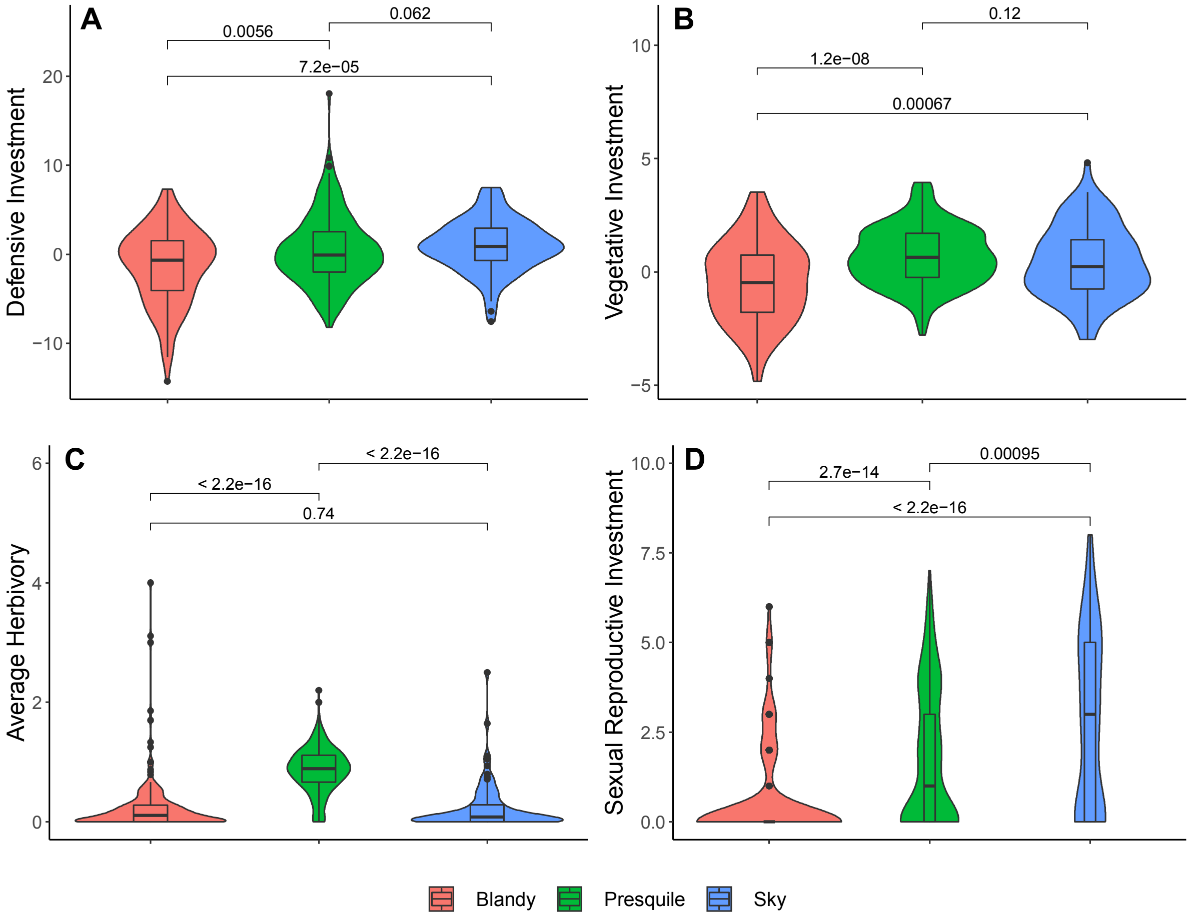

Figure 7. Composite defense and vegetative investment, as well as herbivory intensity and sexual reproductive investment, show significant differences between populations. Plants exhibited significant differences between defensive investment (A), vegetative investment (B), average herbivory (C) and sexual reproductive investment (D) between sites. Plants from Blandy invested less in defensive, vegetative, and sexual reproductive structures compared to other sites. Additionally, these plants experienced less (average) herbivory when compared to Presquile, but not Sky. Individuals from Presquile experienced the most herbivory. In terms of reproductive investment (number of inflorescences), all sites were significantly different, with Sky Meadows exhibiting the highest reproductive investment overall.

Table 5. Post-hoc Tukey tests reveal that sites differ in respect to plant characteristics. Defensive characteristics (cardenolide composition; percent lignin) and vegetative characteristics (apical height; Leaf Mass Area; percent chlorophyll) were z-transformed and summed to create composite scores. Herbivory was calculated based on a modified Daubenmire scale (Blair, John; 2016) per leaf and were then averaged by plant to produce a quantitative assessment of total herbivory. Reproductive investment is characterized by the total number of inflorescences per plant and included both premature and senescing structures. Values in bold represent significant mantel correlations between traits and physical distance.

Population	Defensive	Vegetative	Average Herbivory	Reproductive
Blandy - Presquile	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Blandy - Sky	**< 0.001**	**< 0.001**	0.967	**< 0.001**
Presquile - Sky	0.545	0.374	**< 0.001**	**< 0.001**

Within a site, are plants from different transects phenotypically similar?

Of the four scores examined (defensive, vegetative, average herbivory, and sexual reproductive investment) there were few transects that had spatial structuring (Table 4). Defensive investment and sexual reproductive investment were structured at two of the six transects, but only a single transect (47) was spatially structured in both traits. While transect 46 had the strongest correlation between defensive traits and space (M_R = 0.66) transect 47 showed structure in all characteristics except for average herbivory. In fact, average herbivory never correlated with space regardless of the site. Overall, transects at Blandy had the most spatial structuring of traits, while Presquile did not show structuring in any traits.

Table 6. Summary of fine-scale phenotypic spatial structuring across all three sites. The extent that plant traits correlated with fine-scale physical distance were determined using mantel correlations (cells). Significant mantel correlations are shown in bold. For the six transects in which we had spatial and spectroradiometry data for, half of these transects never showed spatial structuring in any trait (Transects 43, 45, 83). Overall, Blandy showed the most spatial structure. Plants at Presquile did not show spatial structuring regardless of the trait, while plants from Sky were only structured in their sexual reproductive investment. Herbivory intensity (Avg. Herbivory) was variable across space and did not correlate with physical distance on any transect.

Transect	Defensive	Vegetative	Reproductive	Avg. Herbivory	% Structured Traits
Blandy (43)	-0.045	-0.05	-0.03	-0.05	0
Blandy (45)	0.08	0.16	-0.10	-0.08	0
Blandy (46)	**0.66**	0.59	0.43	-0.16	25
Blandy (47)	**0.38**	**0.57**	**0.55**	0.08	75
Sky (73)	0.16	-0.02	**0.18**	0.03	25
Presquile (83)	0.33	-0.05	-0.09	0.02	0
% Structured Transects	33	17	33	0	

<u>Do we find evidence of tradeoffs between growth, reproduction, and defense?</u>

Across all populations, we tested for tradeoffs between growth and defense by looking for relationships between composite scores of these variables. There was no evidence of tradeoffs (negative correlations) between normalized traits. Instead, defensive and vegetative traits are all positively related (Figure 8). Next, we tested for tradeoffs between composite scores and sexual reproduction and herbivory. Again, when all plants were included, sexual reproductive investment was positively correlated with herbivory intensity (Figure 8).

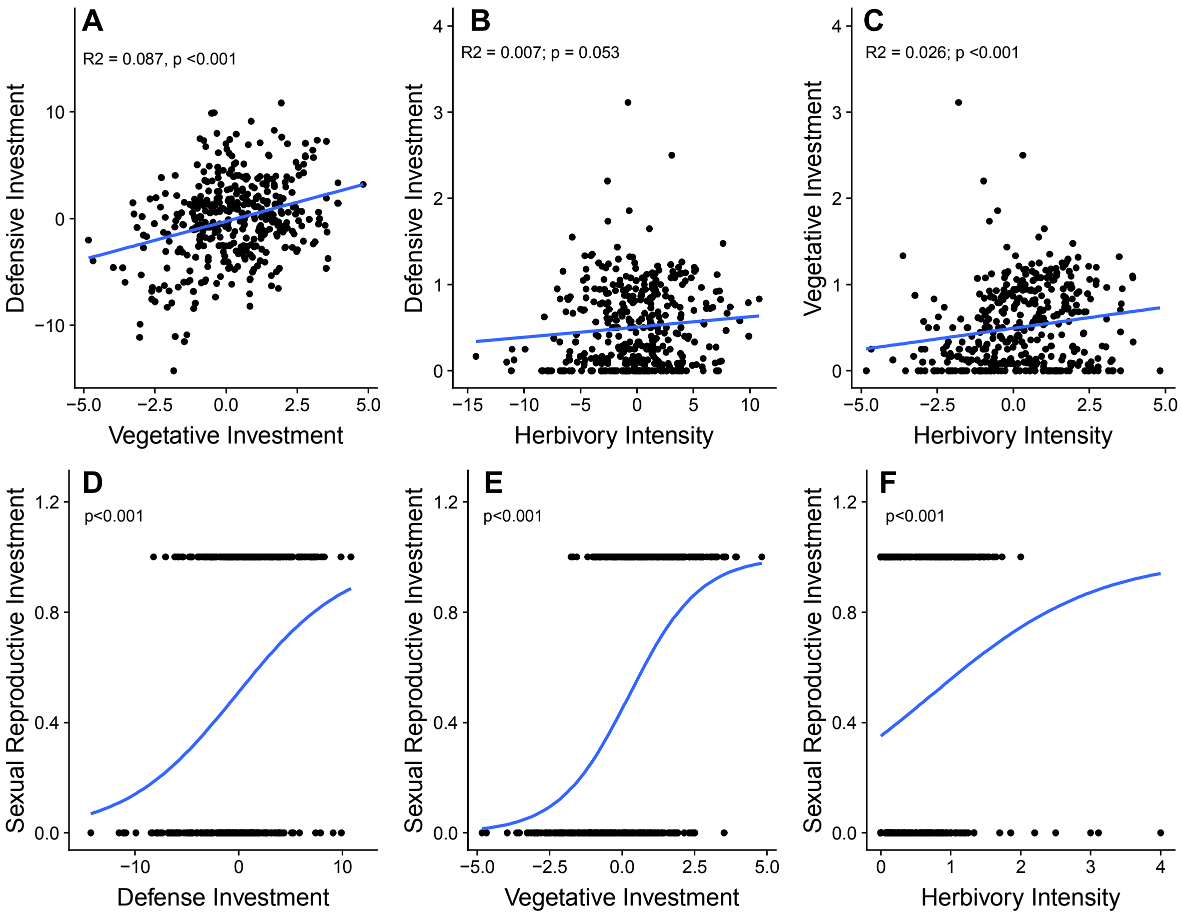

Figure 8. There is no evidence of tradeoffs between plant traits. There were no trade-offs in representative plant traits (defensive, vegetative, and sexual reproduction investment). Instead, plant traits appear to be largely correlated. The strongest of these correlations appears to be between defensive and vegetative investment (A), with marginally less correlation between average herbivory and defensive investment (B) or vegetative investment (C). Sexual reproductive investment (D-F) is also positively correlated with average herbivory and composite traits.

Are individuals that are genetically identical also functionally similar?

Given that clonal individuals share genotypes we sought to test whether clonal individuals were more similar phenotypically than unrelated individuals. This was done using ANOVA's on unique vs clonal pairs of individuals (See Methods). Although these individuals share identical genotypes, clones were not significantly similar in their functional traits (Figure 6).

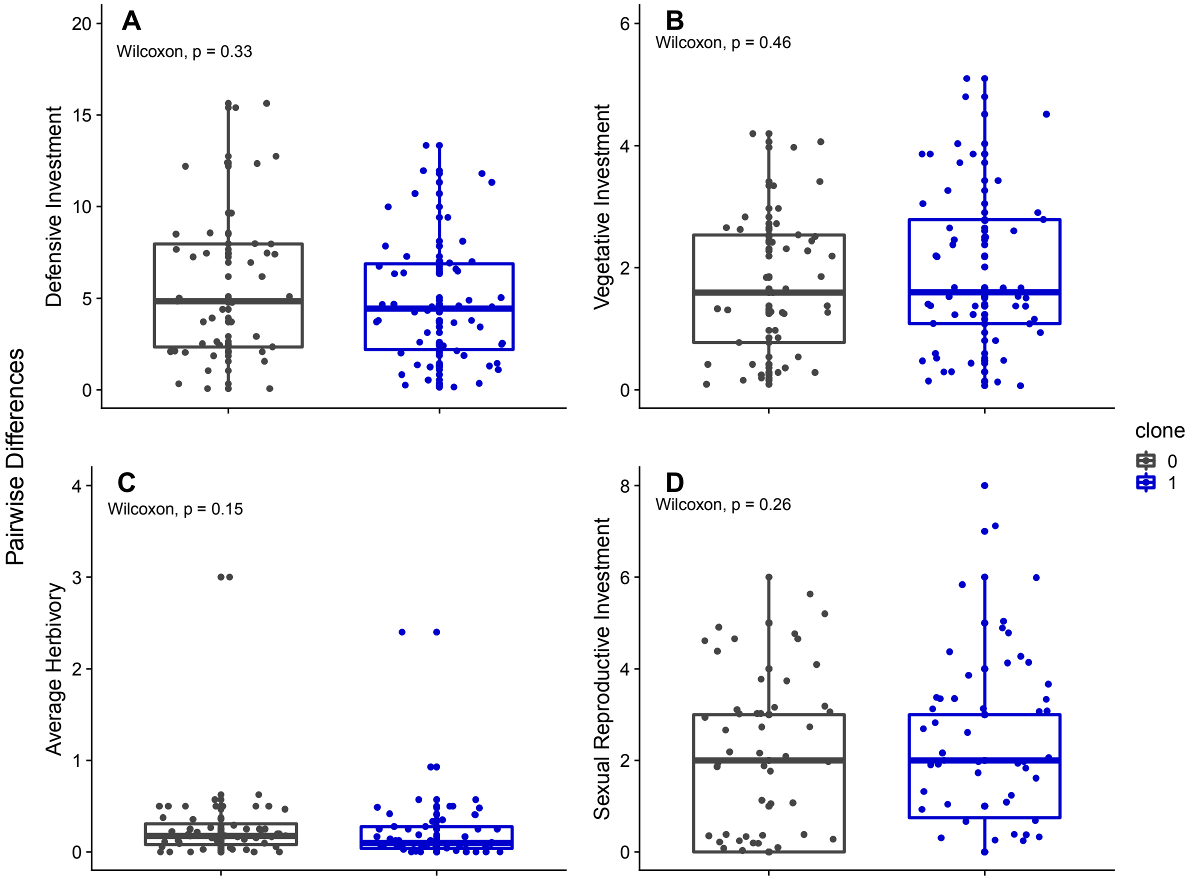

Figure 9. Clonal individuals are not functionally similar. Pairwise comparisons between individuals that do not share genotypes (0) and individuals that are part of the same genotype (1) reveal that clones are not more functionally similar in any plant trait examined. Instead, it appears that environmental variation plays a relatively larger role in functional variation when all sites are considered.

DISCUSSION

Phenotypic variation is the biproduct of environmental and genetic contributions; however, understanding the relative contributions of environment and genetics is difficult. For example, while genetically similar organisms may exhibit a wide range of phenotypic variation due to environmental factors, genetically diverse organisms may also display low levels of phenotypic variation when environments are largely homogeneous. Use of clonal organisms that produce multiple genetically identical individuals within short physical distances provides a unique opportunity to begin to parse apart the relative contribution of genotype and environment.

Increases in clonal reproduction results in both positive and negative consequences. Positively, clonal reproduction provides a sure-fire means to propagate in the absence of mates or when resources do not a favor sexual reproduction. On the downside, increased asexual reproduction lowers genetic diversity and adaptive potential. Although *A. syriaca* has been described as a highly clonal species (Wilber, Henry 1976; Bagi, Istyan 2008) our results indicate otherwise. Overall, the probability of finding a clone -even within two meters or less- was less than ten percent (Figure 5). This was surprising as *A. syriaca* clones have been considered to be within a meter or two of each other (Tecic et al., 1998). However, our findings show that the distance between clones can be much higher than this expectation; in several cases this was between eight and 15 meters (Table 4). Not all sites shared this pattern. For example, clones at Presquile and Sky (as well as Transect 46 from Blandy) were aggregated within approximately three meters of each other. When considering the high proportion of clones found within Sky Meadows (0.60; Table 4) as well the relatively high sexual reproductive investment (Figure 7), it is possible that these plants are taking advantage of higher resources areas and investing these resources in relatively costly flowering structures. This could further explain why we did not see a significant difference in overall investment of vegetative growth or defensive traits at this site.

When examining spatial structuring of plant traits, we found that all sites were significantly different in their sexual reproductive investment (Figure 7; Table 5) but not necessarily in other characteristics. Although Blandy and Sky appear to have similar herbivory pressures, these two populations were significantly different in all other plant traits. This was surprising, as Blandy and Sky are geographically close to one another and therefore one could expect relatively similar environmental conditions. However, abiotic resources are not necessarily consistent within fine-scale physical distance and provide a likely explanation behind this pattern. Examining this further, however, we found little spatial structuring within transects. Instead, Sky Meadows showed spatial structuring of a single trait -sexual reproductive investment- while only a third of the transects at Blandy (33%; Transect 46 and 47) had spatial structuring.

We did not find evidence of tradeoffs between composite traits (Figure 8). Instead, we found that plants that have larger vegetative investment also invest more in defensive traits and also flower more often. Surprisingly, we did not find a significant correlation between herbivory intensity and defensive traits, although there was a weak positive correlation between herbivory intensity and vegetative growth (Figure 8). In addition to a lack of vegetative investment, we also found that plants at Blandy invested significantly less in defensive and reproductive traits. Surprisingly, however, this population did not appear to experience higher herbivory as we would have expected with lower defensive investment. Instead, we found that only plants from Presquile experienced higher herbivory, although this does not appear to be due to differences in defensive investment (Figure 7).

Finally, we found that plants that are genetically identical are not functionally similar (Figure 9) regardless of the trait examined. This was largely different than what we would have expected to find if an individual's genotype was driving functional similarity. Instead, it appears that environmental variation plays a relatively larger role in the functional variation of these populations.

CONCLUSION

Although common milkweed has been generally described as a highly clonal species we found that the probability of finding a clone – even within fine-scale physical distance- was less than ten percent. When examining functional variation, we did not find evidence of tradeoffs between defensive and vegetative investment as has been seen in other species. Spatial structuring of these traits was highly variable between transects, and no one trait appeared to be characteristic of a single population (*i.e.* plants from any given site were not necessarily taller or more toxic than other sites). Finally, variation of traits, either composite traits or individual responses, does not appear to be largely driven by genotypic similarity. Instead, we found that fine-scale environmental variation appears to have a greater influence on the functional and phenotypic variation of these populations.

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APPENDIX

Supplemental Table 1. Age of experimental plants per measurement day and application of fertilizer. Initial harvesting of 99 plants was conducted to determine initial allometric relationships between aboveground and belowground biomass. Final harvest was then completed to determine final aboveground and belowground biomasses.

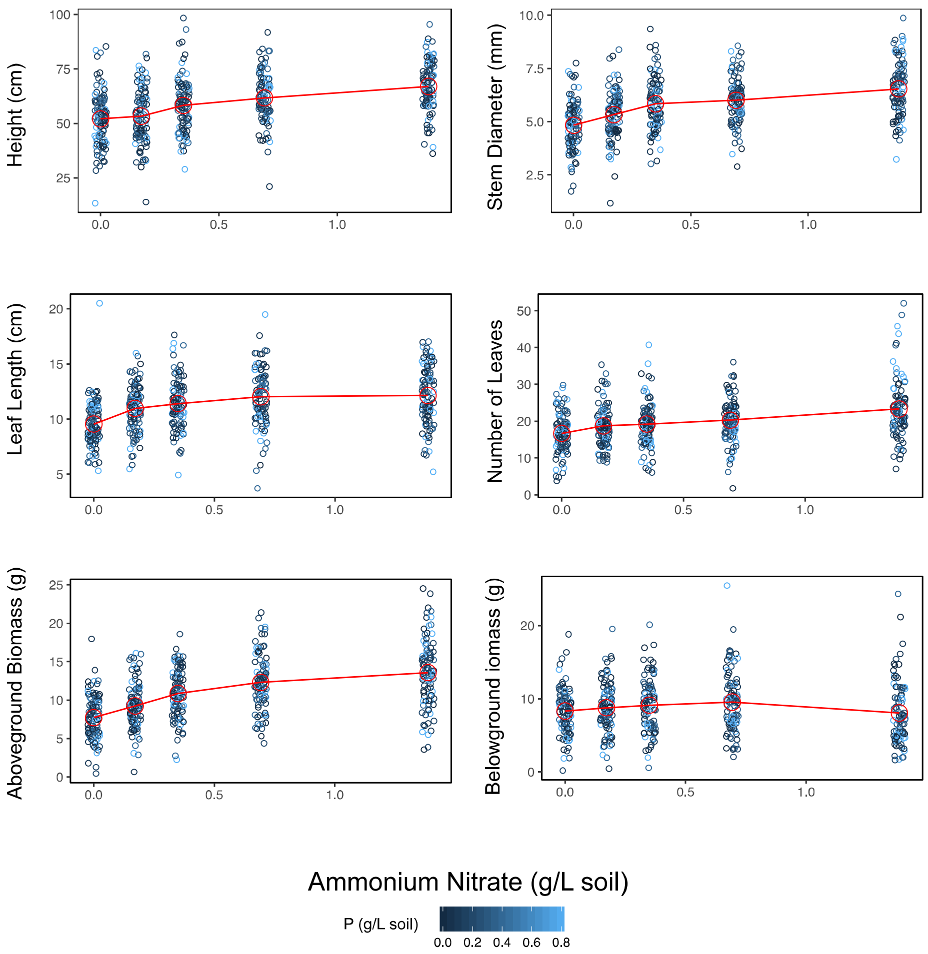
	Date	**Age of plants (days)**
Initial Harvest	6/5/17	21
First Application	**6/6/17**	**22**
Measurement 1	6/19/17	35
Measurement 2	6/27/17	43
Measurement 3	7/5/17	51
Measurement 4	7/12/17	59
Measurement 5	7/18/17	65
Second Application	**6/27/17**	**74**
Measurement 6	7/27/17	74
Measurement 7	8/1/17	78
Final Harvest	8/2/2017	79

Supplemental Table 2. Allometric relationships for above and below ground biomass. Partial regressions of above (left) and belowground (right) biomass for explanatory variables. All variables were included for both final models (above ground biomass = [-0.07 + (0.004*Height) + (0.006*Stem Diameter) + (0.015*Leaf Length) + (0.007*Number Leaves)]; below ground = [-0.023 + (0.001*Height) + (-0.011*Stem Diameter) + (0.010*Leaf Length) + (0.004*Number Leaves)]) as they increased the explanatory power of the models. To ensure that multicollinearity was not affecting our estimates of initial belowground biomass, we calculated Variation Inflation Factors (VIF) for every explanatory variable in our regressions. VIF values provide an index of how much the variance of an estimated regression coefficient is increased due to collinearity. VIF values were not greater than 10, indicating that multicollinearity did not affect our belowground biomass estimates.

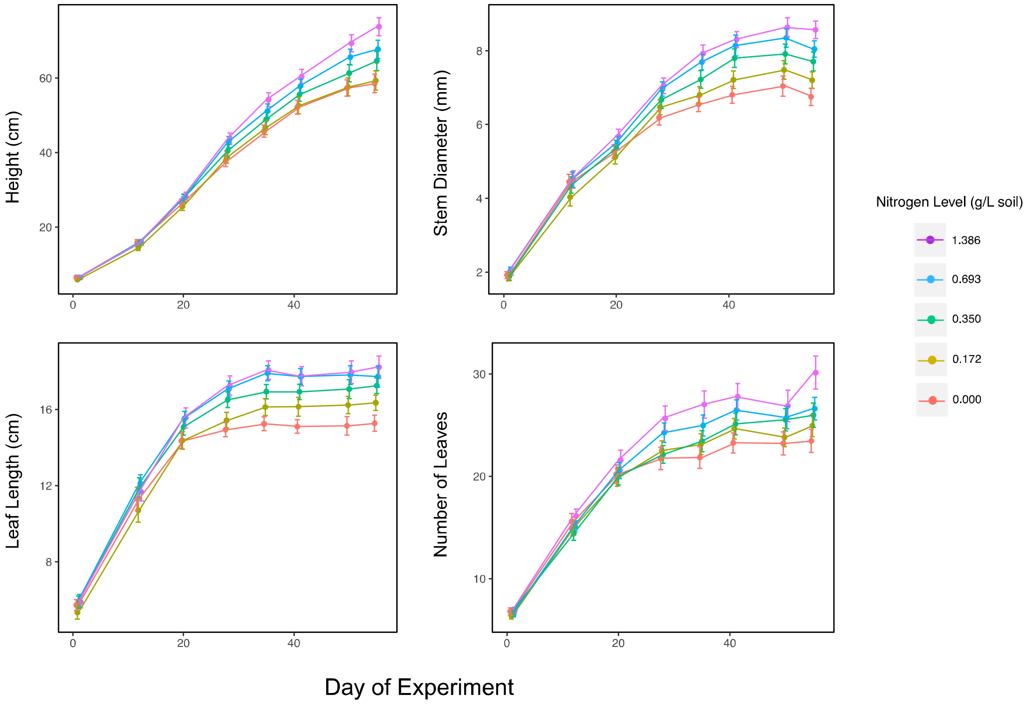
	Intercept (standard error)	**p-value**	**Partial R^2**	**VIF**	**Intercept (standard error)**	**p-value**	**Partial R^2**	**VIF**
Height	0.004 (0.001)	0.010	0.010	1.340	0.001 (0.001)	0.530	0.030	1.340
Stem Diameter	0.006 (0.05)	0.200	0.100	1.270	0.011 (0.004)	0.004	0.030	1.270
Leaf Length	0.015 (0.002)	< 0.001	0.170	1.900	0.010 (0.004)	< 0.001	0.100	1.900
Number of Leaves	0.007 (0.002)	< 0.001	0.130	1.630	0.004 (0.001)	< 0.001	0.030	1.630

Supplementary Table 1. Microsatellite information and GenBank accession numbers. We used seven previously developed primers (Kabet et al., 2010) for genotypic analyses. Note that size ranges, annealing temperature (T_m), and number of cycles reported are based on our own optimization protocols, and therefore differ slightly from previous studies.

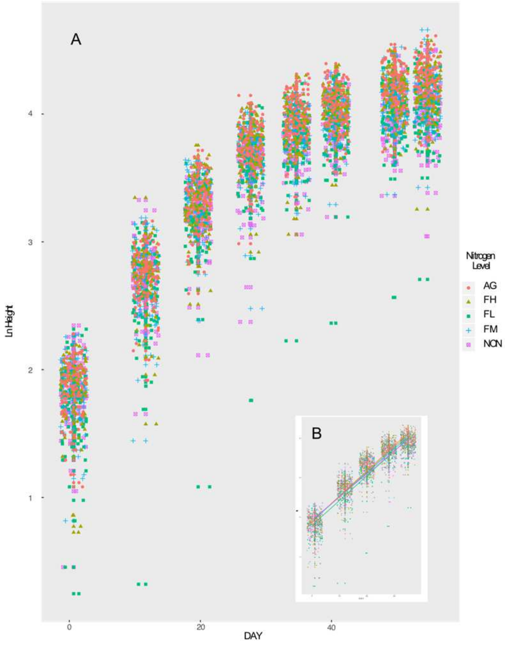
Locus		Sequence	Repeat Motif	Size Range	Tm	# Cycles	GenBank Acc #
ASC5	F:	TTGGAAGCTCAATTCTATACT	$(GAT)_{20}$	174-315	47.5	35	HM004507
	R:	CAAAGATGTAGAGGGTAAGTC					
ASF2	F:	TGAACAAGATCCTGCGAATG	$(AGA)_{10}$	75-123	53	32	HM004509
	R:	TCATTAGCAACAAAGGTATCC					
ASF9	F:	CACAGAAACAAGGTGAAATG	$(AAG)_{9}$	90-143	53	32	HM004508
	R:	TACTTTGCTTAATCAGCTCC					
ASH8	F:	AAATCGCATACAGTGGAAAG	$(AAG)_{11}$	93-180	47.5	35	HM004502
	R:	GACTACTTTCGCTAAATCAG					
ASG6	F:	CTATGCAAACTCCTCATGAT	$(TGG)_{9}$	171-195	53	32	HM004506
	R:	GAAGGCTGTTTCAGATCTTG					
AS94	F:	TTCTTCGAGTAGGTAGGAATG	$(AAG)_{19}$	140-194	53	32	HM004505
	R:	CACCCCTACAAACAATCCT					
ASB5	F:	CCATGAAATTAGCTCAAGATC	$(GAA)_{11}$	189-201	53	32	HM004504
	R:	CAAAGTCCGATTCGGGTAA					



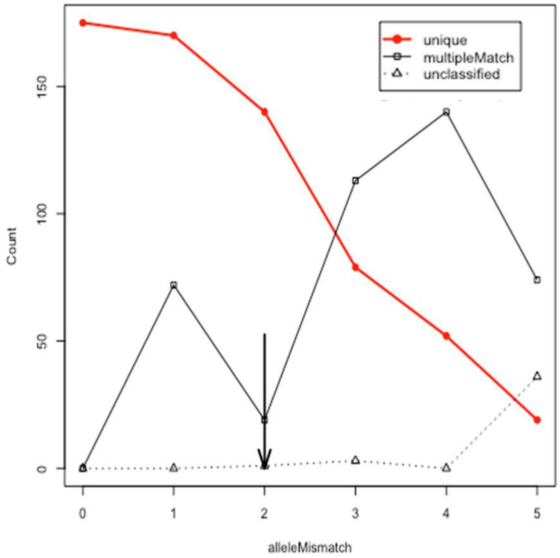
Supplemental Figure 1. Summary of all response variables by nitrogen and phosphorus treatment. Nitrogen (x axis) was treated as a continuous variable (grams per liter of soil); phosphorus concentration is shown in light blue (highest concentration) to dark blue (lowest). Increasing nitrogen concentration appears to increase mean belowground biomass, except at the highest concentration. There appears to be no pattern in phosphorus treatment that explains a change in any of the response variables.



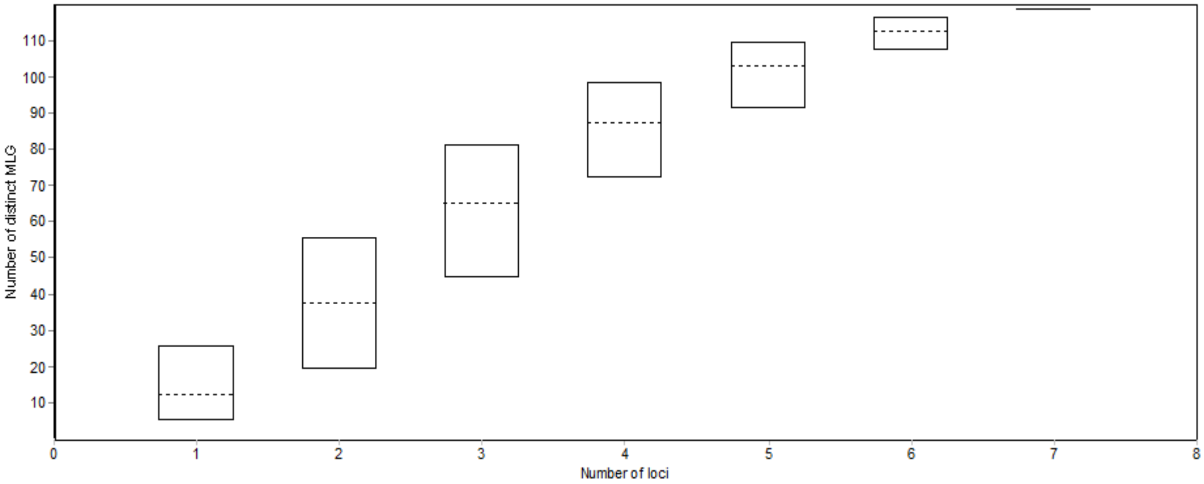
Supplemental Figure 2. Change in phenotypic response variables over time when treated with increasing nitrogen. All phenotypes were measured in cm except for stem diameter (measured in mm). Each line represents the mean value per response variable over time. Error bars are one standard deviation from the mean. All phenotypic response variables share a similar positive pattern in growth over time; however, note that the rate of growth is different between variables.



Supplemental Figure 3. Effect of nitrogen on growth rate and duration on *Asclepias syriaca* in a controlled greenhouse setting. Increasing the amount of available nitrogen appears to increase the duration at which plants were able to grow (A); however, these same treatments did not impact the rate of this growth (B). Axes include the natural log of individual height measurements as a function of time (provided as Measurement day; Supplemental Table 1).



Supplemental Figure 4. Estimating the optimum number of genotyping errors to allow. To avoid over or underestimating the number of unique genotypes present, we first estimated the number of genotyping errors that should be allowed. This was done using the package “allelematch” from RStudio. This criterion is initially based on the assumption that there are no genotyping errors present. Any difference between alleles therefore results in a “unique” genotype. To include the presence of genotyping error, this package then allows for n+1 instances of a mismatched allele. This additionally creates the possibility for individuals to be assigned to multiple genotypes (“multipleMatch”). The optimum number of genotyping errors then falls where the number of multipleMatch(es) reaches (or approaches) zero. For our dataset, we found that the optimal number of genotyping errors, or the number of alleles to allow to mismatch, equaled two (arrow). Based on this estimation, we found 19 individuals that were assigned to multiple genotypes. These individuals were removed from the final dataset.



Supplemental Figure 5. Rarefaction curve of number of unique genotypes as a function of number of loci used. We used GenClone (Arnaud-Haond et al., 2007) to ensure that the number of loci used provided an accurate representation of the total number of MLG's. The number of loci used (seven) in this study appears to provide a good representation of the total number of MLG's possible.