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INTERACTING EFFECTS OF NUTRIENT AVAILABILITY AND ENVIRONMENTAL CHANGE ON GRASSLAND PLANT COMMUNITIES

by

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INTERACTING EFFECTS OF NUTRIENT AVAILABILITY AND ENVIRONMENTAL CHANGE ON GRASSLAND PLANT COMMUNITIES

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University of Nebraska, 2024

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The availability of nutrients plays an important role in shaping the composition, productivity and diversity of plant communities. Such effects are particularly well documented in grassland ecosystems, where both independent studies and collaborations such as the Nutrient Network consistently show increasing productivity and decreasing diversity with the addition of limiting nutrients. The specifics of this pattern, however, vary widely from site to site, and within a single site, they may shift with changes in environmental conditions and disturbance regimes.

My research at Cedar Creek Ecosystem Science Reserve (Minnesota, USA), documents such a pattern through resampling of a long-term experiment on pine encroachment into old field grasslands. In burned plots, where pine encroachment is inhibited, nitrogen addition shifts the herbaceous plant community from one dominated by perennial C4 grasses with native forbs and legumes to one where invasive C3 grasses are abundant. In unburned plots subject to pine encroachment, however, invasive C3 grasses are abundant regardless of nitrogen treatment. This community pattern does not, however, extend to the ecosystem's overall carbon and nitrogen dynamics. While several ecosystem carbon pools respond to fire, they do not respond to nitrogen addition or to fire-nitrogen interactions.

Alongside such measures of species composition and resource cycling, plant functional traits may provide valuable insights into community dynamics. Such traits show considerable intraspecific variability, and understanding this variation is critical to their effective use. At Cedar Point Biological Station (Nebraska, USA), grassland plant species' functional traits show considerable variation with nutrient addition. I found, however, that these traits, and in some cases the strength of their nutrient responses, are also dependent on sampling year. Species display more acquisitive trait values both with nitrogen addition and in high rainfall years, and for leaf dry matter content, these effects interact to produce an elevated nitrogen effect in high rainfall years. Through experimental rainfall manipulation, I found that these patterns can be partially explained by total rainfall but that a substantial component remains to be explained by other components of annual environmental variation.

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Finally, I would like to thank my parents: Jim Wheeler and Ann Behlen Wheeler, for encouraging my curiosity, for fostering my interest in science and nature, and for their continual love and support.

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Summary

GRASSLAND COMMUNITIES’ COMPOSTIONAL AND TRAIT-BASED RESPONSES TO NUTRIENT ADDITION ARE CONTINGENT ON ECOLOGICAL CONTEXT

Nitrogen and other mineral nutrients are important drivers of community and ecosystem dynamics in grassland systems. Understanding the effects of these nutrients is key to answering both conceptual questions related to ecological principles and more applied questions of how ecosystems will respond to eutrophication. Broadly speaking, grassland plant communities are expected to show increased productivity (Fay et al. 2015) and reduced diversity (Harpole et al. 2016) with the addition of limiting nutrients. However, sites can differ dramatically from one another both in the identities of limiting nutrients and in the scale of the effects produced (Fay et al. 2015). Multi-site collaborations, such as the Nutrient Network (Borer et al. 2014) play a valuable role in revealing such commonalities and divergences in nutrient effects across sites.

Just as the effects of nutrient additions can vary from site to site, their effects in a given system may shift as environmental conditions, such as precipitation and fire, change. In Cedar Point Biological Station’s Nutrient Network plots, for instance, nitrogen addition dramatically increases productivity in high-rainfall years, but such effects are largely absent in low-rainfall years (Wang et al. 2017). Understanding such interactions takes on additional importance given anthropogenic changes to climate patterns and disturbance regimes. In this dissertation, I evaluate potentially interactive effects of nutrient addition and environmental changes on two grassland study systems. My first

two chapters examine a pine encroachment experiment in a Minnesota old-field grassland, testing the effects nitrogen and fire on community composition and ecosystem carbon storage. The following two chapters examine the effects of nitrogen in combination with annual and experimental rainfall variation in a Nebraska shortgrass prairie, focusing on intraspecific variation in functional traits.

At Cedar Creek Ecosystem Science Reserve (Minnesota, USA), Eastern White Pine (*Pinus strobus*) is encroaching into old field grassland areas (Dovčiak et al. 2005), consistent with a global trend of woody plant encroachment into grassland systems (Archer et al. 2017, Stevens et al. 2017). In Chapter 1, I describe my resampling of a long-term experiment testing the drivers and consequences of this encroachment, with a focus on herbaceous community composition. As expected, fire dramatically inhibits the encroachment of pines and other woody species into these grasslands. However, changes in herbaceous community composition follow a more complex pattern. Although nitrogen addition at Cedar Creek has well documented effects on community composition (Tilman 1987, Fay et al. 2015), my research shows these effects to be contingent on the fire and thereby the absence of pine encroachment. Among burned plots, nitrogen addition produces a shift from a community dominated by C4 grasses and including a variety of native forbs and legumes to one where introduced C3 grasses are abundant. In unburned plots, overall herbaceous cover is reduced, but the remaining herbaceous community shows no response to nitrogen addition. Instead, C3 grasses are abundant regardless of nutrient treatment. Thus, the exclusion of fire and consequent encroachment of woody species appear to mediate nitrogen effects, potentially through changes in light availability, litter deposition and nutrient uptake.

Woody encroachment into grassland ecosystems can also substantially alter the carbon storage properties of these ecosystems, with generally positive effects on aboveground carbon (McKinley and Blair 2008, Zhou et al. 2022) but with effects on soil carbon varying across environmental gradients (Barger et al. 2011, Liu et al. 2020). In Chapter 2, I extend my analysis of woody encroachment at Cedar Creek to consider potentially interactive fire and nitrogen effects on ecosystem carbon storage. I find that in this system, fire and nitrogen produce no significant effect on soil carbon, whether measured as standing pools or as change over time. Fire dramatically decreases pine and litter carbon, while producing moderate increases in herbaceous plant carbon and decreases in root carbon. Woody encroachment in the absence of fire thus appears to produce an overall increase in ecosystem carbon, but this effect is largely confined to aboveground pools, which are both smaller in magnitude than soil carbon and more susceptible to loss in the event of management changes or disturbance. Despite the previously documented effects of nitrogen on biomass and community composition at this site (Tilman 1987, Fay et al. 2015), it failed to produce a detectable change in total ecosystem carbon.

In addition to community composition and ecosystem properties, grassland systems may also shift though within-species changes. Such changes may be quantified through measurements functional traits, which act as indicators of organisms ecological strategies and interactions and facilitate the description of ecological patterns through general, rather than species-specific rules (McGill et al. 2006). In Chapter 3, originally published in Ecology (Wheeler et al. 2023), I consider the effects of nitrogen-environment interactions on intraspecific change. Intraspecific variation in these traits is known to be common (Siefert et al. 2015), yet variation over time is only rarely considered. At Cedar Point Biological Station (Nebraska, USA), I monitored the values of four functional traits in twelve study species over four years of sampling, finding that three of these four traits varied significantly across study years, with annual variation exceeding fertilization effects for specific leaf area and leaf dry matter content. Additionally, for leaf dry matter content and chlorophyll, the effects of nitrogen addition were contingent on study year, with nitrogen producing stronger leaf dry matter content effects in high-rainfall years.

To evaluate the degree to which these patterns can be explained by rainfall variation, I established a series of rainfall manipulation treatments, in association with the NPKD-Net global collaboration. I then measured the same functional traits across three study years, focusing on 6 study species in order to improve species-level replication. The results of this experiment, described in Chapter 4, show that experimental rainfall manipulation alters functional trait values in a number of study species, but that these effects are smaller in magnitude and less consistent than the variation observed across study years. For both specific leaf area and leaf dry matter content, half of species respond to experimental rainfall manipulation, while all species respond to nutrient addition and sampling year, and while nutrient by year interactions are common nutrient by rainfall treatment interactions are not statistically supported. An overall interaction between rainfall and nutrient treatments can be observed for plant height, with reduced nutrient effects under experimental drought, but chlorophyll content shows no response to rainfall manipulation. These results confirm a causal effect of total precipitation on trait values in some cases, but suggest that other variables such as humidity, temperature, and the timing of rainfall events may also play important roles in explaining annual patterns of trait variation.

These studies illustrate several instances where nitrogen's effects on a community are dependent on environmental conditions. At Cedar Creek, the effects of nitrogen addition on community composition are contingent on fire (and thereby the absence of pine encroachment). At Cedar Point, the effects of nitrogen of leaf dry matter content are enhanced in high rainfall years, while nutrient effects on plant height are enhanced by experimental water addition. In other cases, however, the effects of nitrogen and environmental change produce purely additive effects, as with specific leaf area from 2017-2020 and for many trait-species combinations under rainfall manipulation. And in the case of ecosystem carbon at Cedar Creek, nitrogen produces no detectable effect despite observations to the contrary.

While changes in nutrient effects should not be expected as a universal effect of environmental changes, the potential for such interactions will be an important consideration for studies focused on nutrient effects. When nutrient responses are considered from the perspective of species interactions and ecological theory, the potential context dependence of these responses should be acknowledged and, when feasible, tested. Likewise, when nutrient deposition is considered as a component of anthropogenic change, co-occurring changes to climate and land management should be considered as potential confounding factors. These nutrient effects and interactions may occur through intraspecific change as well as through species turnover, and the relevant patterns of intraspecific change may themselves vary from species to species. As relevant environmental factors are likely to vary from site to site, and both local expertise and large-scale studies of environmental gradients are likely to aid in identifying potential interactions. In evaluating such effects, a combination of observational and experimental approaches may be ideal, in order to test causal relationships while accounting for the more complex nature of ongoing environmental changes relative to experimental manipulations.

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Chapter 1

NITROGEN EFFECTS ON COMMUNITY COMPOSITION ARE CONTINGENT ON FIRE IN A GRASSLAND UNDERGOING ENCROACHMENT BY EASTERN WHITE PINE (*PINUS STROBUS*)

Abstract

In addition to altering ecosystem states, anthropogenic changes may also alter the drivers of community dynamics within these ecosystems. As changes to disturbance regimes facilitate woody encroachment into grasslands, the nutrient responses of these grasslands may also change. Previous research has shown nitrogen as a key driver of community dynamics in grassland ecosystems, including those present at Cedar Creek Ecosystem Science Reserve (Minnesota, USA). As part of an investigation into the drivers and consequences of Eastern White Pine (*Pinus strobus*) encroachment at this site, we surveyed pine abundance and herbaceous community composition following 18 years of experimental fire, nitrogen and herbivore manipulation. While pine abundance varied widely in unburned plots, it did not significantly respond to nitrogen addition or herbivory. As expected, pine encroachment was dramatically inhibited in burned plots. Species richness in the herbaceous community did not differ significantly between treatments. The Shannon diversity index responded interactively to fire and nitrogen, with nitrogen addition decreasing diversity in unburned plots but increasing diversity in burned plots. Nitrogen's effects on the overall composition of the herbaceous plant community were contingent upon fire. Within the burned treatment, nitrogen addition led to an increase in the cover of invasive C3 grasses. Within the unburned treatment,

nitrogen had no consistent effect on herbaceous species composition. Despite research showing nitrogen as a key driver of community dynamics in the grasslands of our study site, we found that this effect is contingent on the presence of fire and absence of woody encroachment. With this variation in nitrogen effects, we see that a factor playing a major role in structuring a community can cease to play that role as disturbance regimes and ecosystem states are altered.

Introduction

Theory predicts that transitions from one stable community state to another will occur when threshold environmental conditions are reached (May 1977, Bestelmeyer et al. 2011), and if drivers of change are ongoing, alternate community states may be persistent and ecologically relevant even when not stable in a mathematical sense (Hansen et al. 2013). As dramatically altered landscapes become more common, understanding their functioning will be increasingly important to addressing ecological and management questions. Anthropogenically driven state changes are ongoing in ecosystems as divergent as coral reefs undergoing bleaching (Hughes et al. 2017), drying montane cloud forests (Helmer et al. 2019, Zinnert et al. 2021), salt marshes supplanted by mangrove forests (Cavanaugh et al. 2014, Zinnert et al. 2021), and kelp forests transitioning to urchin barrens (Filbee-Dexter and Scheibling 2014). Here, we focus on the encroachment of woody plant species into grasslands, a phenomenon ongoing in grasslands worldwide (Archer et al. 2017, Stevens et al. 2017), seeking to understand whether variables that drive community dynamics in grasslands continue to have the same effects as these ecosystems transition to savanna or forest states.

Woody plant encroachment appears to be partially driven by global factors (Wigley et al. 2010, Stevens et al. 2016, Venter et al. 2018), including the tendency for elevated CO_2 levels to favor C3 woody species over C4 grasses (Morgan et al. 2007). However, more localized factors also play important roles, with fire suppression in particular facilitating increased growth of woody species. In the central great plains of North America, fire frequencies below one in three years often trigger transitions to shrubland or woodland, potentially with self-reinforcing feedbacks if burns are not directly managed, as wooded areas are less prone to frequent fires (Ratajczak et al. 2014). The restoration of fire may prevent further woody encroachment but not necessarily restore a grassland state (Miller et al. 2017). Introductions of grazing herbivores may also increase woody encroachment by suppressing grass biomass and fire intensity, though such effects are inconsistent across systems (Archer et al. 2017). Increases to browser populations may have the opposite effect, including at our study site, Cedar Creek Ecosystem Science Reserve (Minnesota USA), where browsing by deer reduces growth of woody plant species (Inouye et al. 1994, Ritchie et al. 1998). The effects of herbivory may be enhanced by, or contingent on, an interaction with fire (Briggs et al. 2002, O'Connor et al. 2019).

Woody plant encroachment into grasslands tends to reduce community diversity, particularly at high rainfall sites where productivity of encroaching woody species is highest (Ratajczak et al. 2012). While this effect is not universal, community composition may be altered even if overall diversity is unchanged (Matson and Bart 2014). Beyond its direct impacts, however, it is unclear how woody encroachment will alter the effects of other variables, such as nutrient enrichment, relative to the effects observed in the original grassland and to those observed in other forested ecosystems.

In ecosystems currently in a grassland state, responses to nutrient enrichment are widely studied, with large scale collaborative work (Borer et al. 2014a) integrating and supplementing single-site research. In general, the addition of limiting nutrients results in an increase in productivity but a loss of diversity (Harpole and Tilman 2007, Harpole et al. 2016). Nitrogen is the nutrient most commonly associated with these effects, though many sites also respond to phosphorus, potassium, or some combination of these nutrients (Fay et al. 2015). Nitrogen effects are a prominent driver of community composition in the old field grasslands of Cedar Creek Ecosystem Science Reserve (Isbell et al. 2013). With nitrogen addition to grasslands, perennial, native and nitrogen-fixing species are particularly likely to be lost (Suding et al. 2005), potentially through several mechanisms, including reduced niche dimensionality (Harpole et al. 2016), increased light limitation (Borer et al. 2014b), and nitrogen-induced stresses such as ammonia toxicity and soil acidification (Band et al. 2022). Nitrogen effects are often enhanced by fire, due to volatilization of pre-existing nitrogen and removal of light limitation (Blair 1997), though even the reduced effects observed in the absence of fire can be significant (Seastedt et al. 1991).

In forest systems, nutrient based studies are less abundant, and many rely on observational data rather than experimental addition (Verheyen et al. 2012, Perring et al. 2018). Meta analyses of this more limited data set, however, suggest that nitrogen responses may be less consistent in forests than grasslands (Midolo et al. 2019). Diversity effects seem to scale strongly with the rate of nitrogen addition, with significant diversity losses occurring only at high levels of nitrogen addition (de Schrijver et al. 2011). De Schrijver's analysis also detected increased graminoid abundance as a relatively consistent response across studies, though this effect may be contingent on interactions between light availability and nitrogen addition rate (Perring et al. 2018). Communities where increased graminoid cover has been observed include pine-dominated communities, with grasses, shrubs and creeping herbs increasing in abundance with nitrogen addition to Argentinian loblolly pine (*Pinus taeda*) plantations and non-pine tree species tending to decline (Trentini et al. 2018). High levels of nitrogen addition do also have the potential to drive pine mortality, as observed with red pines (*Pinus resinosa*) at the Harvard Forest, Massachusetts LTER site, though such effects required nitrogen addition levels an order of magnitude greater than atmospheric deposition (Magill et al. 2004).

Plant community monitoring in grassland communities undergoing woody encroachment provides an opportunity to evaluate whether nutrient response patterns are altered by community state changes. Through a long-term experiment examining Eastern White Pine (*Pinus strobus)* encroachment in the old field grasslands of Cedar Creek Ecosystem Science Reserve, we examined both the drivers of woody encroachment and its effects on the composition and nutrient responses of the herbaceous plant community. We expected hypothesized that we would observe the following patterns:

1.) Pine encroachment will respond strongly to fire, with trees establishing in unburned plots but failing to do so in burned plots. Among unburned plots, deer herbivory may decrease pine encroachment, while nitrogen fertilization may increase it.

2.) Herbaceous community diversity will decline with nitrogen addition. This effect will be weaker in the presence of pine encroachment, due to reduced levels of total herbaceous cover and the potential for stresses associated with pine cover and litter buildup to supersede nitrogen limitation.

3.) Herbaceous community composition will respond strongly to nitrogen addition, with low nitrogen plots being dominated by strong nitrogen competitors. This effect will likewise be partially suppressed by pine encroachment.

Methods

Our study site, Cedar Creek Ecosystem Science Reserve, is located in East Bethel, Minnesota (USA,42º25'N, 93º10'W) and features a mixture of wetlands, forests, savannas and old-field successional grasslands. Our study site is located in Field B in a grassland area abandoned from agriculture in 1957 and now undergoing encroachment by Eastern White Pine (*Pinus strobus*). At the time of the experiment's establishment, such encroachment was common in central Minnesota, with local climate fluctuations likely acting as a contributing factor (Dovčiak et al. 2005). By the time of our surveys, adult pines were common, some occurring as isolated trees in grassland areas, and others forming areas of closed forest canopy (Image S1).

Cedar Creek is characterized by sandy soils with low nitrogen content, and the productivity of its grasslands increases with nitrogen addition (Fay et al. 2015). Nitrogen availability is also a key determinant of species composition (Tilman 1987), with strong nitrogen competitors acting as dominant species under ambient conditions and weak nitrogen competitors gaining an advantage with nitrogen addition (Harpole and Tilman 2006). Nitrogen addition at this site also results in a persistent reduction of biodiversity (Isbell et al. 2013). While these effects are dependent on the rate of nitrogen addition, some changes in productivity and community composition can be detected at annual rates as low as 1g/m^2 (Wilcots et al. 2021). Within Field B, fire has limited direct effects on community composition (Knops 2006, Li et al. 2013), though these effects have not been tested in the context of pine encroachment or nitrogen addition.

Treatment and Sampling Methods

Our study plots were established in 2000. Each of the 32 treatment plots measures 20x20m, with a central 10x10m area used for most sampling to minimize any edge effects. To evaluate the effects of fire, nitrogen addition, and herbivore exclusion, each was applied as a treatment to half of the study plots, following a fully factorial design. Fire was applied through prescribed burns in the years 2002, 2003, 2006, 2009, 2017 and 2018 in the assigned plots. Burning treatments took place in late spring (May 1 in 2018), prior to the growing season for most herbaceous species. Nitrogen was added in the form of ammonium nitrate, at an annual rate of 4g fertilizer/m^2 (1.4 g N/m^2). This rate is comparable to the anthropogenically increased rate of atmospheric deposition occurring in Minnesota (~0.9g/m^2; Ackerman et al. 2019) and to the lower rates of experiments testing multiple nitrogen addition rates at Cedar Creek (Isbell et al. 2013, Wilcots et al. 2021). Herbivore exclosure fences were 6 feet in height and constructed using hardware cloth with 2x4 in. openings, excluding white-tailed deer (*Odocoileus virginianus*), but allowing access for smaller herbivores. As deer hunting was reinstated at Cedar Creek six years prior to the establishment of the experiment, the population of excluded herbivores was likely already reduced relative to previous experiments at this site. Modeling by the Minnesota Department of Natural Resources from 2012-2018 estimated regional deer populations of 7-8 individuals per square kilometer (Norton 2018), compared to populations of 10-29 individuals per square kilometer observed during aerial surveys in the 1970 and 1980s (Inouye et al. 1994).

In 2018, we surveyed the vegetation in each treatment plot. To evaluate woody plant abundance, we conducted a census of all woody stems with a basal diameter of at least 2cm. For each such individual, we recorded basal diameter, location, and species identity. *Pinus strobus* represented 90.3% of these stems and 97.8% of their total basal area, with the remainder including shrubs such *Frangula alnus* and trees of less locally abundant genera such as *Quercus* and *Populus*. This census took place from May 9 through June 6. To evaluate the composition of the herbaceous community, we conducted cover surveys in 10 subplots per treatment plot, with each cover subplot measuring 0.5x1m. These subplots were established and permanently marked at regular intervals around the inner edge of each plot’s 10x10 m core area during the establishment of the experiment in 2000 (see Image S2), with adjustments to avoid the inclusion of woody stems taller than 0.5m. During surveys, we visually estimated the percent cover of each species rooted in the plot, along with litter and bare ground. Consistent with past research at this site, cover was estimated on a relative basis, with total cover of all species, litter and bare ground summing to 100% (Tilman et al. 1996, Knops et al. 1999, Fargione and Tilman 2005).

Though our focus in these surveys was on herbaceous species, woody species were included in instances where seedlings were rooted in the survey plots. For analysis of the cover data, we averaged the 10 subplot-level cover estimates to produce treatment plot-level values.

Statistical Analysis

For our statistical analyses, we used R version 4.0.5 (R Core Team 2021). To evaluate treatment effects on pine encroachment, we conducted an ANOVA, treating total basal area in the inner 10x10m area of each treatment plot ($m^2/100m^2$) as a response to the three treatment variables and their interactions. Basal area is a common metric of woody plant abundance (Nowacki and Abrams 1997, Hughes et al. 2006, Brudvig et al. 2011), with the benefit of reflecting patterns of change in both the size and number of woody individuals (Hanberry et al. 2014). In addition to basal area, we analyzed pine stem count, which, as count data, could be modeled using a generalized linear model with a negative binomial distribution, better accounting for the large number of zero values (plots without pine stems) in the data set.

To evaluate treatment effects on diversity, we calculated species richness, based on the number of species present in any cover plot, and the Shannon Diversity Index, with average cover as our metric of abundance for each treatment plot. These plot-level diversity metrics were likewise treated as ANOVA response variables. Additionally, we used full plot presence-absence data and subplot-level diversity calculations to test the sensitivity of these analyses to the scale of our diversity measurements.

To evaluate changes to community composition more broadly, we applied a PERMANOVA test, with the treatment plot level average cover values for each species as response variables. For visualization purposes, we also conducted an NMDS analysis of species cover by treatment plot. For these analyses, we used the R package vegan (Oksanen 2022). Following this multivariate analysis, we applied an indicator species analysis (Dufrêne and Legendre 1997) using the R package indicspecies (De Caceres and Legendre 2009) to identify taxa important in distinguishing treatments from one another. Following these tests, we conducted a series of functional group comparisons to examine the role of functional group changes in explaining the observed differences. For these purposes, we categorized species as C3 graminoids, C4 graminoids, forbs (non-leguminous), legumes, or woody plants. For each functional group, we used t-tests to compare total cover in nitrogen addition vs. unfertilized control plots within a given burn treatment. As this was a planned set of ten comparisons, we used a Bonferroni adjusted alpha value of $P = 0.05 * 1/10 = 0.005$ to account for multiple comparisons.

Results

Deer exclusion had no significant effect on pine encroachment (Table S1.1), species diversity (Table S1.2), nor on species composition (Table S1.3). Clear differences were, however, apparent between burned and unburned plots and in some cases between nitrogen addition and unfertilized plots.

Pine encroachment responded strongly to fire. While *Pinus strobus* varied dramatically in abundance among unburned plots, it was almost entirely absent from burned plots, with basal area averaging only 2% of unburned levels (Figure 1.1a, Table S1.1a). Nitrogen addition had no significant effect on basal area, nor was there a significant fire x nitrogen interaction. Pine stem count showed a similar response pattern, with a clear response to fire, only a marginal nitrogen effect and no significant fire x nitrogen interaction (Figure 1.1b, Table S1.1b). Including the full 20x20 m plot area (thus expanding the sampling area but increasing the potential for edge effects; Tables S1c, S1dS1.1c,S1.1d) and including woody species other than *Pinus strobus* (Tables S1.1e-S1.1h) did not qualitatively alter the results, except for the disappearance of nitrogen's marginal effect.

Herbaceous community diversity, measured by the Shannon Index showed an interactive response to fire and nitrogen addition (Figure 1.2a, Table S1.2a). Among unburned plots, nitrogen addition reduced the Shannon Index from a mean of 1.81 to 1.34, while among burned plots, nitrogen addition increased the mean Shannon Index from 1.34 to 1.76. Transforming the Shannon Index values to their exponential form, or Hill number q=1 (Hill 1973), for analysis did not alter this pattern (Table S1.2b). Species richness, in contrast to Shannon diversity, was highest in unfertilized burned plots, with a mean value of 17.6 recorded species in the $5m^2$ total survey area and lowest in unburned nitrogen addition plots, with a mean of 12.5 species (Figure 1.2b). However, these differences were not statistically significant (Table S1.2c).

Diversity measurements calculated at the subplot level before averaging showed a similar pattern, with species richness showing no response to nitrogen and Shannon diversity showing an interactive effect of fire and nitrogen. A richness analysis based on species' presence or absence in the 20 x 20m treatment areas likewise showed no statistically significant differences. Analysis at this scale did also show a significant main effect of fire on richness, which was lower in unburned plots, likely as a result of lower overall herbaceous cover (Table S1.3a-d, Figures S1.1-S1.3).

Herbaceous community composition showed a strong overall response to fire (F = 11.7, d f=1 , *p* = 0.001) and a distinctive fire x nitrogen interaction (F = 2.6, df = 1, *p* = 0.041, Figure 1.3, Table S1.4). Unburned plots were clearly differentiated from burned plots, showing greater abundance of woody seedlings and lower overall vegetative cover (30.0% in unburned plots, compared to 68.8% in burned plots). While unburned plots showed a high degree of overall compositional variation, they showed no differentiation on the basis of nitrogen addition. Among burned plots, however, nitrogen addition produced a clear response. Invasive C3 grasses such as *Poa pratensis* and *Elymus repens* were abundant in burned nitrogen addition plots, as well as in unburned plots. Burned plots without nitrogen addition had particularly high cover of *Schizachyrium scoparium*, an abundant C4 grass known to be a dominant nitrogen competitor (Tilman and Wedin 1991, Wedin and Tilman 1993). Native forbs and legumes such as *Achillea millefolium*, *Lespedeza capitata*, *Solidago nemoralis*, and *Oligoneuron rigidum* were significant indicator species for this treatment, though low in absolute abundance (Table 1.1).

Our functional group analyses showed a 5-fold increase in C3 graminoid cover with nitrogen addition among burned plots (t = -5.4, df = 7.9, *p* = 0.0007; Figure 1.4) but no nitrogen effect on C3 cover in unburned plots (t = -0.17, df = 13.9, *p* = 0.865). C4 and legume cover appeared to decrease with nitrogen addition in burned plots, but these effects were not significant at the adjusted alpha value of 0.005, nor were there any significant effects of nitrogen addition on the cover of forbs or woody species (Table S1.5).

Discussion

Despite extensive research showing the importance of nitrogen in Cedar Creek's old field grassland communities (Tilman 1987, Harpole and Tilman 2006, Isbell et al. 2013), we found that this nutrient's effects on community composition in our plots were highly contingent on the presence of fire. While burned plots were highly differentiated from one another on the basis of nitrogen addition, no such differentiation was apparent among unburned plots. This effect is likely mediated by pine encroachment into unburned plots. While *Pinus strobus* was excluded almost entirely from burned plots, its encroachment into many unburned plots was extensive. Pine encroachment did not itself significantly respond to nitrogen. Deer herbivory, despite its strong effects in previous research at Cedar Creek (Inouye et al. 1994, Ritchie et al. 1998), did not alter pine encroachment or community composition given the current population size and management regime. While plant community diversity responded interactively to nitrogen and fire, the pattern of variation in the Shannon Index diverged from our expectations. Where we would have expected nitrogen addition to reduce diversity in burned plots dominated by grassland species (Harpole and Tilman 2007, Harpole et al. 2016), we instead saw the Shannon index increase with nitrogen addition among burned plots. This change appears to be driven somewhat idiosyncratically by a small number of prominent grass species. Among burned nitrogen addition plots, the invasive C3 grasses *Elymus repens* and *Poa pratensis* were abundant, but *Schizachyrium scoparium*, the most common species at the site overall, remained common. Among unfertilized burned plots, invasive C3 grasses were rare, and the dominance of *Schizachyrium scoparium* resulted in low species evenness and thus a low Shannon index, despite a higher abundance of native forb and legume species relative to other treatments. Our study's low rate of nitrogen addition may also contribute to the lack of diversity loss with this treatment. In previous research at Cedar Creek, Isbell and colleagues (2013) detected no diversity changes at 0-1.0g/m^2 nitrogen addition, but significant diversity losses with nitrogen addition rates of 2.0-5.4g/m^2 and higher, with correspondingly higher increases in C3 biomass (rates between 1 and 2 g/m^2, like those used in our study, were not tested).

Focusing on changes in species composition more broadly, the strong main effect of fire can be largely explained by the absence of pine encroachment in burned plots. These plots exist in a grassland state and have high cover values for common grass and forb species. Unburned plots are more variable in their ecosystem structure, with some remaining in a grassland or savanna state and others developing into pine forest with minimal herbaceous cover. The lower cover values for common herbaceous species are thus expected. The effect of nitrogen in unburned plots likewise matches our expectations. Past research at Cedar Creek has shown that nitrogen addition leads to increased cover of invasive C3 grasses at the expense of C4 and legume species (Tilman 1987, Harpole and Tilman 2006), and our observed pattern closely matches this trend. We would expect these effects to be enhanced by burning, given the tendency for fire to increase the effects of nitrogen addition, particularly in the year following a burn treatment (Seastedt et al. 1991, Blair 1997).

While we expected that these nitrogen effects would be weaker under fire suppression and pine encroachment, their complete disappearance is striking. Unburned plots retain, on average, 42% of the total vegetative cover of burned plots, yet this remaining community shows no differentiation on the basis of nitrogen addition. A number of mechanistic explanations for this lack of effect are possible. Pine encroachment, even at low levels, may affect the herbaceous community through shading or litter deposition, potentially in ways that supersede nitrogen effects. Additionally, in this system, both fire suppression (Li et al. 2013) and nitrogen addition (Tilman 1987) are associated with increases in the cover and biomass of C3 grasses, which may make their effects difficult to discern in an interactive context.

Changes to the soil microbial community, which have previously been documented between oak forests and savannas at Cedar Creek (Dickie et al. 2009), could also play a role in mediating nitrogen effects. Woody encroachment can dramatically alter soil microbial community composition, particularly through increases in ectomycorrhizal fungi, which commonly associate with woody species, and decreases in arbuscular mycorrhizal fungi, which frequently associate with herbaceous species (Becklin et al. 2012, Sepp et al. 2021), and microbial community composition can in turn shape plant community compositions through their effects on competitive dynamics (Tedersoo et al. 2020). The greater host specificity and more frequent allelopathic effects of ectomycorrhizal fungi (Tedersoo et al. 2020) may inhibit the establishment of later successional grassland species, which play a key role in distinguishing fertilized and unfertilized burned plots, and which tend to have less negative plant-soil feedbacks than early successional species (Bauer et al. 2015).

It is also possible that nitrogen uptake by pines or by soil microbes could limit the effects of nitrogen addition on actual nitrogen availability to the herbaceous community. Sampling in these study plots in 2010 (Li et al. 2014) showed an increase in gross ammonification with nitrogen addition but no other significant effects (Table S1.6). *Pinus strobus* appears to gain a competitive advantage in this system through its long nitrogen residence time, which may result in reduced N availability to herbaceous species (Laungani and Knops 2009). Soil pH shows only minimal changes with pine abundance at Cedar Creek (S. Hobbie, unpublished data) and is thus unlikely to be an important explanatory factor.

Despite their uncertain mechanistic cause, these results make clear that the effects of nitrogen addition under pine encroachment do not match the effects of nitrogen addition in burned, pine-suppressed grasslands at Cedar Creek. Neither do they match the patterns often seen with nitrogen addition to forests in temperate regions, such as increased graminoid cover (de Schrijver et al. 2011). The observed lack of nitrogen response in unburned plots is thus distinct from what would be predicted based either on this site's prior dynamics or on the dynamics of existing forest and savanna sites. As ecosystems are increasingly shifted towards novel states by anthropogenic pressures, such changes to the drivers of their community dynamics may become increasingly common.

Figure 1.1: Pine Encroachment by Treatment. Each point represents pine basal area (m^2 basal area in a $100m^2$ sampling area, Panel a,) or stem count (Panel b) in a single treatment plot. Horizontal lines represent the mean values for each treatment, with vertical lines showing the standard errors of those means. Significance of fire and nitrogen effects is indicated at the top left of each panel, with and ● indicating marginal significance ($p < 0.1$) and *** indicating significance at the $p < 0.001$ level.

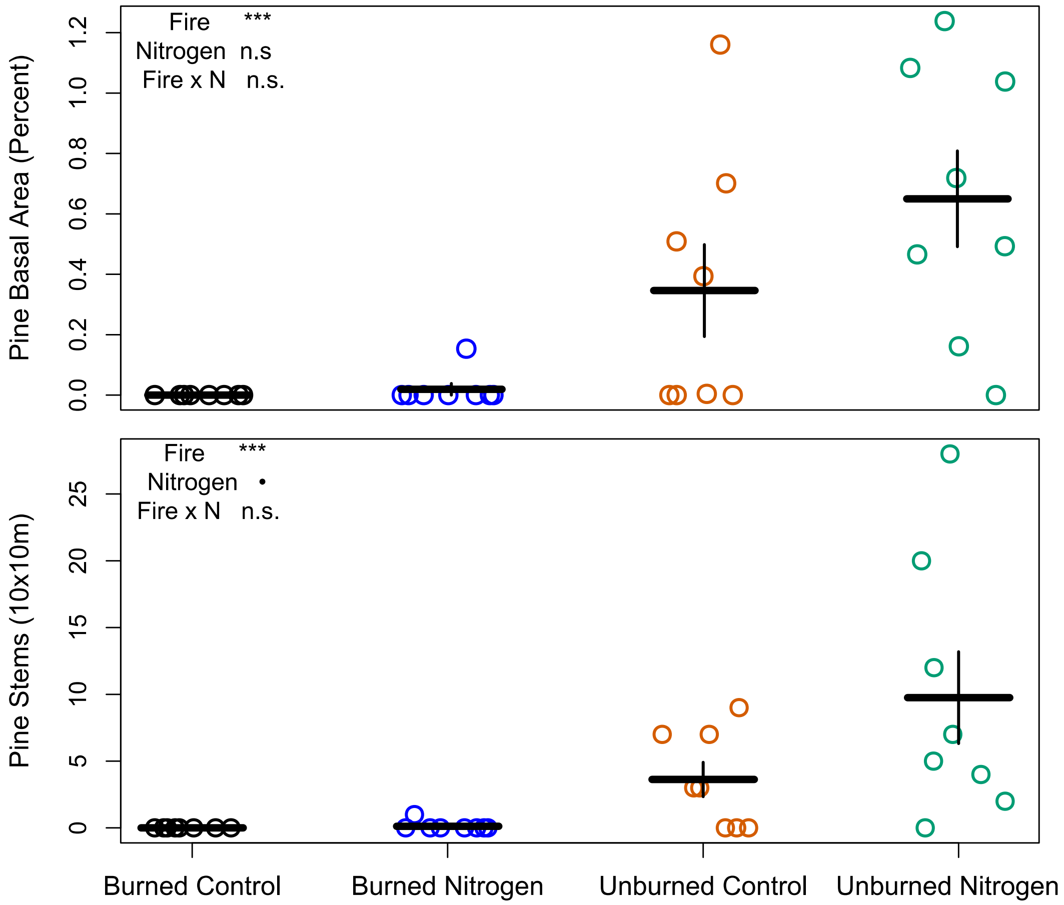


Figure 1.2: Diversity by Treatment Each point represents diversity of species rooted in the ten herbaceous cover plots of a single treatment plot. Horizontal lines represent the mean values for each treatment, with vertical lines showing the standard errors of those means. The interactive effect of fire and nitrogen on the Shannon diversity index is highly significant ($p < 0.01$).

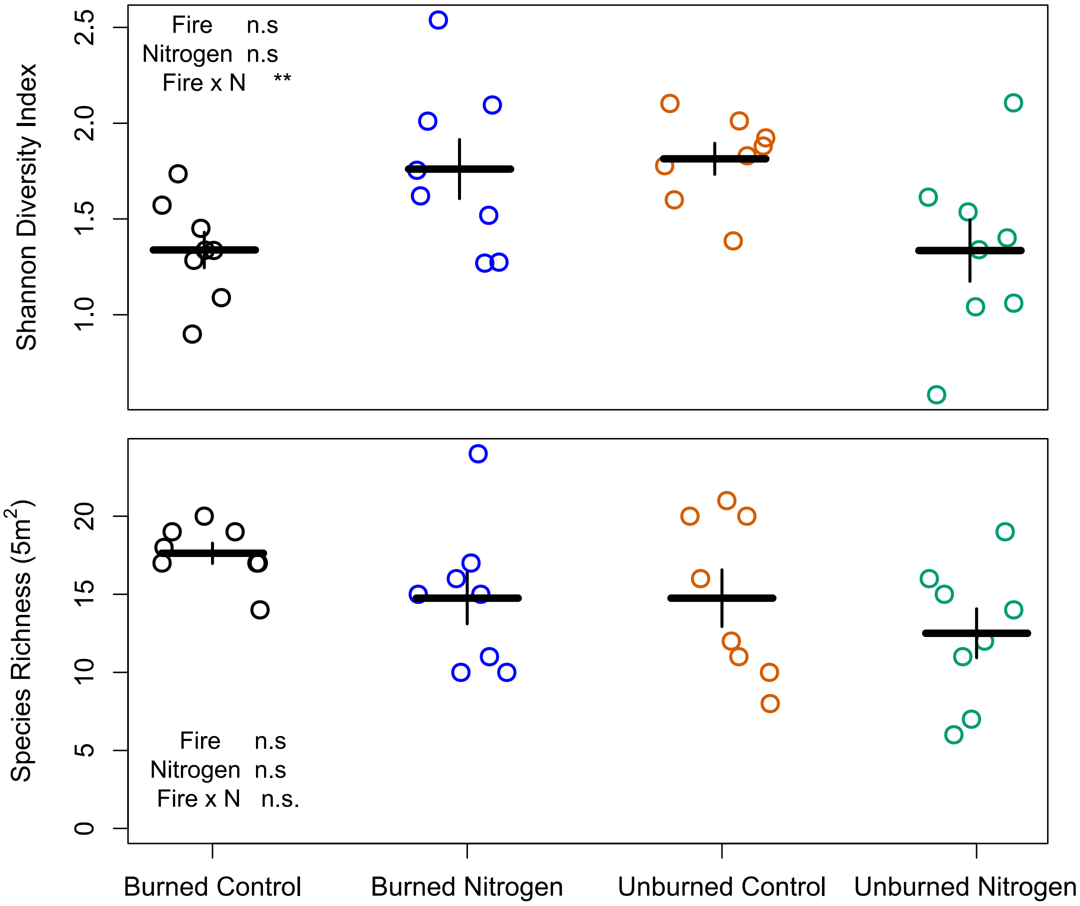


Figure 1.3: Compositional Response to Fire and Nitrogen: Each point represents a single treatment plot, with cover values averaged across 10 sampling plots. Point color indicates fire and nitrogen treatment, with fill corresponding to total vegetative cover. Ellipses indicate the standard error around the midpoint for each treatment. Smaller labeled points represent species weightings for highly significant indicator species (p<0.01) and species with greater than 2% mean cover, with labels corresponding to the first three letters of each genus and species name (see full names in Table 1.1).

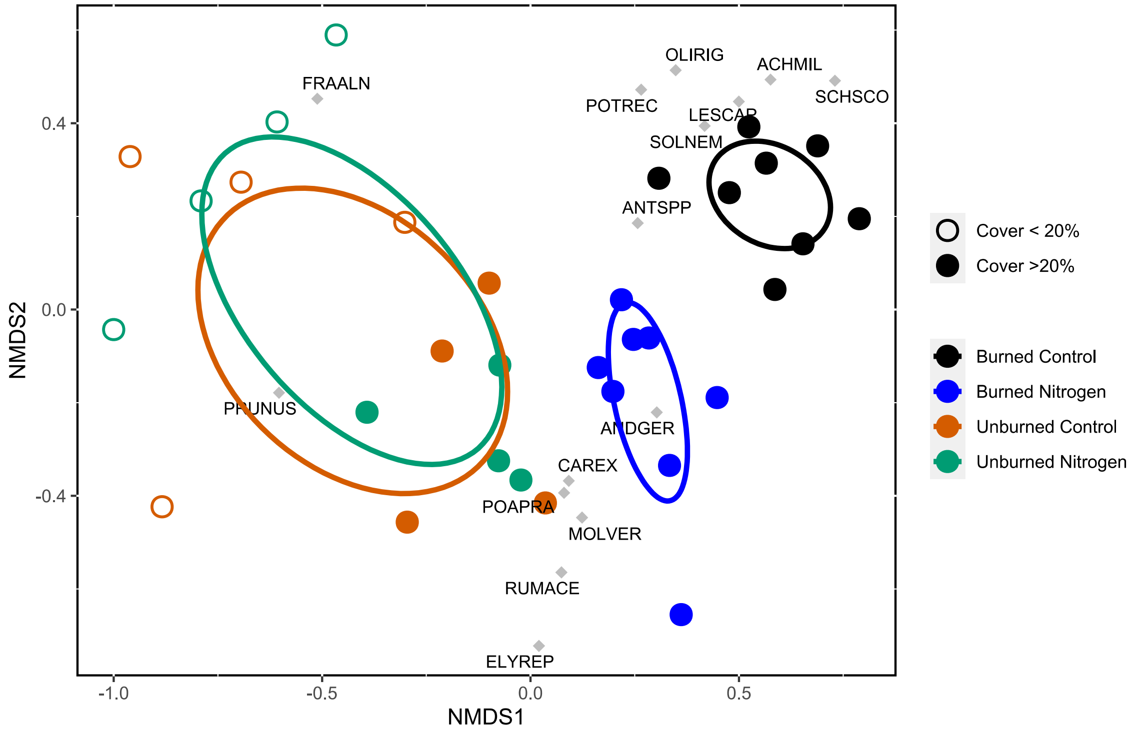
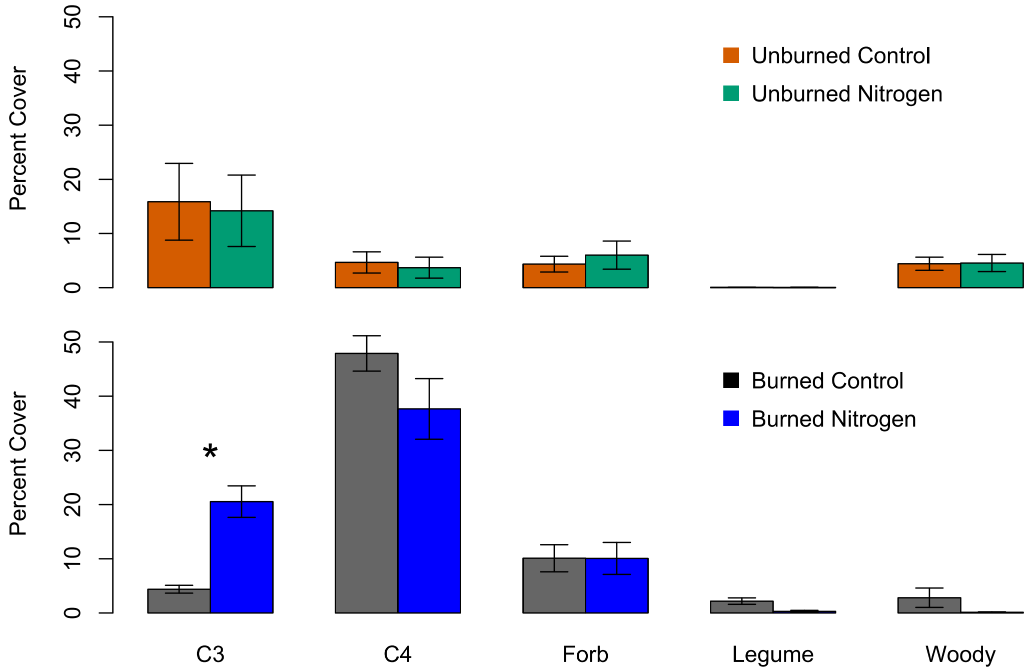


Table 1.1: Indicator and Abundant Species
Species shown are those detected as significant treatment indicators ($p<0.05$), as well as those with an overall average cover of at least 2% across all plots, regardless of indicator status.

Species	**Average Cover Percent**	**Associated Treatment(s)**	**Association Statistic**	***p* value**
Achillea millefolium	0.74	Burned Control	0.802	0.0001
Solidago nemoralis	0.08	Burned Control	0.716	0.0001
Lespedeza capitata	0.41	Burned Control	0.703	0.0005
Oligoneuron rigidum	0.30	Burned Control	0.699	0.0003
Potentilla recta	0.10	Burned Control	0.617	0.0014
Hieracium longipilum	0.02	Burned Control	0.515	0.0422
Dichanthelium villosissimum	0.06	Burned Control	0.448	0.0474
Rudbeckia hirta	0.002	Burned Control	0.430	0.0417
Equisetum sp.	0.02	Burned Control	0.390	0.0492
Anemone cylindrica	0.004	Burned Control	0.375	0.0467
Antennaria sp.	0.24	Burned Control	0.329	0.0079
Ambrosia artemisiifolia	0.57	Burned Nitrogen	0.516	0.0119
Mollugo verticillata	0.03	Burned Nitrogen	0.500	0.0021
Andropogon gerardii	5.68	Burned Nitrogen	0.493	0.0232
Carex sp.	2.78	Burned Nitrogen	0.460	0.0425
Acer rubrum	0.18	Unburned Control	0.541	0.0101
Prunus sp.	0.15	Unburned Control	0.519	0.0076
Solanum dulcamara	0.03	Unburned Control	0.317	0.0454
Schizachyrium scoparium	17.19	Burned Control/ Nitrogen	0.828	0.0001
Cyperus sp.	0.24	Burned Control/ Nitrogen	0.465	0.0226
Frangula alnus	1.16	Unburned Control/Nitrogen	0.580	0.0042
Elymus repens	5.52	None Significant	0.405	0.1043
Poa pratensis	4.52	None Significant	0.248	0.5801
Rumex acetosella	2.03	None Significant	0.259	0.5370

Figure 1.4: Functional Group Response to Fire and Nitrogen Bars indicate the mean cover of each functional group in the corresponding experimental treatment, with error bars indicating the standard errors of those means. Asterisks indicate significant differences between control and nitrogen addition plots within a burn treatment, using the Bonferroni adjusted *p* value of 0.005.



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Chapter 1 Supplementary Material

Image S1.1a. Site map and photograph. Treatment plots are marked on a Google Earth image. Note that pine cover is variable in unburned plots but almost entirely absent from burned plots. The location of the photograph shown below is indicated by a star.

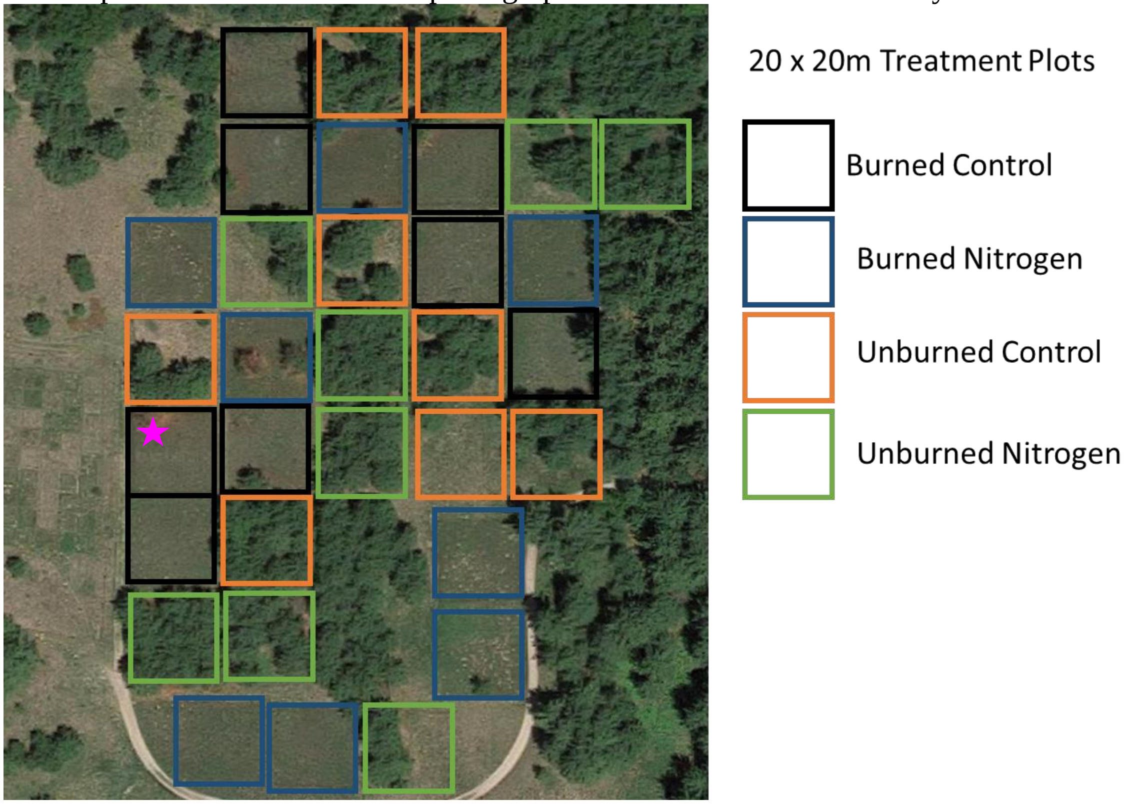


Image S1.2: Treatment Plot Layout

Fire, nitrogen and herbivore fencing treatments were applied to the full 20x20m area. Herbaceous cover surveys were conducted in the 0.5m^2 subplots. Woody stems were censused in the full 20x20m treatment area, with those in the inner 10x10m area used for the main analysis (Table S1.1a/b) and those outside it used in the supplemental analysis (Table S1.1c/d).

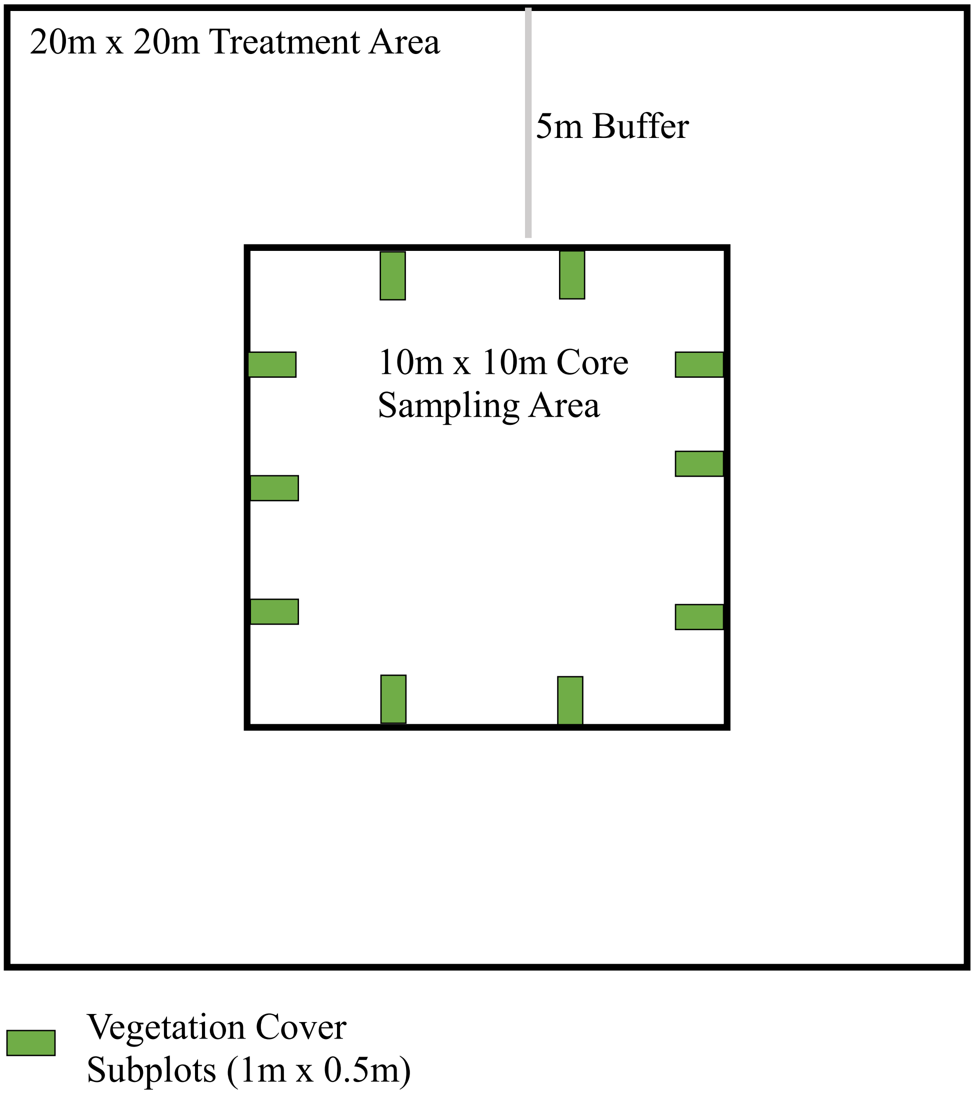


Table S1.1a: Pine Basal Area ANOVA results (10x10m)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	**1**	**17.486**	**0.0003**
Nitrogen	1	1.911	0.180
Fencing	1	0.549	0.466
Fire * Nitrogen	1	1.484	0.235
Fire * Fencing	1	0.332	0.570
Nitrogen * Fencing	1	0.073	0.789
Fire * Nitrogen * Fencing	1	0.011	0.946

Table S1.1b: Pine Stem Count GLM results (10x10m)

	Degrees of Freedom	**LR ChiSq**	***p* value**
Fire	**1**	**46.886**	**<0.0001**
Nitrogen	1	3.392	0.065
Fencing	1	0.793	0.373
Fire * Nitrogen	1	0.391	0.531
Fire * Fencing	1	0.626	0.429
Nitrogen * Fencing	1	1.012	0.314
Fire * Nitrogen * Fencing	1	0.000	0.999

Table S1.1c: Pine Basal Area ANOVA results (20x20m)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	**1**	**47.709**	**<0.0001**
Nitrogen	1	0.481	0.495
Fencing	1	0.000	0.992
Fire * Nitrogen	1	0.420	0.523
Fire * Fencing	1	0.003	0.960
Nitrogen * Fencing	1	0.000	1.000
Fire * Nitrogen * Fencing	1	0.006	0.939

Table S1.1d: Pine Stem Count GLM results (20x20m)

	Degrees of Freedom	**LR ChiSq**	***p* value**
Fire	**1**	**108.178**	**<0.0001**
Nitrogen	1	1.462	0.227
Fencing	1	0.016	0.899
Fire * Nitrogen	1	0.099	0.753
Fire * Fencing	1	0.061	0.804
Nitrogen * Fencing	1	0.284	0.594
Fire * Nitrogen * Fencing	1	0.002	0.962

Table S1.1e: Total Basal Area ANOVA results (10x10m)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	**1**	**20.803**	**0.0001**
Nitrogen	1	1.192	0.286
Fencing	1	0.193	0.664
Fire * Nitrogen	1	0.857	0.364
Fire * Fencing	1	0.075	0.787
Nitrogen * Fencing	1	0.346	0.562
Fire * Nitrogen * Fencing	1	0.179	0.678

Table S1.1f: Total Stem Count GLM results (10x10m)

	Degrees of Freedom	**LR ChiSq**	***p* value**
Fire	**1**	**37.419**	**<0.0001**
Nitrogen	1	1.725	0.1891
Fencing	1	0.183	0.6688
Fire * Nitrogen	1	0.831	0.3620
Fire * Fencing	1	0.275	0.6001
Nitrogen * Fencing	1	2.287	0.1305
Fire * Nitrogen * Fencing	1	2.143	0.1432

Table S1.1g: Total Basal Area ANOVA results (20x20m)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	**1**	**49.771**	**<0.0001**
Nitrogen	1	0.319	0.578
Fencing	1	0.026	0.874
Fire * Nitrogen	1	0.298	0.590
Fire * Fencing	1	0.005	0.944
Nitrogen * Fencing	1	0.009	0.926
Fire * Nitrogen * Fencing	1	0.0001	0.992

Table S1.1h: Pine Stem Count GLM results (20x20m)

	Degrees of Freedom	**LR ChiSq**	***p* value**
Fire	**1**	**93.409**	**<0.0001**
Nitrogen	1	0.002	0.965
Fencing	1	0.733	0.392
Fire * Nitrogen	1	2.555	0.110
Fire * Fencing	1	2.553	0.110
Nitrogen * Fencing	1	0.880	0.348
Fire * Nitrogen * Fencing	1	1.598	0.206

Table S1.2a: Shannon Diversity ANOVA Results

	Degrees of Freedom	**F statistic**	***p* value**
Fire	1	0.042	.839
Nitrogen	1	0.052	.822
Fencing	1	1.034	.319
Fire * Nitrogen	**1**	**13.135**	**.001**
Fire * Fencing	1	0.052	.821
Nitrogen * Fencing	1	3.909	.059
Fire * Nitrogen * Fencing	1	0.395	.535

Table S1.2b: Hill Number (q=1) ANOVA Results

	Degrees of Freedom	**F statistic**	***p* value**
Fire	1	0.013	.911
Nitrogen	1	0.050	.825
Fencing	1	1.009	.325
Fire * Nitrogen	**1**	**10.643**	**.003**
Fire * Fencing	1	0.023	.881
Nitrogen * Fencing	1	3.717	.066
Fire * Nitrogen * Fencing	1	0.025	.877

Table S1.2c: Species Richness ANOVA Results

	Degrees of Freedom	**F statistic**	***p* value**
Fire	1	2.673	.115
Nitrogen	1	2.673	.115
Fencing	1	0.269	.609
Fire * Nitrogen	1	0.040	.844
Fire * Fencing	1	0.460	.504
Nitrogen * Fencing	1	0.574	.456
Fire * Nitrogen * Fencing	1	0.078	.782

Diversity Analyses by Scale: Diversity measurements were produced by an algorithmic process (see Diversity Scale.Rmd at https://figshare.com/s/bcaf81e6b3b2080b0cb2), calculating diversity for every possible combination of n subplots within a given plot and then averaging those results to produce plot level measurements. At a subplot count of n=10, these calculations are equivalent to our original, treatment plot level scale of analysis, with species abundances aggregated across all 10 subplots before being used to calculate diversity (as presented in tables S2a-S2c). At a subplot count of n= 1, diversity is calculated individually for each subplot and then averaged to produce plot level measurements (Tables S3a-S3c). Intermediate subplot counts are presented graphically, with Diversity Scale.R supporting the replication of statistical testing.

Table S1.3a: Shannon Diversity ANOVA Results (Subplot Scale)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	1	3.156	0.088
Nitrogen	1	0.796	0.381
Fencing	1	2.783	0.108
Fire * Nitrogen	**1**	**4.464**	**0.045**
Fire * Fencing	1	1.566	0.223
Nitrogen * Fencing	1	0.697	0.412
Fire * Nitrogen * Fencing	1	0.313	0.581

Table S1.3b: Hill Number (q=1) ANOVA Results (Subplot Scale)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	1	2.501	0.126
Nitrogen	1	1.36	0.254
Fencing	1	2.904	0.101
Fire * Nitrogen	1	3.916	0.059
Fire * Fencing	1	1.292	0.267
Nitrogen * Fencing	1	0.649	0.428
Fire * Nitrogen * Fencing	1	0.075	0.787

Figure S1.1: Shannon Diversity by Treatment and Scale: Each data point represents the mean diversity measurement for a given treatment plot, based on the algorithmic process described above, with “scale” denoting the number of subplot count used for each calculation. Lines connect the mean values for each of the four treatment groups.

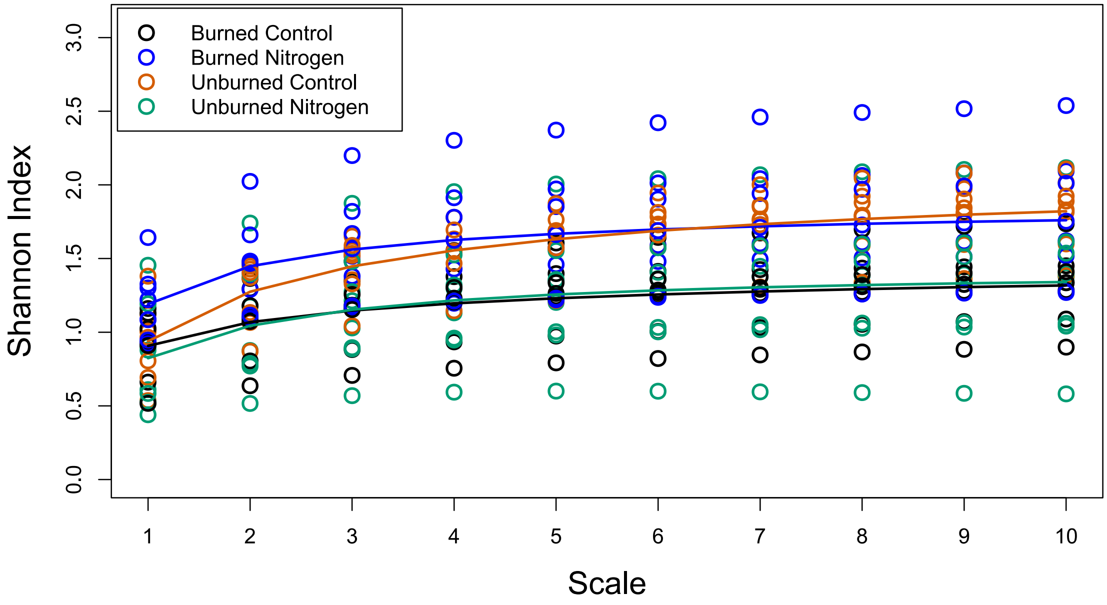


Table S1.3c: Species Richness ANOVA Results (Subplot Scale)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	**1**	**28.546**	**<0.0001**
Nitrogen	1	2.922	0.100
Fencing	1	4.133	0.053
Fire * Nitrogen	1	0.146	0.705
Fire * Fencing	1	0.110	0.743
Nitrogen * Fencing	1	0.285	0.599
Fire * Nitrogen * Fencing	1	0.499	0.487

Figure S1.2: Species Richness by Treatment and Scale: Each data point represents the mean diversity measurement for a given treatment plot, based on the algorithmic process described above, with “scale” denoting the number of subplot count used for each calculation. Lines connect the mean values for each of the four treatment groups.

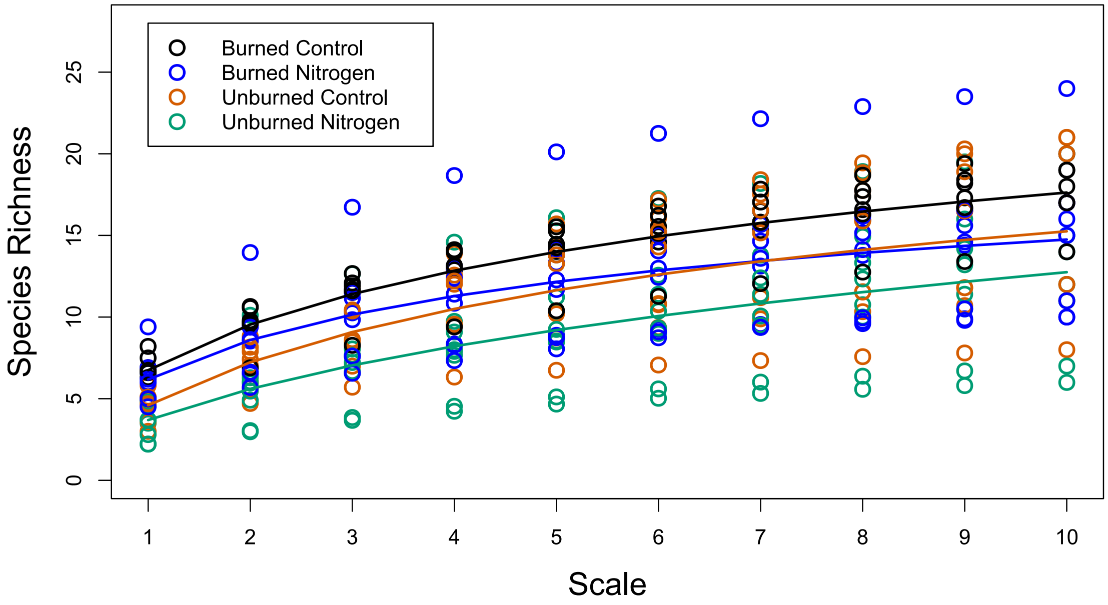


Table S1.3d: Species Richness ANOVA Results (20x20m presence absence data)

	Degrees of Freedom	**F statistic**	***p* value**
Fire	1	1.400	.248
Nitrogen	1	2.488	.128
Fencing	1	0.622	.438
Fire * Nitrogen	1	0.350	.560
Fire * Fencing	1	0.523	.477
Nitrogen * Fencing	1	0.004	.948
Fire * Nitrogen * Fencing	1	1.400	.248

Figure S1.3: Species Richness (20x20m) by Treatment: Each point represents the number of species found in a presence area of a plot's full 20x20m treatment area. Horizontal lines represent the mean values for each treatment, with vertical lines showing the standard errors of those means.

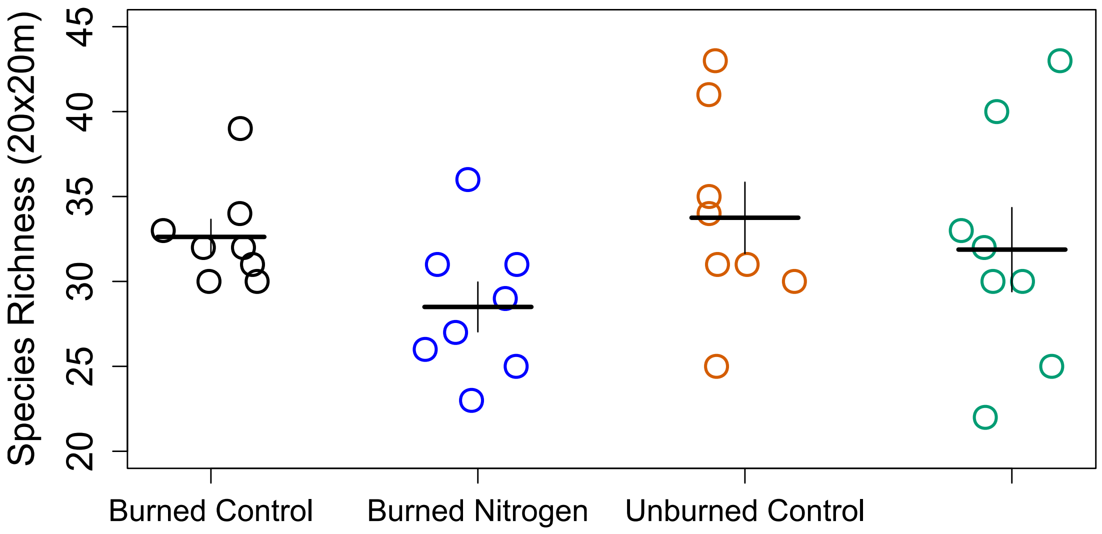


Table S1.4: Community Composition PERMANOVA Results

	Degrees of Freedom	**F statistic**	***p***
Fire	**1**	**11.652**	**.011**
Nitrogen	1	2.121	0.058
Fencing	1	0.738	0.634
Fire * Nitrogen	**1**	**2.571**	**0.031**
Fire * Fencing	1	0.695	0.648
Nitrogen * Fencing	1	0.802	0.548
Fire * Nitrogen * Fencing	1	0.862	0.491

Table S1.5: Functional Group Cover Comparisons

Functional Group	**Treatments**	**t**	**Degrees of Freedom**	***p***
C3	**Burned Control vs. Burned Nitrogen**	**-5.393**	**7.869**	**0.0007**
	Unburned Control vs. Unburned Nitrogen	0.173	13.926	0.865
C4	Burned Control vs. Burned Nitrogen	1.579	11.262	0.142
	Unburned Control vs. Unburned Nitrogen	0.352	13.999	0.730
Forb	Burned Control vs. Burned Nitrogen	0.008	13.631	0.994
	Unburned Control vs. Unburned Nitrogen	-0.559	10.98	0.588
Legume	Burned Control vs. Burned Nitrogen	3.050	8.425	0.0149
	Unburned Control vs. Unburned Nitrogen	0.102	12.916	0.920
Woody	Burned Control vs. Burned Nitrogen	0.824	9.987	0.429
	Unburned Control vs. Unburned Nitrogen	-1.457	7.477	0.188

Nitrogen Mineralization
Data for these analyses were collected in conjunction with sampling in an adjacent field. For methodological details see Li, W., J. J. M. H. Knops, X. Zuo, and R. Laungani. 2014. Carbon and Nitrogen Cycling are Resistant to Fire in Nutrient-Poor Grassland. Soil Science Society of America Journal 78:825–831.

Table S1.6a: 2010 Nitrogen Mineralization Analysis: Gross Ammonification

	Estimated Value	**Degrees of Freedom**	**F statistic**	***p* value**
Intercept	**0.751**	-	-	-
Fire (Unburned Effect)	0.378	1	.314	.580
Nitrogen (Addition Effect)	**0.751**	**1**	**6.090**	**.021**
Fencing (Unfenced Effect)	0.059	1	0.576	.455
Fire * Nitrogen	0.094	1	0.667	.422
Fire * Fencing	-0.115	1	1.540	.227
Nitrogen * Fencing	0.120	1	0.585	.452
Fire * Nitrogen * Fencing	-0.996	1	1.015	.324

Table S1.6b: 2010 Nitrogen Mineralization Analysis: Net Ammonification

	Estimated Value	**Degrees of Freedom**	**F statistic**	***p* value**
Intercept	-.667	-	-	-
Fire (Unburned Effect)	0.478	1	1.474	0.237
Nitrogen (Addition Effect)	0.410	1	0.006	0.940
Fencing (Unfenced Effect)	0.104	1	0.142	0.710
Fire * Nitrogen	-0.708	1	1.223	0.280
Fire * Fencing	-0.193	1	0.210	0.651
Nitrogen * Fencing	-0.482	1	0.177	0.678
Fire * Nitrogen * Fencing	0.687	1	1.089	0.307

Table S1.6c: 2010 Nitrogen Mineralization Analysis: Gross Nitrification

	Estimated Value	**Degrees of Freedom**	**F statistic**	***p* value**
Intercept	0.702	-	-	-
Fire (Unburned Effect)	-0.143	1	2.192	0.101
Nitrogen (Addition Effect)	3.312	1	3.486	0.074
Fencing (Unfenced Effect)	0.087	1	0.457	0.506
Fire * Nitrogen	-2.856	1	2.209	0.150
Fire * Fencing	-0.028	1	0.249	0.622
Nitrogen * Fencing	-1.847	1	0.605	0.444
Fire * Nitrogen * Fencing	1.479	1	0.269	0.608

Table S1.6d: 2010 Nitrogen Mineralization Analysis: Net Nitrification

	Estimated Value	**Degrees of Freedom**	**F statistic**	***p* value**	
Intercept	0.326	-	-	-	
Fire (Unburned Effect)	0.099	1	0.205	0.655	
Nitrogen (Addition Effect)	-0.085	1	0.178	0.677	
Fencing (Unfenced Effect)	0.067	1	2.997	0.096	
Fire * Nitrogen	-0.568	1	0.855	0.364	
Fire * Fencing	-0.008	1	0.220	0.643	
Nitrogen * Fencing	0.377	1	2.004	0.170	
Fire * Nitrogen * Fencing	0.393	1	0.240	0.629	

Chapter 2

PINUS STROBUS ENCROACHMENT OVER 17 YEARS INCREASES ABOVEGROUND CARBON BUT DOES NOT ALTER SOIL CARBON IN MINNESOTA OLD FIELD GRASSLANDS

Abstract

Woody encroachment into grassland ecosystems is a globally widespread phenomenon. In addition to localized biodiversity effects, such transitions also have the potential to alter ecosystem carbon storage. Past research, however, has shown that such effects can vary dramatically in magnitude and direction. In this study we sought to determine the effects of pine encroachment on ecosystem carbon in Minnesota old field grasslands. Following 20 years of experimental manipulation, we sampled above and belowground carbon pools in unburned, pine-encroached plots and in burned plots where pine encroachment was suppressed. We found that unburned plots exceeded burned plots in total ecosystem carbon. This effect, however, was driven primarily by aboveground carbon storage in litter and pine trees. Soil carbon, by far the largest ecosystem pool overall, showed no significant differences across experimental treatments. Nitrogen addition and deer herbivory did not significantly alter pine encroachment or ecosystem carbon pools. These results indicate that carbon storage changes driven by pine encroachment onto old field grassland ecosystems are likely to be small in magnitude and contingent upon the continued absence of fire.

Introduction

Anthropogenic drivers, both local and global, are contributing to widespread transitions between ecosystem types (Zinnert et al. 2021). In addition to local effects on conservation and land use, managing these transitions may alter ecosystems' carbon storage, either accelerating increases in atmospheric CO_2 or contributing to natural climate solutions (Griscom et al. 2017, Fargione et al. 2018). Understanding carbon storage during ecosystem transitions thus plays an important role in accurately projecting changes to global carbon balance and in limiting increases to atmospheric CO_2 and associated climate effects.

In evaluating the effects of ecosystem transitions on carbon storage, it is essential that the full range of carbon pools be considered. While aboveground biomass is subject to the most obvious changes as ecosystems transition from one state to another, belowground biomass frequently exceeds aboveground biomass, and root/shoot ratios can vary dramatically across ecosystem types (Jackson et al. 1996). Belowground biomass is also more likely than aboveground biomass to be converted to soil carbon (Jackson et al. 2017, Sokol and Bradford 2019), which itself dwarfs plant carbon as a component of ecosystem carbon storage (Batjes 1996, Amundson 2001) and can differ by more than an order of magnitude across ecosystem types, with estimates ranging from 1.4kg/m^2 in temperate deserts to over 70 kg/m^2 in wetlands (Post et al. 1982). Estimates of soil carbon are, unfortunately, subject to substantial uncertainty. While the majority of soil carbon sampling occurs in the upper 10-30 cm of soil, deeper soils may contain as much as half of soil carbon (Knops and Bradley 2009) and may show distinct responses from shallow soils (Yang and Knops 2023), making them essential to accurately quantifying soil carbon pools.

Woody encroachment is a widespread form of ecosystem transition, occurring globally across a broad range of grass-dominated ecosystems (Archer et al. 2017, Stevens et al. 2017), with satellite data suggesting a 7% increase in global tree cover since 1982 (Song et al. 2018). While global factors, including the effects of atmospheric CO_2 on competition between C3 trees and C4 grasses (Morgan et al. 2007), appear to contribute to this phenomenon (Wigley et al. 2010, Stevens et al. 2016, Venter et al. 2018), more local factors can act as important controls. Fire limits the capacity of woody plants to successfully invade many ecosystems, and its suppression can enable or accelerate woody encroachment (Ratajczak et al. 2014, Miller et al. 2017). Herbivory regimes may also have significant effects, either independently (Inouye et al. 1994, Ritchie et al. 1998), or interactively with fire (Briggs et al. 2002, O’Connor et al. 2019). Woody encroachment into grassland ecosystems tends to increase aboveground standing biomass and thus aboveground carbon storage (McKinley and Blair 2008, Zhou et al. 2022), though the amount of carbon stored and the rate at which it accumulates will vary by species (Cannell 1996). Global comparisons also indicate generally higher root biomass in forests than grasslands (Jackson et al. 1996), though when comparing sites on a local basis, woody encroachment may reduce root biomass and carbon (Liu et al. 2020).

Woody encroachment also has the potential to alter soil carbon, with a number of studies finding evidence of increasing soil carbon with these transitions. In Zambian savannas (Blaser et al. 2014) and Oklahoma (USA) tallgrass prairies, soil carbon increases in the immediate vicinity of woody plants (Biral et al. 2019). In comparisons of management areas with and without encroachment, woody encroachment appears to increase soil carbon in the Hebei province of China (Wang et al. 2013) and the Flint Hills of Kansas, USA (McKinley and Blair 2008). At the latter site, this effect has been associated with fire control of woody vegetation, and isotopic analysis indicates that the increased carbon in unburned areas is derived from C3 plants (Connell et al. 2020). This pattern is not universal, however. In a long-term manipulation of fire regimes in South Africa's Kruger National Park, Zhou and colleagues (2002) found no significant changes in soil organic carbon and thus a smaller than expected change in total ecosystem carbon. Similarly, experimental removal of encroaching redcedar (*J. occidentalis*) in Oregon rangelands has no effect on soil carbon, nor on total ecosystem carbon despite opposing changes in aboveground and root biomass (Abdallah et al. 2020). And while woody encroachment is often inhibited by fire, fire itself can reduce soil carbon when occurring at high frequencies (Pellegrini et al. 2018, 2020).

Site to site variation in the effects of woody encroachment on soil carbon may, in part, be explained by water availability. High precipitation sites often show losses of soil carbon with woody encroachment, while lower precipitation sites tend to gain soil carbon (Jackson et al. 2002, Barger et al. 2011). High precipitation sites, however, tend to show greater increases in aboveground biomass and productivity with woody encroachment (Barger et al. 2011, Liu et al. 2020). Despite the potentially offsetting effects of soil carbon, the magnitude of aboveground carbon increases suggests that mesic grasslands, such as those in the eastern portions of the North American Great Plains region, are likely to act as carbon sinks when undergoing woody encroachment (Barger et al. 2011).

Effects of woody encroachment on ecosystem carbon may also be dependent on nitrogen availability. While resource limitation patterns vary across grasslands, nitrogen limitation is common to many sites, including our study site, Cedar Creek Ecosystem Science Reserve (Tilman 1987, Harpole and Tilman 2006, Isbell et al. 2013, Fay et al. 2015). At such sites, productivity, biomass and plant carbon can be expected to increase with nitrogen availability. Nitrogen effects in forest systems are less frequently tested experimentally (Verheyen et al. 2012, Perring et al. 2018), but it appears that the effects of this nutrient may be less consistent than in grasslands (Midolo et al. 2019). Nitrogen availability also appears to limit the accumulation of soil carbon (Tilman et al. 1996, Knops and Tilman 2000), with water and nitrogen playing interactive roles in some systems (Harpole et al. 2007).

A key limitation of existing research into the effects of woody encroachment on ecosystem carbon is the prevalence of observational studies often making comparisons across a small number of study sites. Here, we explore these dynamics in a well replicated field experiment, factorially manipulating fire, nitrogen and herbivory across a total of 32 study plots, and conducting comprehensive measurements of ecosystem carbon and nitrogen following 20 years of treatment. Through this experiment, we aimed to better quantify the effects of woody encroachment, specifically the encroachment of *Pinus strobus* (Eastern White Pine) into Minnesota (USA) old field grasslands, on ecosystem carbon storage, and to test whether these effects are contingent on nitrogen availability or herbivore access.

Methods

Study Site

Our experiment was conducted at Cedar Creek Ecosystem Science Reserve, East Bethel, Minnesota (USA,42º25’N, 93º10’W) in Field B, a successional grassland abandoned from agriculture in 1957. Cedar Creek’s soils, derived from glacial outwash, are sandy and low in nitrogen (Grigal et al. 1974), with an estimated 89% and 75% of original soil carbon and nitrogen, respectively, lost during agriculture (Knops and Tilman 2000). Within Field B, fire treatment does not significantly alter soil carbon (Li et al. 2014) and has minimal effects on plant community composition (Knops 2006, Li et al. 2013), but these effects have not previously been tested in areas undergoing pine encroachment.

Experimental Design

Our experiment was established in 2000, with thirty-two 20x20m plots assigned to factorial combinations of burning, nitrogen and herbivory treatments. Prescribed burning in designated plots began in 2002 and was repeated in 2003, 2006, 2009, 2017 and 2018. Nitrogen was added annually as ammonium nitrate, at a rate of 4g fertilizer/m^2 (1.4gN/m^2), which is among the lower rates of nitrogen addition used in experiments at this site (Tilman 1987, Isbell et al. 2013, Wilcots et al. 2021) but comparable to the rate of atmospheric nitrogen deposition in the region (Ackerman et al. 2019). Herbivore fences, constructed with 2x4in. opening hardware cloth to a height of 6 feet, were designed to exclude White-tailed Deer (*Odocoileus virginianus*) but allow smaller herbivores to access the plots.

In 2018, we conducted measurements designed to estimate all pools of ecosystem carbon and nitrogen (hereafter, CN). To estimate the CN content of woody plants, we censused all woody stems in the treatment plots with a basal diameter of at least 2cm, recording basal diameter and DBH. Carbon content in kilograms for each stem was estimated as $0.005(\text{Basal Diameter})^{1.96}$, with nitrogen content in grams as $0.05(\text{Basal Diameter})^{1.88}$, based on allometric equations calibrated by destructive sampling (J.M.H. Knops, unpublished data). These estimates were then summed within each treatment plot to produce plot-level estimated of woody carbon and nitrogen.

For litter and herbaceous plant CN, we clipped standing biomass and collected litter in two transects per treatment plot, each measuring 10cm in width by 3m in length. Clipped biomass was sorted to functional group, dried at 60ºC and weighed. Following weighing, a subsample from each plot-functional group combination was ground and analyzed for percent CN content using a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies, Valencia, CA). We multiplied these percentages by the biomass measurements to compute total carbon and nitrogen in the clipped samples. In cases where plot level CN percentages were not available (some samples were too small for grinding and analysis), we instead used average values for the corresponding functional group. Samples requiring such extrapolation represented only 1.6% of biomass. For belowground plant CN, we collected 10cm diameter root cores at three points on each biomass transect. At each coring point, samples were collected for 5 depth categories: 0-

10cm, 10-20cm, 20-40cm, 40-60cm and 60-100cm. After washing to remove soil, the samples of each depth were aggregated by treatment plot and then dried, weighed and analyzed in the same manner as the aboveground biomass samples.

To calculate soil CN, we collected soil cores at 9 points in each treatment plot. Each core was 1cm in diameter and 1m in depth, partitioned into the same five depth categories as the root samples. Each sample was individually sieved, ground and analyzed for percent CN content using the Costech elemental analyzer. To calculate mass on a per volume basis, we estimated soil bulk density (g/cm^3) as 1.509 - 0.000103* soil nitrogen (mg/kg), a formula previously calibrated for Cedar Creek's soils (Wedin and Tilman 1990).

For analysis purposes measurements of each carbon and nitrogen pool were standardized to grams of carbon or nitrogen per square meter and averaged at the treatment plot level. We then applied ANOVAs treating each carbon and nitrogen pool as a response variable, along with total ecosystem carbon and the corresponding C:N ratios. For these analyses, soil and root carbon were summed across all depths. Our three treatment variables (fire, nitrogen and herbivory) and their interactions served as predictor variables.

To evaluate the effects of depth and depth by treatment interactions on soil and root carbon, we constructed linear mixed models treating depth as a categorical variable interacting with the three treatment variables as fixed effects, with a random effect of plot to account for correlations in carbon values at different soil and root depths in the same plot. To evaluate carbon changes over time, we used unpublished woody census data from 2001 and soil CN data from 2002 and 2009, collected using the same methods described above. We then calculated woody and soil carbon change over time, which we treated as an ANOVA response variable. For this analysis, only soils from 0-40cm depth were considered, as deeper soils were not sampled in 2002 or 2009. For all analyses we used R version 4.0.5 (R Core Team 2021), including the lme4 package used for mixed models (Bates et al. 2015).

Results

Total ecosystem carbon averaged 6800 g/m^2 in unburned plots, compared to 5700g/m^2 in burned plots (F=14.5, df=1, p=0.0009). Burning reduced woody carbon from 380g/m^2 to 18g/m^2 (F= 49.5, df=1, p<0.001) litter carbon from 480g/m^2 to 1.5g/m^2 (F=50.5, df=1, p<0.001) and root carbon from 480g/m^2 to 320g/m^2 (F=20.0, df=1, p=0.0002) relative to unburned plots. Burning increased herbaceous plant carbon from an average of 24g/m^2 to 66g/m^2 (F=24.2, df=1, p<0.001), while total soil carbon showed no significant variation across treatments, with an average value of 4700g/m^2. No carbon pool responded significantly to nitrogen addition or herbivory, nor to any interaction between the treatment variables (Figure 2.1).

These patterns of soil and woody carbon response remained consistent when considering carbon change over time. The woody carbon change in unburned plots, at +360g/m^2 total or +21g/m^2 per year dramatically exceeded woody carbon change in burned plots at -6.5g/m^2 total (F=53.5, df=1, p<0.001). Soil carbon increased by an average of 470g/m^2 (29g/m^2 per year) between 2002 and 2016, with no significant treatment differences in accumulation rate (Figure 2.3).

Incorporating depth into soil carbon analyses revealed a significant interaction between depth and fire treatment, with burned, grass dominated plots showing higher carbon measurements than unburned plots in shallow soils but lower carbon measurements in deeper soils (χ^2=18.8, df=4, *p*=0.0009), corresponding with a similar pattern in root carbon (χ^2=51.8, df=4, *p*<0.001, Figure 2.4). No other treatment interacted significantly with depth.

As with carbon, nitrogen was elevated in unburned plots in litter (F=95.8, df=1, *p*<0.001) and woody plants (F=49.0, df=1, *p*<0.001). Total root nitrogen increased both in the absence of fire (F=26.7, df=1, *p*<0.001) and with nitrogen addition (F=11.5, df=1, *p*=0.002). Soil nitrogen (F=3.37, df=1, *p*=0.079) and total ecosystem nitrogen (F=3.99, df=1, *p*=0.057) showed higher average values in nitrogen addition plots, though these patterns were only marginally significant. Herbaceous nitrogen did not vary across experimental treatments (Figure 2.5).

In addition to carbon and nitrogen themselves, C:N ratios varied with fire and nitrogen addition treatments for several of the ecosystem pools (Figure 2.6). Soil carbon responded interactively to these variables (F=4.6, df=1, *p*=0.04), with the highest C:N ratios occurring in unfertilized, unburned plots. Herbaceous biomass (F=5.3, df=1, *p*=0.04), roots (F=7.6, df=1, *p*=0.01), and litter (F=5.0, df=1, *p*=0.04) likewise showed interactive responses, with nitrogen addition reducing C:N ratios in burned plots.

Discussion

We found that limitation by fire of pine encroachment into old field grasslands had no significant effect on soil carbon, which constitutes by far the largest carbon pool in our study ecosystem. Unburned plots did, however, surpass burned plots in litter, root and aboveground plant carbon, though the comparatively small scale of these carbon pools means that ecosystem carbon differed by less than 20% between these treatments. No carbon pool responded to herbivory or nitrogen addition, either independently or interactively with fire.

The changes in aboveground carbon match our expectations based on changes in the plant community. Burned plots in this experiment are dominated by herbaceous vegetation while many unburned plots have transitioned to a savanna or forest state (see Chapter 1). The greater herbaceous plant carbon observed in burned plots and greater pine carbon observed in unburned plots matches expectations based on these community effects, with the latter pool greatly exceeding the former due to the accumulation of carbon in woody tissue. Interestingly, carbon in plant litter plays a similarly large role in distinguishing the ecosystems. These differences were likely exaggerated by the timing of the 2018 burn treatment, which consumed most litter in burned plots. However, over 80% of litter in unburned plots was made up of pine needles and woody debris, which we would expect to be largely absent from burned plots even in sampling years without fire.

The lack of change in total soil carbon contrasts with those studies finding increased levels of soil carbon with woody encroachment (McKinley and Blair 2008, Wang et al. 2013, Blaser et al. 2014, Biral et al. 2019) but consistent with studies that do not detect such an effect (Abdallah et al. 2020, Zhou et al. 2022). Though some research suggests woody encroachment may reduce soil carbon at higher rainfall sites (Barger et al. 2011, Liu et al. 2020), we do not find evidence of such a pattern when considering total soil carbon to a depth of 100cm. Although total soil carbon did not vary across treatments, the depth distribution of that carbon did vary, with more carbon present in shallow soils in burned plots dominated by herbaceous vegetation and more carbon present in deeper soils in the unburned plots undergoing pine encroachment. Root carbon follows a similar pattern, suggesting that carbon introduced to soil through root exudates and turnover may explain the pattern of carbon distribution across soil depths.

Despite the expectation that soil carbon is limited by soil nitrogen (Tilman et al. 1996, Knops and Tilman 2000, Harpole et al. 2007) and that productivity at Cedar Creek is nitrogen limited (Tilman 1987, Harpole and Tilman 2006, Isbell et al. 2013, Fay et al. 2015), we did not detect an effect of nitrogen addition on any ecosystem carbon pool. The effects of increased ecosystem nitrogen appear to have manifested primarily as reductions in plant and soil C:N ratios rather than increased carbon storage. While it is possible that higher rates of nitrogen addition might lead to increases in ecosystem carbon, the rates used in this experiment are comparable to atmospheric nitrogen deposition rates (Ackerman et al. 2019) and may thus be more relevant in the context of predicting ecosystem dynamics than the higher rates used in many ecological studies.

Herbivory likewise failed to generate significant effects on carbon storage. While past research had found that deer herbivory decreases woody encroachment at Cedar Creek (Inouye et al. 1994, Ritchie et al. 1998), management changes prior to the start of this experiment appear to have reduced the size of the deer population and its effect on plant community dynamics (Wheeler et al. in review). In the absence of effects of woody plant abundance, deer herbivory did not significantly alter ecosystem carbon.

It should also be noted that the effects of woody encroachment may be contingent on the encroaching species and the composition of the grassland community. The results of our study align closely with research at Cedar Creek focusing on carbon storage in monocultures (Laungani and Knops 2009). This study found that *Pinus strobus* decreased soil carbon by 16%, while sequestering carbon in aboveground tissues. A similar decline in soil carbon, however, was detected when *Schizachyrium scoparium*, a perennial C4 grass among the site’s strongest nitrogen competitors (Wedin and Tilman 1993, Fargione and Tilman 2005), was grown in monoculture. In our experiment *Schizachyrium scoparium* was the most abundant species in burned plots, representing 46% of surveyed plant cover. This comparison suggests that the lack of soil carbon response in our experiment may be driven largely by species-specific dynamics and that *Pinus strobus* invasions into more diverse grasslands might result in a net loss of soil carbon.

Overall, we detected no effect of pine encroachment on soil carbon, and patterns of change at shallow soil depths and comparisons to monoculture studies suggest that if effects occur at magnitudes too small to be detected by our sample size or in communities less heavily dominated by *Schizachyrium scoparium*, they are more likely to be negative than positive. Unburned, pine encroached plots stored substantially more carbon aboveground, though it should be noted that carbon stored in aboveground pools is more vulnerable to loss through management changes or wildfire (Anderegg et al. 2020, Wu et al. 2023). The variable ecosystem carbon effects detected by studies of woody encroachment into grasslands suggest that these effects must be understood in light of local conditions and species characteristics. With pine encroachment into Minnesota old-field grasslands, additional carbon storage will largely be limited to that occurring in aboveground tissues and litter.

Figure 2.1. Carbon variation across treatments. Each panel shows 2018 measurements of the corresponding carbon pool, standardized to units of g/m^2. Note the differences in scale among panels. Data points represent measurements for individual treatment plots, colored according to their fire and nitrogen treatments. Horizontal represent mean values for each fire x nitrogen treatment combination, with vertical lines representing standard errors of those means.

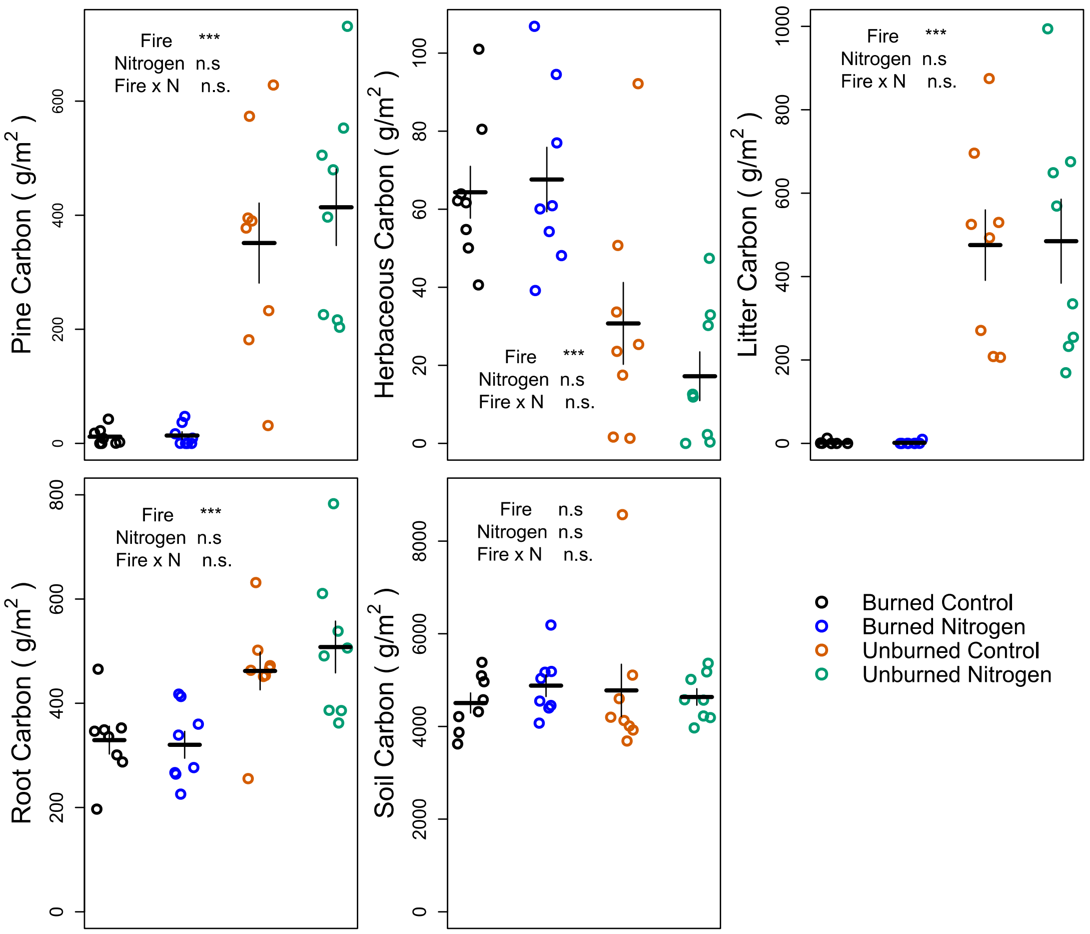


Figure 2.2. Total ecosystem carbon across treatments. Standardized carbon measurements from each pool in Figure 2.1 are shown on a single axis. Note that, while most carbon resides in belowground pools, the differences between burned and unburned plots are driven by pine and litter carbon.

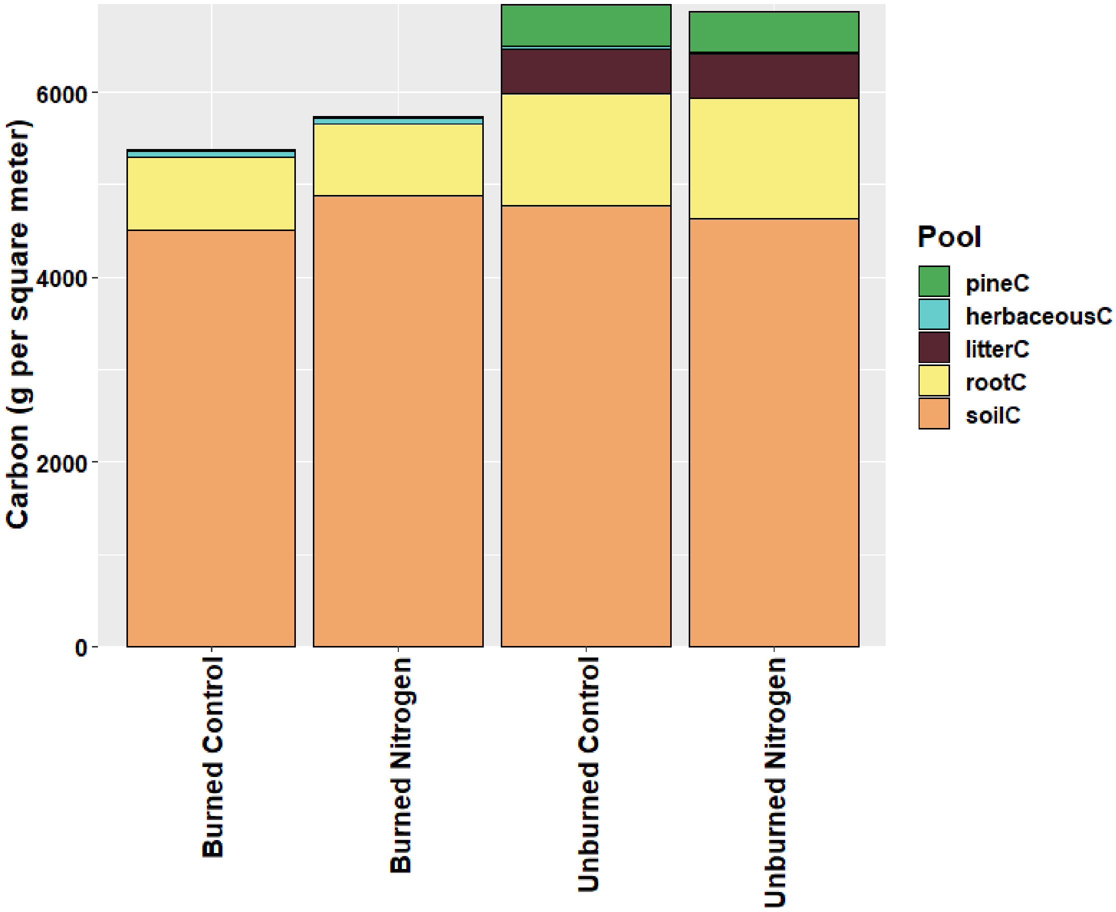


Figure 2.3. Woody and soil carbon change over time. Each point represents carbon measurements for a single treatment plot in the corresponding year and treatment (note that pine measurements are in 2001 and 2018). Pine carbon increases over time only in unburned plots, while trends in soil carbon do not significantly vary across treatments.

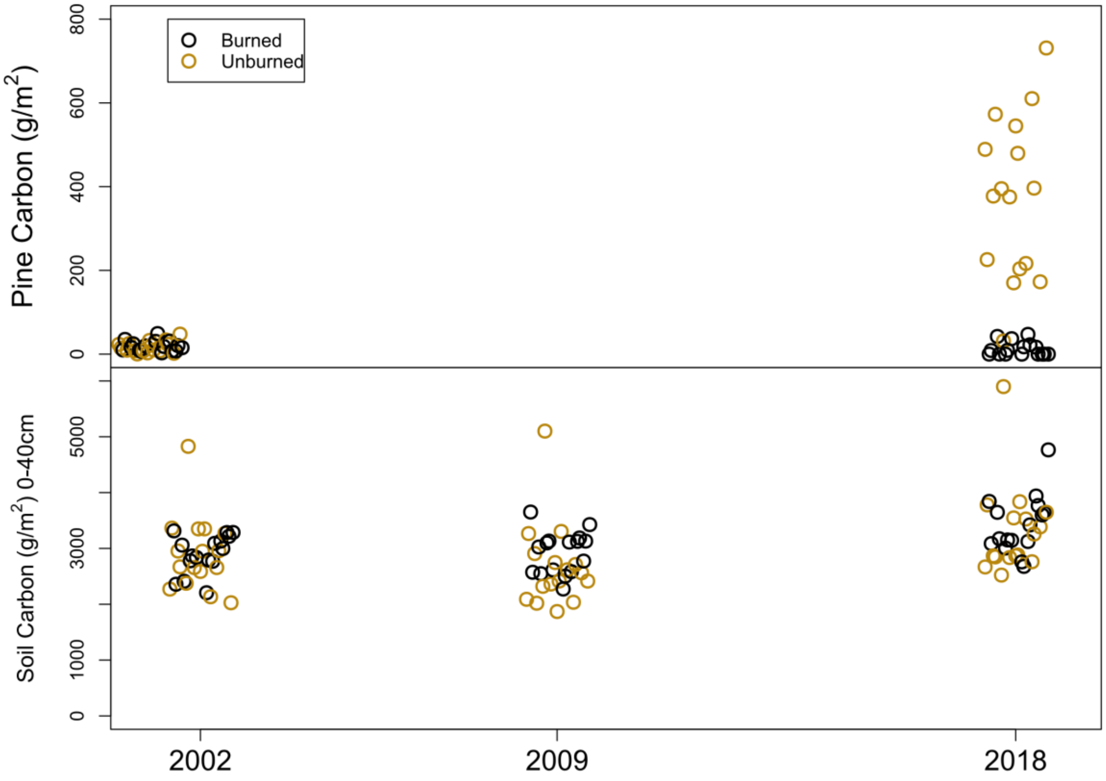


Figure 2.4. Soil and root carbon by depth. Each point corresponds to 2018 carbon measurements in a single plot, averaged across 9 samples for soil or 2 transects for roots at each depth. Point spacing on the x-axis corresponds to the midpoint of each depth category. For both soil and roots, carbon in burned plots is elevated at shallow depths.

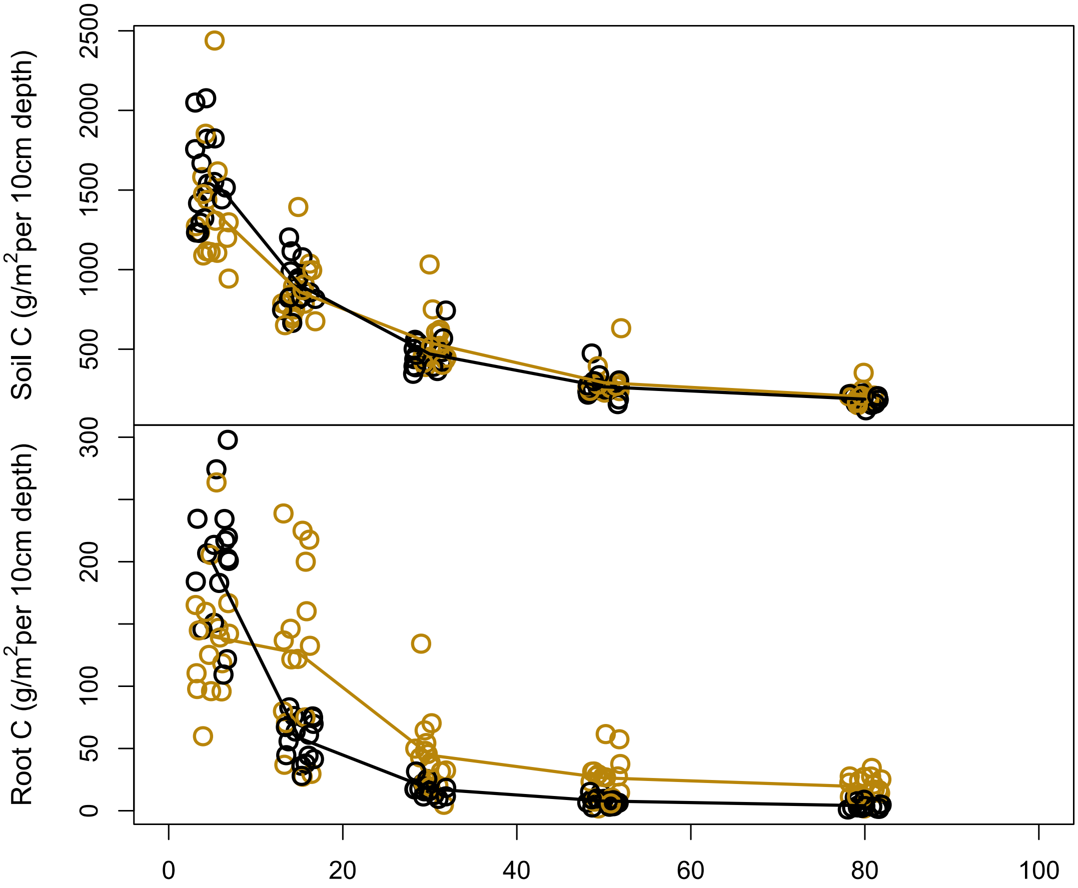


Figure 2.5: Nitrogen variation across treatments. Each panel shows 2018 measurements of the corresponding nitrogen pool, standardized to units of g/m^2. Note the differences in scale among panels. Data points represent measurements for individual treatment plots, colored according to their fire and nitrogen treatments. Horizontal represent mean values for each fire x nitrogen treatment combination, with vertical lines representing standard errors of those means.

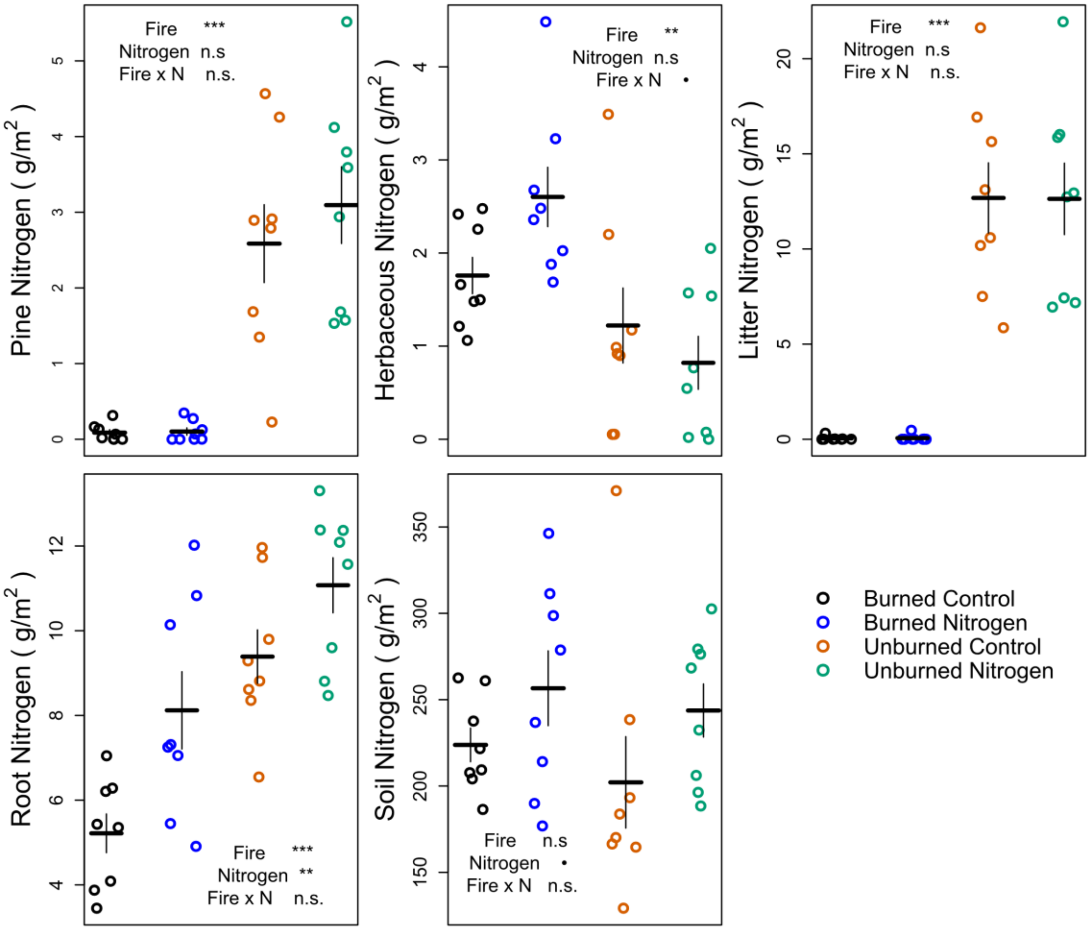
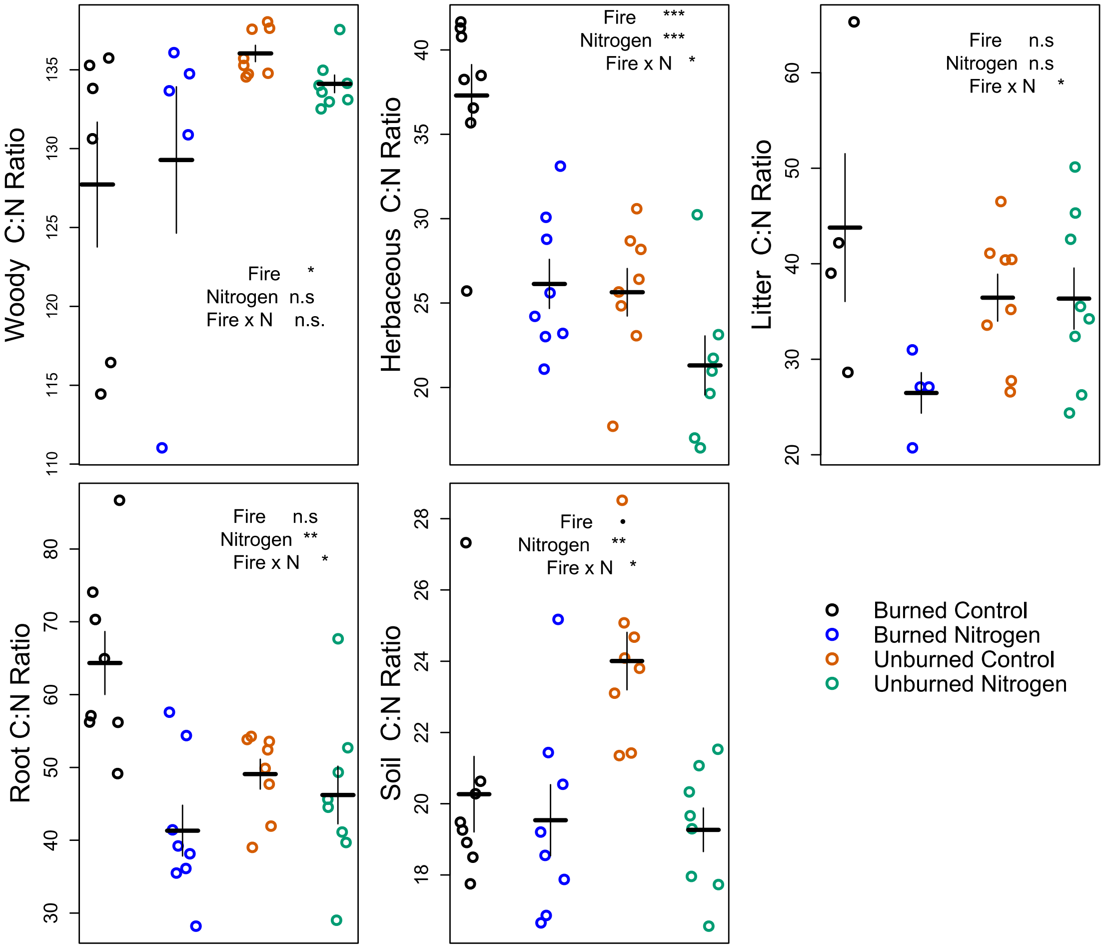


Figure 2.6: Carbon:nitrogen ratios across treatments. Data points represent 2018 measurements for individual treatment plots, colored according to their fire and nitrogen treatments. Horizontal represent mean values for each fire x nitrogen treatment combination, with vertical lines representing standard errors of those means.



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Chapter 3

FUNCTIONAL TRAITS' ANNUAL VARIATION EXCEEDS NITROGEN-DRIVEN VARIATION IN GRASSLAND PLANT SPECIES

Abstract

Effective application of functional trait approaches to ecological questions requires understanding the patterns of trait variation within species as well as between them. However, few studies address the potential for intraspecific variation to occur on a temporal basis, and thus for trait-based findings to be contingent upon sampling year. To quantify annual variation in the functional traits of grassland plant species, we measured specific leaf area, leaf dry matter content, plant height, and chlorophyll content in twelve shortgrass prairie plant species. We repeated these measurements across four years, both in long-term nitrogen addition plots and in corresponding control plots. Three of the four traits showed significant year-to-year variation in a linear mixed model analysis, generally following a pattern of more acquisitive leaf economics spectrum traits in higher rainfall years. Furthermore, two of the measured traits responded interactively to nitrogen addition and sampling year, though only one, leaf dry matter content, showed the expected pattern of stronger nitrogen responses in high rainfall years. For leaf dry matter content and specific leaf area, trait responses to sampling year were larger than responses to the nitrogen addition treatment. These findings illustrate that species' functional traits can respond strongly to environmental changes across years, and thus that trait variation

in a species or community is likely to extend beyond the values and patterns observed in any single year.

Introduction

Functional trait measurements have emerged as a powerful tool in addressing a broad range of ecological questions. Considered at the species level, functional traits can predict the distributions of organisms and the interactions between them. Considered at the community level, these same traits can predict a wide range of ecosystem functions (Funk et al. 2017). As such patterns are not restricted to particular taxa, analyses based on functional traits may prove a more powerful avenue towards finding generalizable ecological rules than "nomenclatural", or species-based comparisons (McGill et al. 2006).

Functional traits' associations with ecological trade-offs suggest that organisms with particular combinations of traits should thrive under corresponding sets of environmental conditions. The leaf economics spectrum in plants, described at a biogeographical level by Wright and colleagues (2004) and quickly extended to smaller-scale comparisons (Garnier et al. 2007, Gross et al. 2007, McIntyre and Lavorel 2007), is arguably the best studied example of such a pattern: under cool, dry and nutrient-poor conditions, plants tend to produce slow growing, long-lived leaves. This strategy and its associated trait values, including low nitrogen content, low specific leaf area (SLA), and high leaf dry matter content (LDMC) are described as conservative, while the opposing strategy and associated trait values, found under warmer, wetter and more nutrient-rich conditions, are described as acquisitive.

Applications of functional traits to ecological questions depend largely on trait comparisons at the species level. For these purposes, species average trait values may be either calculated locally or drawn from global databases such as TRY (Kattge et al. 2011). However, such comparisons are complicated by the large portion of functional trait variation that occurs within species. According to a meta-analysis by Siefert and colleagues (2015), intraspecific trait variation makes up 25% of functional trait variation within plant communities and 32% of the functional trait variation between communities. Whether through plastic environmental response (Reich et al. 1996, Vitasse et al. 2010), selection on genetic variants, or a combination of both processes (Olsen et al. 2013), intraspecific variation can be expected to produce a pattern of individual-level trait values better suited to local conditions than species-level averages. For instance, nutrient addition experiments (La Pierre and Smith 2015, Siefert and Ritchie 2016, Tatarko and Knops 2018) show that individuals of a given species exhibit more acquisitive leaf economics spectrum traits under nutrient enrichment treatments than conspecifics under ambient conditions. Similarly, intraspecific trait variation often corresponds to patterns of water availability or precipitation (Olsen et al. 2013, Guo et al. 2017, Nunes et al. 2017, Yue et al. 2019).

As environmental conditions vary across years, functional trait measurements may be expected to vary on an annual basis, even when sampling areas and methodology remain constant. A study by Garnier and colleagues (2001) focused on quantifying trait variation across temporal and spatial scales, and several subsequent studies have documented trait differences between pairs of study years (Tatarko and Knops 2018, Chen et al. 2019, Luo et al. 2019). Though the causes of such changes can rarely be directly tested, climatic variation appears the most likely cause.

Annual variation in species’ traits may play an important role in their interactions with one another and with the broader environment. If not properly accounted for, however, this variation could undermine the assumptions trait-based analysis depends upon (Shipley et al. 2016), resulting in mistaken extrapolations and causing important community dynamics to be overlooked. Analyses extrapolating from a single set of species-level trait measurements would, for instance, fail to detect the plastic effects that contribute to community level change (La Pierre and Smith 2015) if that change occurred on a temporal basis. Even explicit measurements of trait variability could be substantially underestimated if derived based on sampling at a single point in time, and comparisons of trait values between sites could be contingent on sampling conditions. Temporal variation might also have interactive effects with other variables of interest, analogous to biomass effects in water/nutrient colimited systems (Wang et al. 2017).

More detailed knowledge of year-to-year trait variation will allow us to better understand how much variation is undetected in single-year sampling and how likely such variation is to alter ecological processes and analyses. Currently, however, studies of year-to-year intraspecific variation are rare, with existing multi-year studies considering only two years of trait data (Garnier et al. 2001, Tatarko and Knops 2018, Chen et al. 2019) or focusing their data collection and analysis on community-weighted, rather than intraspecific change (Tatarko and Knops 2018, Chen et al. 2019, Luo et al. 2019). A larger temporal sample should allow for variability estimates less sensitive to the individual years chosen, as well as more detailed comparisons of trait differences to possible explanatory factors. We therefore chose to monitor intraspecific trait variation across four study years in a North American grassland site. Through this sampling, we sought to answer the following ecological questions:

1.) How variable are plant species' functional traits across years? Based on existing two-year studies, we predicted that trait variation between years could approach, or even exceed, the level of variation seen between fertilized and unfertilized study plots.
2.) Does intraspecific trait variation correspond to environmental variation? We anticipated that trait values would respond to growing season precipitation, with plant species displaying more conservative trait values under low rainfall conditions.
3.) Do trait responses to nutrient availability depend on sampling year? Based on productivity responses observed at our study site (Wang et al. 2017), we expected that nitrogen addition and study year would affect species' traits interactively, with stronger nitrogen effects apparent in high rainfall years.

Methods

To address these questions, we conducted repeated surveys of functional traits in study plots at Cedar Point Biological Station in western Nebraska, USA (41°12' N, 101°38' W). These study plots occur in shortgrass prairie on bluffs overlooking Lake Ogallala and were established in 2007 in association with the Nutrient Network global collaboration (Borer et al. 2014). The site shows a distinct pattern of resource limitation by water and nitrogen. In dry years, productivity shows no significant response to nutrient addition. In wet years, overall productivity increases, and nitrogen addition leads to a further productivity increase, driven primarily by responsive annual species. Thus, productivity appears to be limited by water in dry years but by nitrogen in wet years (Wang et al. 2017). This pattern creates ideal circumstances for testing effects of both nutrient and water availability, as well as any interactions between them. Over the course of our study 2 years, 2017 and 2020 had below average growing season precipitation (179 and 154 mm respectively, between April 1 and June 30, compared to an average of 206mm, Figure S3.1) while the other two had above average precipitation (322mm in 2018 and 214 mm in 2019). Previous trait sampling at this site, focused on community-level variation (Tatarko and Knops 2018), shows functional trait responses to nitrogen, as well as temporal variation over two study years.

Sampling Methods

To assess year-to-year intraspecific trait variation, we surveyed plants within twelve 5x5m study plots, repeating our sampling in each year from 2017 to 2020. We surveyed only control and nitrogen addition plots, as past research has shown other nutrients do not alter functional traits (Tatarko and Knops 2018) or productivity (Wang et al. 2017) at this site. Nitrogen was applied to the addition plots at a rate of $10gN/m^2$/year in the form of time-release urea, and each addition plot was associated with a control plot in one of the site's six experimental blocks.

For our trait sampling, we selected twelve species common at our site and spanning a range of functional groups (Table 3.1). For each species-plot combination, we measured

SLA, LDMC, maximum height, and chlorophyll content. While some species were absent in specific plots or years, we were able to collect 383 sets of trait measurements across the four study years. To produce plot-level height measurements, we measured three individuals per species-plot combination and averaged their measured heights. For measurements of the remaining traits, we collected aggregated leaf samples, drawing from individuals distributed throughout the study plots. All leaves collected were fully expanded, non-senesced, and showed minimal herbivore/pathogen activity. Where possible, we collected enough leaves to constitute one gram of total fresh biomass. We measured chlorophyll content of three leaves per species-plot sample using a CCM-300 Chlorophyll Content Meter (Opti-Science, Hudson, New Hampshire, USA), averaging three readings per leaf to improve measurement precision. As with height, we averaged these three measurements to generate a plot-level measurement.

To compute SLA and LDMC, we measured the fresh mass of each sample to the nearest 0.01g and measured the total leaf area using an LI-3000A leaf area meter (LI-COR Lincoln, Nebraska, USA). For species with leaves too narrow to be accurately measured in this manner (*Artemisia filifolia*, *A. frigida*, *Bromus tectorum*, and *C. filifolia*), we instead imaged the leaves in a flat-bed scanner with a resolution of 600dpi and calculated total area using ImageJ software (NIH, Bethesda, Maryland, USA). We then dried the samples in a forced air oven at 60ºC for a minimum of 48 hours before making dry mass measurements. For each species-plot combination, we calculated SLA as total leaf area divided by total dry mass and LDMC as total dry mass divided by total fresh mass.

Data Analysis

To test for year and nutrient effects on species' functional traits, we developed linear mixed effects models in R version 3.6.2 (R Development Core Team 2019), treating sampling year and nutrient addition as fixed effects. We treated species as a random effect, as our interest was in overall patterns of intraspecific variation, rather than the responses of individual species. We likewise treated experimental block as a random effect, though we omitted this factor from the multivariate and chlorophyll models, where its effect was extremely small and prevented model convergence. Height and SLA were log transformed to better conform to the assumptions of linear modeling, while chlorophyll and LDMC were analyzed with their original units.

To prevent the per-family Type I error rate from increasing due to multiple comparisons with the four response traits, we first conducted MANOVA testing of year and nutrient effects on the full array of functional traits and then applied an adjusted alpha of 0.05/(4-1) = 0.0167 for subsequent univariate tests (Frane 2015). In order to incorporate species random effects in multivariate model fitting, we used the MCMCglmm function (Hadfield 2010). We evaluated the significance of year, nutrient and interactive effects by comparing Deviance Information Criterion (DIC) values of models including and omitting each of these effects. Contingent on the significance of this multivariate analysis, we proceeded to fit models for each individual functional trait, using the lme4 package (Bates et al. 2015). For these models, we applied ANOVA tests to evaluate year, nutrient and interactive effects, followed by Tukey tests (using the multcomp R package) for post-hoc comparisons.

Results

The multivariate analysis provided evidence of an interactive effect of sampling year and nitrogen treatment on the measured functional traits, with the interactive model outperforming an additive version (ΔDIC=8.7). Both additive and interactive nutrient/year models outperformed models incorporating only a single fixed effect predictor variable (Table 3.2), providing strong support for further analysis of these variables' main and interactive effects on individual traits, given the use of an adjusted alpha value (Bird and Hadzi-Pavlovic 2014).

In the analyses of individual traits, the largest SLA differences were due to species identity (with a variance of 0.539 log units), but highly significant responses could be attributed to both sampling year ($\chi^2 = 82.8$, df=3, $P < 0.001$; Table S3.1) and nutrient treatment ($\chi^2 = 24.4$, df=1, $P < 0.001$). Leaves measured in 2018 and 2019 showed higher SLA values than those measured in 2017 and 2020, with a difference of 0.45 log units between the most distinct years (2018 and 2020), representing a difference between 49.0 and 74.1 cm^2/g after reverse transformation (Table S3.2 for full post-hoc comparisons). These patterns align with weather data, in that 2017 and 2020 had below average growing season precipitation while 2018 and 2019 had above average precipitation (322 and 214 mm). Nitrogen addition led to an increase in species' SLA values by only 0.16 log units, representing an increase from 58.9 to 69.1 cm^2/g. Contrary to expectations, there was no significant interaction between nutrient treatment and sampling year ($\chi^2 = 1.42$, df=1, p = 0.700; Figure 3.1a).

LDMC measurements showed a similar response pattern to SLA, with more acquisitive values recorded in high rainfall years (2018 and 2019), and in nutrient addition plots. In contrast to SLA, nutrient effects on LDMC varied significantly from year to year (χ^2 = 17.7, df=3, *P* = 0.001; Figure 3.1b; Table S1.1), with large nitrogen effects observed in wet years (4.4 and 3.9 percentage points in 2018 and 2019, respectively) and smaller, non-significant differences observed in dry years. Among control plots, the largest year to year difference, 4.9 percentage points, occurred between 2019 and 2020, while for nitrogen plots, the largest year to year difference was 8.9 percentage points, between 2018 and 2020 (see Table S1.3 for full post hoc comparisons).

Height measurements differed from SLA and LDMC in that trait differences between years were not statistically significant (χ^2 =7.55, df=3, *P* = 0.056; Table S3.1). Plant heights did increase significantly with nutrient addition (χ^2 = 85.7, df=1, $P < 0.001$), from an average of 24.3cm to 30.4cm after reverse transformation. There was no significant nutrient-year interaction (χ^2 = 3.19, df=3, p = 0.363) (Figure 3.1c).

Chlorophyll, like LDMC, responded interactively to nutrient addition and year (χ^2 = 10.3, df=3, *P* = 0.0164; Figure 3.1d; Table S3.1). In this case, however, the largest nitrogen effect occurred in 2017, increasing chlorophyll content by 113.2 mg/m^2, while the smallest nitrogen effect, a non-significant difference of 40.2 mg/m^2, occurred in 2018. Leaves sampled in 2017 showed the lowest chlorophyll levels overall, while those sampled in 2019 showed the highest levels, with an average difference of 110.9 mg/m^2 for control plots and 69.2 mg/m^2 for nitrogen addition plots sampled in these years (see Table S3.4 for full post-hoc comparisons).

Overall, three of four traits shifted towards more acquisitive values in high rainfall years and, with the exception of chlorophyll in 2020, showed more conservative values in dry years (Figure 3.2). While functional groups differed in the strength of some effects, these differences were not consistent from trait to trait, and accounting for them did not alter the overall significance of year and nitrogen effects (Figure S3.3; Table S3.5). Across traits, responses to sampling year were most consistent for SLA and LDMC, the study traits most closely associated with the leaf economics spectrum. Height, and to a lesser extent chlorophyll content responded more clearly to nutrient addition than to sampling year, a pattern illustrated by principal components based visualization of the data (Figure 3.3).

Discussion

These results illustrate that variation across years is an important component of intraspecific variation in plant functional traits. Three of our four studied traits showed significant year-to-year variation, and for SLA and LDMC, annual variation exceeded variation in response to nitrogen addition, as well as annual change observed in previous, shorter-term studies (Garnier et al. 2001, Tatarko and Knops 2018). We also observed that annual variation in these traits was consistent with expected water availability responses, with more acquisitive trait values occurring in high rainfall years. Finally, while interactions between year and nitrogen effects were less consistent in strength and direction than overall annual changes, trait responses to nitrogen did vary across years for both LDMC and chlorophyll content.

Comparisons of year and nutrient effect sizes are particularly striking in that this study's year-to-year comparisons reflect only ambient levels of variation, while its nitrogen effects reflect a decade long experimental manipulation. Though effect sizes are presumably site dependent, these results do suggest that annual intraspecific variation may often exceed variation driven by natural, or ambient anthropogenic, levels of nutrient enrichment. Nitrogen deposition rates in western North America are expected to remain below 2g/m^2/year through 2050 (Galloway et al. 2004), while our addition treatment added nitrogen at five times this rate. Effects of annual variation could be further enhanced in settings with greater annual variation in conditions. Such sites, and those with weaker nutrient limitation, could see annual effects exceed nutrient effects by substantial margins. This would result in a significant underestimates of intraspecific trait variation when such estimates are based on sampling conducted in a single year.

Though we were not able to directly test mechanisms underlying the observed annual trait variation, the observed patterns match our expectations for a scenario where trait responses are driven by rainfall. The low SLA, high LDMC and low chlorophyll content (indicative of low leaf N) observed in the drier conditions of 2017 and 2020 are all associated with the conservative leaf economics spectrum strategies we would expect to observe in resource-poor conditions (Wright et al. 2004, Garnier et al. 2007, Gross et al. 2007, McIntyre and Lavorel 2007), a pattern also consistent with the results observed in shorter-term studies (Garnier et al. 2001, Tatarko and Knops 2018, Chen et al. 2019). More direct tests of this causal mechanism would, however, be valuable, as annual effects on ecological variables may stem from other factors even in settings where precipitation plays an important role (Groves and Brudvig 2019). Future analyses incorporating experimental rainfall manipulation will aid in uncovering what portion of annual trait variation can be attributed to precipitation. Longer-term observational studies will also be valuable, generating data sets with sufficient sample size and precipitation variation for regression-based analyses.

To the extent that annual trait variability is a response to precipitation, these measurements will be valuable in projecting changes to trait values under future climatic changes. In western North American grasslands, global climate change is expected to result in more frequent and intense drought (Dai 2013, Cook et al. 2015). While the most extreme future conditions may be beyond the range of current measurements, current studies can nonetheless provide valuable insight into species' capacities for intraspecific change. Similar approaches could be applied in settings where temperature or other environmental variables drive trait responses.

A surprising aspect of our results is that, while we expected to find stronger nitrogen effects in high rainfall years, corresponding to the productivity effects observed by Wang and colleagues (2017), our data did not consistently show such a pattern. The effects of nitrogen addition and sampling year on height and SLA instead followed a purely additive pattern. Chlorophyll content did show an interactive response to these variables, but did not follow the expected pattern, instead showing a stronger nitrogen response in one of the two low rainfall years, a weaker nitrogen response in one of the two high rainfall years, and intermediate nitrogen responses in the two remaining years. Only LDMC showed the expected pattern of larger nitrogen effects corresponding to high rainfall conditions. The divergence of these traits' patterns of variation from one another and from the site's pattern of productivity response (Wang et al 2017) emphasizes that trait values and productivity can both respond to complex arrays of variables and thus may not always be correlated as expected.

In those cases where traits do respond additively to nitrogen and sampling year, the lack of interaction, though unexpected, is potentially a valuable finding. In such cases, one year of trait sampling should be sufficient for the purpose of evaluating nutrient effects on functional traits. While extrapolation of such response patterns between sites will not always be reliable, and while multi-year data will remain valuable in evaluating other study questions, a single sampling year approach holds obvious advantages in speed and in required labor. Confirming the circumstances under which such an approach is likely to yield representative findings would therefore be of great benefit in planning future research.

Analysis of annual variation may substantially improve ecological predictions in many contexts, including species and community responses to climate change. A large scale analysis of tundra sites (Bjorkman et al. 2018) illustrates the potential for intraspecific variation to shift functional trait averages to a greater degree than species turnover alone would predict. Such shifts may substantially alter ecosystem function parameters, and annual variation will influence estimates of intraspecific variability in evaluating such scenarios. Intraspecifically driven changes to community traits may also have consequences beyond the communities in question. For instance, accounting for temperature-induced changes to boreal forests' needle lifespan can improve productivity and carbon cycling estimates in climate models (Reich et al. 2014). Changes to productivity or decomposition rates, mediated by leaf economics spectrum traits, could have similar effects, and incorporating year-to-year variability into estimates of potential intraspecific variation could strongly influence estimates of these effects' magnitude.

Intraspecific trait variation, also holds substantial implications for species coexistence. Most trait-based analyses of coexistence, such as those proposed by Adler and colleagues (2013), rely on species-level trait values, which may uncover informative patterns (Stubbs and Wilson 2004, Kraft et al. 2008, Catford et al. 2019) but does risk misrepresenting trait differentiation by failing to account for overlap between species' trait ranges (Violle et al. 2012). Interactions between individuals will ultimately be determined by the traits of those individuals rather than by species averages, and accounting for intraspecific trait variation allows more precise detection of niche differentiation in trait-based studies (Paine et al. 2011, Siefert 2012, Lasky et al. 2014). Incorporating annual variation could provide further precision.

Intraspecific variation can also act itself as a mechanism promoting or inhibiting coexistence. Mathematical modeling suggests intraspecific variation may aid coexistence in specific scenarios (Uriarte and Menge 2018, Crawford et al. 2019), but in general, intraspecific variation can be expected to interfere with coexistence by increasing interspecific competition and decreasing intraspecific competition (Barabás and D'Andrea 2016, Hart et al. 2016, Turcotte and Levine 2016), thereby inhibiting stabilizing mechanisms of coexistence, as defined by Chesson (2000). Whether responses to annual variation enhance such barriers to coexistence will depend on the precise nature of species’ responses. If all species respond in the same manner, trait differences between species will be maintained, and changes to coexistence dynamics should be minimal. Such responses are often discussed in terms of consistency of rank order, and several studies have found these orders to be relatively consistent, even as environmental change shifts species’ traits (Garnier et al. 2001, Kazakou et al. 2014, Fajardo and Siefert 2016). However, even shifts that retain rank order could substantially impact species’ niche overlap with one another and thus their coexistence dynamics. Greater species-level analysis of annual trait effects could help predict the frequency of such changes.

In summary, we find that annual variability is a major component of intraspecific trait variation and is therefore likely to have a substantial impact on trait-based ecological interactions, as well as on the results of trait-based research. The latter effect may be particularly pertinent to studies aimed at quantifying populations’ trait variation or attempting to extrapolate trait values across time or space. While annual trait variation appears correlated with water availability at our study site, further experimental manipulation will be needed to clearly establish the relevant causal mechanisms. It is also likely that annual intraspecific trait responses will, to some degree, vary with the species, trait, and geographic location considered. Studies evaluating trait variation in other settings would be of great value in determining which patterns of year-to-year trait variation can be extrapolated across sites and which patterns are more contingent upon experimental context. Such research could be particularly valuable at long-term research sites where many future trait-based studies could be informed by locally focused analyses. While it is neither feasible nor necessary that all trait-based studies measure annual variability, it is critical that the potential effects of such variation be considered during the design and interpretation of trait-based experiments.

Table 3.1: Study Species Twelve species were included in trait sampling, representing a range of functional groups. Cover values represent mean abundance from 2017 to 2020 across all Cedar Point Nutrient Network plots.

Species	**Functional Group**	**Lifespan**	**Photosynthetic Pathway**	**Provenance**	**Mean Percent Cover**
Artemisia filifolia	Shrub	Perennial	C3	Native	3.6
Artemisia frigida	Forb	Perennial	C3	Native	5.4
Bouteloua gracilis	Graminoid	Perennial	C4	Native	7.9
Bromus tectorum	Graminoid	Annual	C3	Introduced	28
Carex filifolia	Graminoid	Perennial	C3	Native	14
Dichanthelium oligosanthes	Graminoid	Perennial	C3	Native	2.0
Helianthus annuus	Forb	Annual	C3	Native	1.8
Hesperostipa comata	Graminoid	Perennial	C3	Native	9.8
Lithospermum incisum	Forb	Perennial	C3	Native	0.27
Pascopyrum smithii	Graminoid	Perennial	C3	Native	2.2
Psoralidium tenuiflorum	Legume	Perennial	C3	Native	0.57
Sphaeralcea coccinea	Forb	Perennial	C3	Native	0.39

Table 3.2: Multivariate Model Comparison. Each linear mixed model incorporates year and/or nitrogen addition as fixed effects with species identity as a random effect and the four measured functional traits as response variables. For each model, deviance, effective parameters, and deviance information criterion are calculated using the MCMCglmm function (Hadfield 2010), with ΔDIC values calculated by subtracting the DIC value of the most predictive model, which includes an interactive effect of year and nutrient treatment.

Model	**Deviance**	**Effective Parameters**	**DIC**	**ΔDIC**
Year * Nitrogen	3684.0	31.9	3747.8	0
Year + Nitrogen	3695.6	30.5	3756.5	8.7
Year	3707.6	30.0	3767.6	19.7
Nitrogen	3801.3	29.0	3859.2	111.3

Figure 3.1. Trait responses to year and nutrient treatment. Colored points represent species means for each treatment-year combination, with the associated vertical lines showing standard errors of those means. Black, horizontal lines represent the expected values for each treatment-year combination, generated by linear mixed modeling (Table S3.1). Standard errors for these overall means are not shown, as they largely reflect the variation accounted for by the models' species terms. Overall significance of year (Y), nutrient (N) and interactive (YxN) effects is indicated at the top right of each panel. For traits with significant year effects or treatment-year interactions, significant differences between individual years or treatment-year combinations ($P<0.05$ in a Tukey post-hoc comparison) are indicated by letters above the plotted points. Blue text indicates years with above average rainfall, with red text indicating below average rainfall.

Figure 3.2. Modeled trait responses to sampling year and nitrogen addition. Bars represent expected mean trait values across all species, based on the linear mixed model for each trait, with non-significant effects omitted from calculations. Red bars indicate low rainfall years, with blue bars indicating high rainfall years.

Figure 3.3. Principal component analysis of trait variation. In panel a, data points are grouped by nutrient treatment and sampling year, with years combined for clarity into wet years (2018 and 2019) and dry years (2017 and 2020). In panel b, data points are grouped by species. Each point represents a plot-level trait measurement, while ellipses represent standard errors around each group's midpoint.

Figure 3.1. Trait responses to year and nutrient treatment.

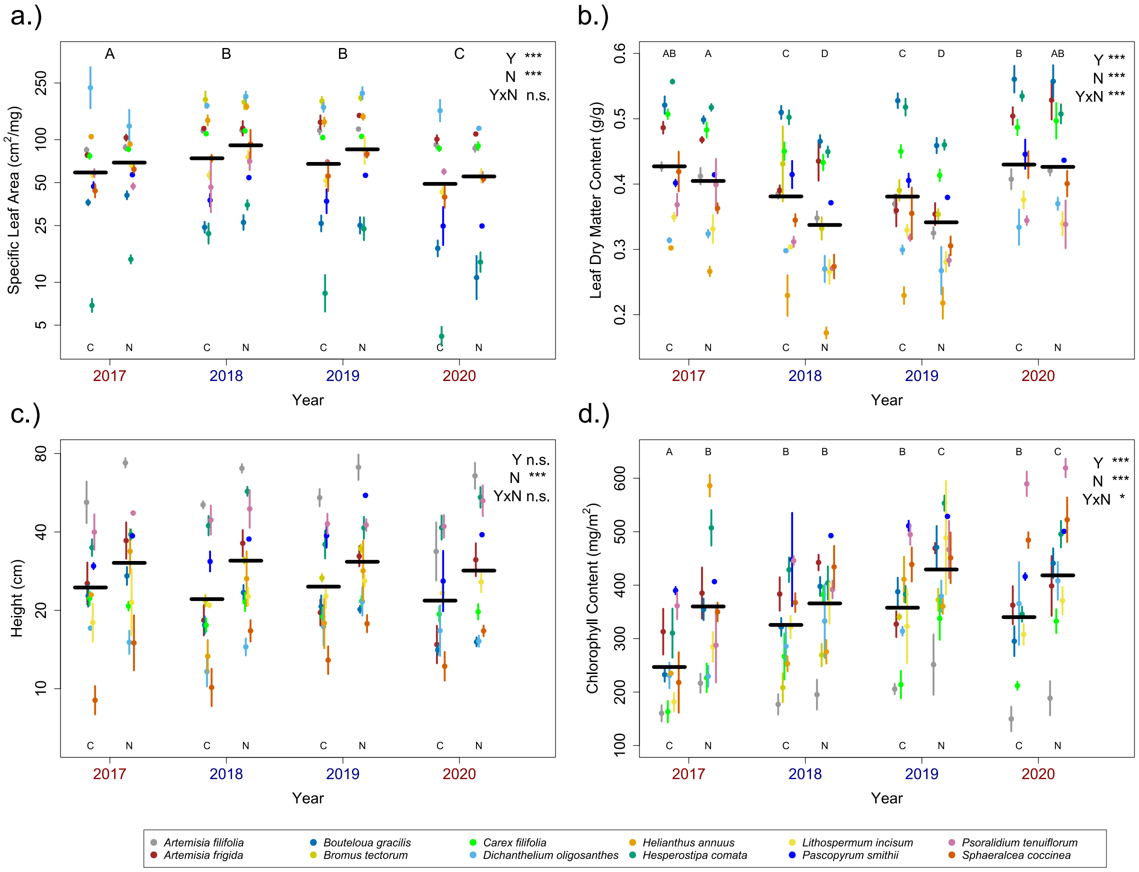


Figure 3.2. Modeled trait responses to sampling year and nitrogen addition.

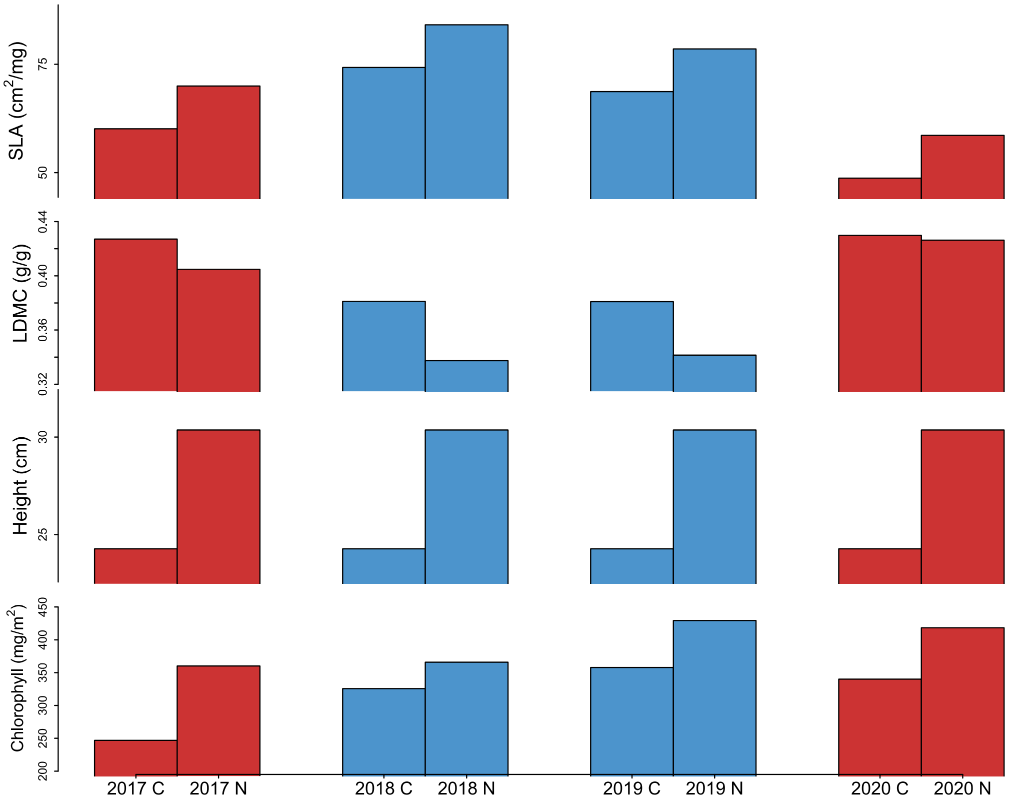
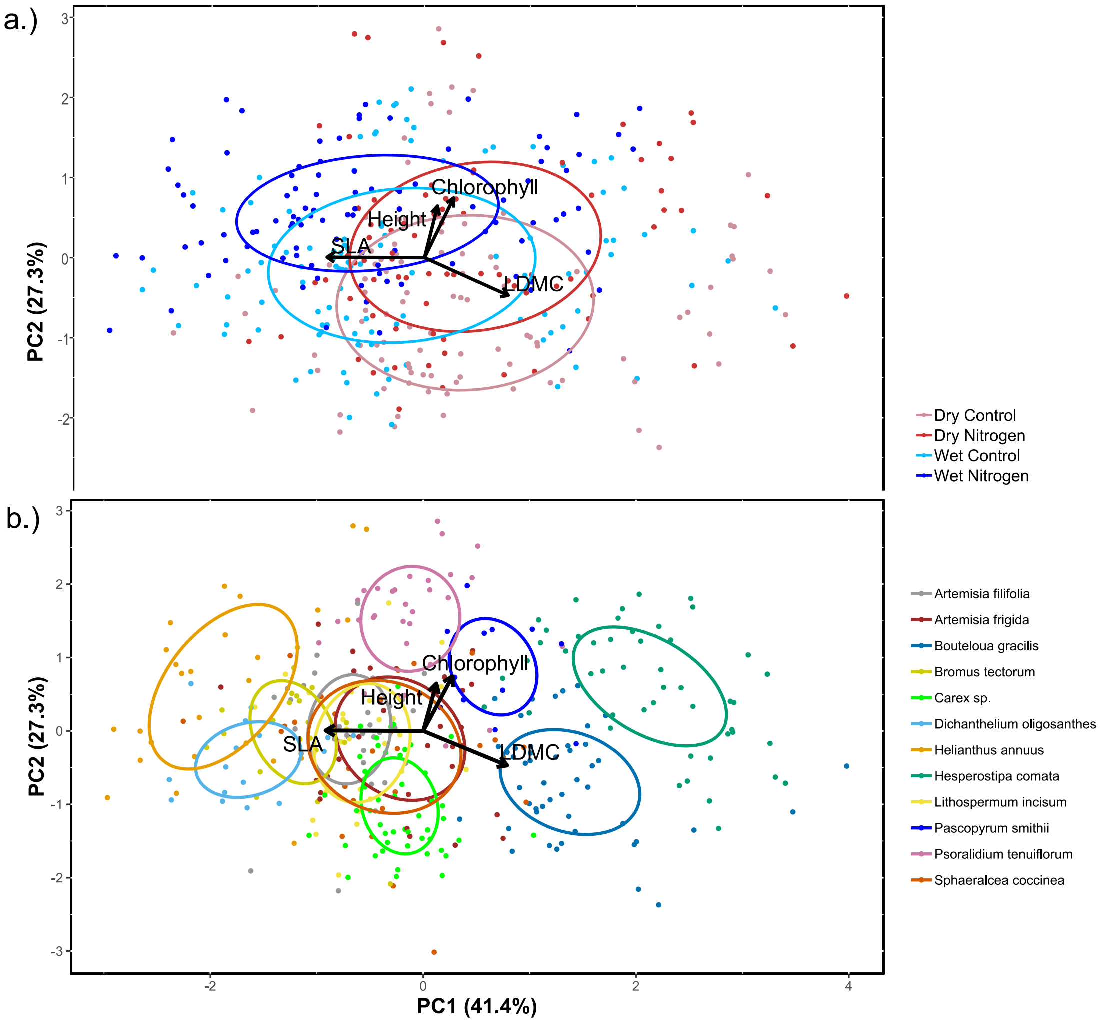


Figure 3. Principal component analysis of trait variation.



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Chapter 3 Appendix

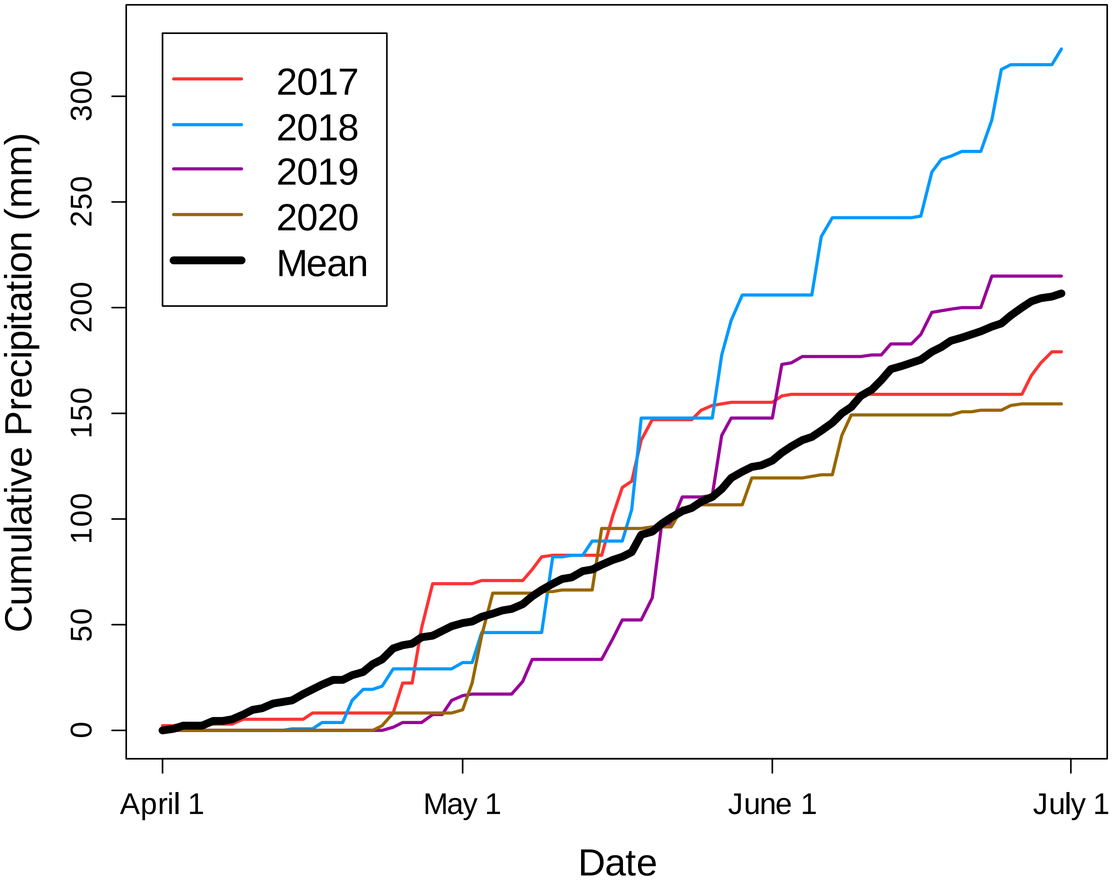

Figure S3.1. Cumulative precipitation from April 1, shown in each of the four study years, along with mean accumulation over all years since weather station establishment in 1997. Note below average precipitation by season end in 2017 and 2020 and above average precipitation in 2018 and 2019.

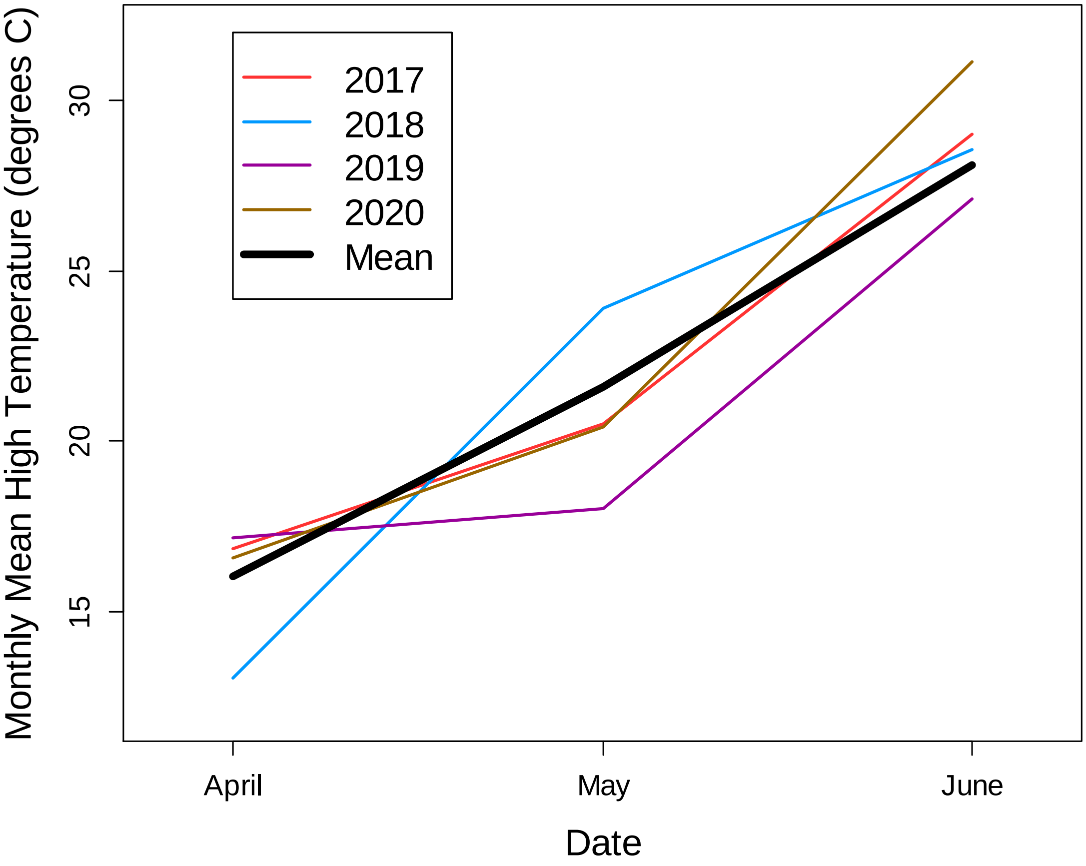

Figure S3.2. Monthly mean of daily high temperatures in the growing season of each study year, shown with mean conditions since weather station establishment in 1997.

Table S3.1: Linear mixed model output for single trait analyses. See methods for model descriptions.

	Fixed Effects				**Random Effects**		
Specific Leaf Area	***Nutrient***	***Year***	*Nutrient *Year*		**Species**	**Block**	**Residual**
χ2	**24.37**	**82.81**	1.423	Standard Deviation	0.73 (n=12)	0.07 (n=6)	0.36
df	**1**	**3**	3				
p	**7.9e-07**	**< 2.2e-16**	0.7002				
Leaf Dry Matter Content	***Nutrient***	***Year***	***Nutrient *Year***		**Species**	**Block**	**Residual**
χ2	**56.647**	**247.9**	**17.670**	Standard Deviation	0.078 (n=12)	0.007 (n=6)	0.036
df	**1**	**3**	**3**				
p	**5.2e-14**	**< 2.2e-16**	**0.00051**				
Height	***Nutrient***	***Year***	*Nutrient *Year*		**Species**	**Block**	**Residual**
χ2	**85.71**	7.55	3.1891	Standard Deviation	0.42 (n=12)	0.08 (n=6)	0.28
df	**1**	3	3				
p	**< 2e-16**	0.056	0.363				
Chlorophyll	***Nutrient***	***Year***	***Nutrient *Year***		**Species**	**Block**	**Residual**
χ2	**81.24**	**73.58**	**10.270**	Standard Deviation	80.0 (n=12)	**-**	78.9
df	**1**	**3**	**3**				
p	**< 2.2e-16**	**7.3e-16**	**0.0164**				

Table S3.2 Tukey post-hoc comparisons of SLA across sampling years. See Table S3.1 for primary analysis.

Years	Estimated Difference	Standard Error	z	p
2017:2018	**0.25256**	**0.05243**	**4.817**	**< 0.001**
2017:2019	**0.17334**	**0.0525**	**3.302**	**0.00513**
2017:2020	**-0.20124**	**0.05393**	**-3.732**	**< 0.001**
2018:2019	-0.07923	0.04984	-1.59	0.3843
2018:2020	**-0.4538**	**0.05344**	**-8.491**	**< 0.001**
2019:2020	**-0.37458**	**0.05343**	**-7.011**	**< 0.001**

Table S3.3 Tukey post hoc comparisons of LDMC across nitrogen treatments and sampling years. See Table S3.1 for primary analysis.

Years/ Treatments	Estimated Difference	Standard Error	z	p
2017C:2017N	-0.02231	0.007706	-2.894	0.073
2017C:2018C	**-0.04599**	**0.00727**	**-6.326**	**<0.001**
2017C:2018N	**-0.08974**	**0.007464**	**-12.024**	**<0.001**
2017C:2019C	**-0.04622**	**0.007203**	**-6.418**	**<0.001**
2017C:2019N	**-0.08562**	**0.007548**	**-11.342**	**<0.001**
2017C:2020C	0.00276	0.007347	0.376	1
2017C:2020N	-0.00081	0.007895	-0.103	1
2017N:2018C	**-0.02368**	**0.007527**	**-3.146**	**0.0351**
2017N:2018N	**-0.06744**	**0.007638**	**-8.829**	**<0.001**
2017N:2019C	**-0.02392**	**0.00748**	**-3.197**	**0.0301**
2017N:2019N	**-0.06331**	**0.007719**	**-8.202**	**<0.001**
2017N:2020C	**0.025066**	**0.007674**	**3.266**	**0.0238**
2017N:2020N	0.021494	0.008129	2.644	0.1395
2018C:2018N	**-0.04375**	**0.007168**	**-6.104**	**<0.001**
2018C:2019C	-0.00024	0.006967	-0.034	1
2018C:2019N	**-0.03963**	**0.007245**	**-5.47**	**<0.001**

Table S3.3 continued

Years/ Treatments	Estimated Difference	Standard Error	z	p
2018C:2020C	**0.048748**	**0.007248**	**6.726**	**<0.001**
2018C:2020N	**0.045177**	**0.00778**	**5.807**	**<0.001**
2018N:2019C	**0.043519**	**0.007108**	**6.122**	**<0.001**
2018N:2019N	0.004126	0.007307	0.565	0.9992
2018N:2020C	**0.092502**	**0.007438**	**12.436**	**<0.001**
2018N:2020N	**0.088931**	**0.007912**	**11.24**	**<0.001**
2019C:2019N	**-0.03939**	**0.007189**	**-5.48**	**<0.001**
2019C:2020C	**0.048984**	**0.007175**	**6.827**	**<0.001**
2019C:2020N	**0.045413**	**0.00772**	**5.883**	**<0.001**
2019N:2020C	**0.088377**	**0.007525**	**11.745**	**<0.001**
2019N:2020N	**0.084805**	**0.007991**	**10.613**	**<0.001**
2020C:2020N	-0.00357	0.00785	-0.455	0.9998

Table S3.4 Tukey post-hoc comparisons of chlorophyll content across nitrogen treatments and sampling years. See Table S3.1 for primary analysis.

Years/ Treatments	Estimated Difference	Standard Error	z	p
2017C:2017N	**113.179**	**16.806**	**6.735**	**<0.01**
2017C:2018C	**78.735**	**15.869**	**4.962**	**<0.01**
2017C:2018N	**118.962**	**16.274**	**7.31**	**<0.01**
2017C:2019C	**110.927**	**15.717**	**7.058**	**<0.01**
2017C:2019N	**182.406**	**16.459**	**11.082**	**<0.01**
2017C:2020C	**93.279**	**16.038**	**5.816**	**<0.01**
2017C:2020N	**171.405**	**17.212**	**9.958**	**<0.01**
2017N:2018C	-34.445	16.416	-2.098	0.4143
2017N:2018N	5.782	16.672	0.347	1
2017N:2019C	-2.252	16.314	-0.138	1
2017N:2019N	**69.226**	**16.846**	**4.109**	**<0.01**
2017N:2020C	-19.9	16.742	-1.189	0.9352
2017N:2020N	**58.226**	**17.746**	**3.281**	**0.0227**
2018C:2018N	40.227	15.638	2.572	0.1657
2018C:2019C	32.193	15.208	2.117	0.4028
2018C:2019N	**103.671**	**15.807**	**6.558**	**<0.01**

Table S3.4 continued

Years/ Treatments	Estimated Difference	Standard Error	z	p
2018C:2020C	14.544	15.816	0.92	0.9842
2018C:2020N	**92.67**	**16.959**	**5.464**	**<0.01**
2018N:2019C	-8.034	15.505	-0.518	0.9996
2018N:2019N	**63.444**	**15.956**	**3.976**	**<0.01**
2018N:2020C	-25.683	16.222	-1.583	0.7601
2018N:2020N	**52.443**	**17.263**	**3.038**	**0.0485**
2019C:2019N	**71.478**	**15.685**	**4.557**	**<0.01**
2019C:2020C	-17.648	15.659	-1.127	0.9509
2019C:2020N	**60.478**	**16.831**	**3.593**	**<0.01**
2019N:2020C	**-89.127**	**16.413**	**-5.43**	**<0.01**
2019N:2020N	-11.001	17.435	-0.631	0.9985
2020C:2020N	**78.126**	**17.125**	**4.562**	**<0.01**

Figure S3.3: Trait responses to year and nutrient treatment by functional group. Colored points represent functional group means for each treatment-year combination with the associated vertical lines showing standard errors of those means. Graminoids show greater annual variability in SLA than non graminoids, but lower variability in LDMC. Nitrogen increases the height of non graminoids more strongly than graminoids, while chlorophyll content responds more strongly to nitrogen in graminoids than in non graminoids.

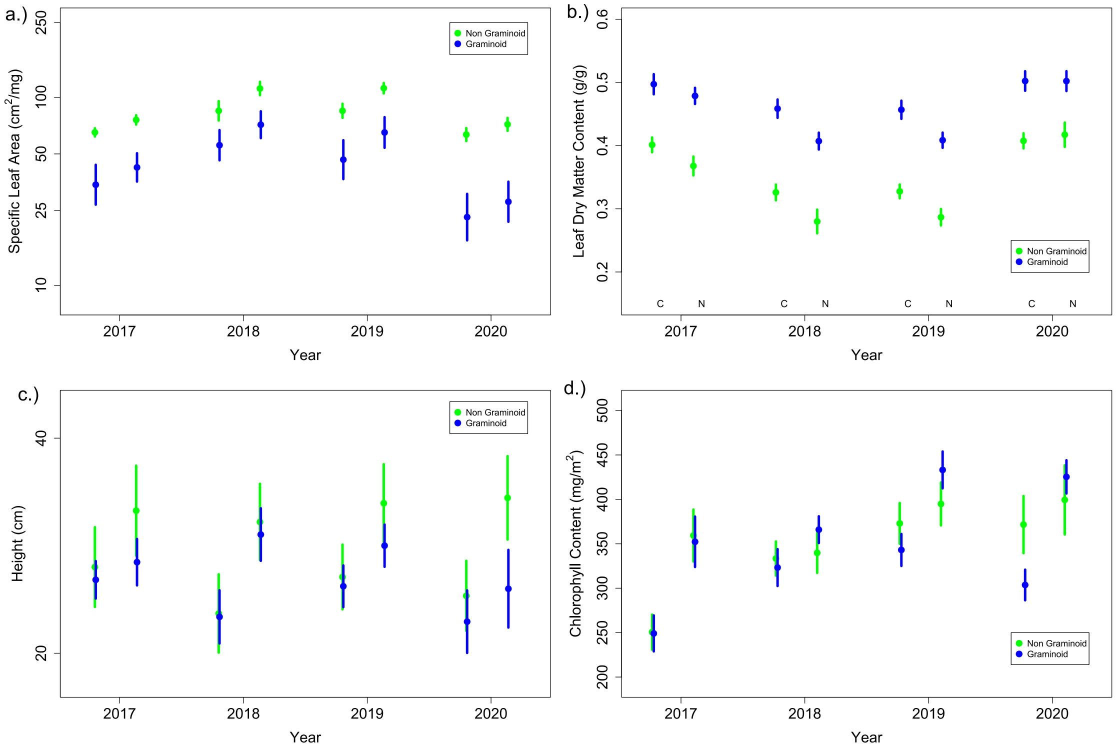

Table S3.5: Linear mixed model results with functional group effect.

	Year	**Nutrient**	**Functional Group**	**Year * Nutrient**	**Year * Functional**	**Nutrient * Functional**	**Year * Nutrient * Functional**
Specific Leaf Area							
χ2	**84.2771**	**25.430**	0.7802	1.0087	**18.4061**	0.4741	1.4748
df	**3**	**1**	1	3	**3**	1	3
p	**< 2.2e-16**	**4.587e-07**	0.37709	0.79916	**0.00036**	0.49113	0.68809
Leaf Dry Matter Content							
χ2	**248.3399**	**57.0421**	**4.9976**	**19.1562**	**12.3881**	0.8096	0.8907
df	**3**	**1**	**1**	**3**	**3**	1	3
p	**< 2.2e-16**	**4.266e-14**	**0.02538**	**0.00025**	**0.00617**	0.36824	0.82768
Height							
χ2	7.1830	**77.771**	0.0929	2.9894	0.5648	**13.9342**	1.3081
df	3	**1**	1	3	3	**1**	3
p	0.0663	**< 2.2e-16**	0.76058	0.3933	0.90445	**0.00020**	0.72721
Chlorophyll Content							
χ2	**73.9876**	**82.678**	0.0019	**11.092**	7.5426	**6.5063**	4.8960
df	**3**	**1**	1	**3**	3	**1**	3
p	**5.971e-16**	**< 2.2e-16**	0.96499	**0.0112**	0.05647	**0.01075**	0.17958

Chapter 4

EXPERIMENTALLY MANIPULATED PRECIPITATION PARTIALLY EXPLAINS ANNUAL VARIATION IN GRASSLAND PLANT SPECIES' FUNCTIONAL TRAITS

Abstract

Plant functional traits show extensive intraspecific variation, and understanding this variation is essential to using these traits effectively and reliably in ecological research and analyses. A potentially important, though rarely studied, component of intraspecific variation is variation over time. At our study site, grassland plant species show year to year trait variation greater than their variation in response to nitrogen addition. The patterns of variation appear consistent with rainfall driven responses, with more acquisitive leaf economics spectrum trait values occurring in high rainfall years. Thus far, however, this connection has been supported only by observational comparisons across a small number of study years. In this study, we sought to test the effects of rainfall variation on species' functional traits experimentally. To do so, we used rainfall shelters and rainfall addition treatments to manipulate water availability in a factorially combined set of rainfall and nutrient treatment plots. In these plots, we collected functional trait data on six common plant species over three study years. We found that three of our four study traits responded to rainfall manipulation, with both year and shelter effects occurring interactively with species identity and nutrient treatment. Four of six species showed a significant specific leaf area or leaf dry matter response to rainfall treatment, while rainfall and nitrogen treatments produced a small overall effect

on plant height. These responses were, however, less consistent and smaller in magnitude than sampling year responses, which occurred for all traits and species. These results lead us to conclude that total rainfall is one component in a broader array of variables driving year to year trait variation, with other aspects of rainfall variation, such as intervals between rainfall events and changes to vapor pressure deficit likely playing important roles. The relative weight of these variables may vary depending on the species, sites, and traits considered.

Introduction

Functional traits are a valuable tool in an extensive array of ecological analysis types, including predicting species distribution and abundance (Green et al. 2022), linking communities to ecosystem services (Lavorel and Grigulis 2012), and elucidating the mechanisms behind biological patterns (Adler et al. 2013). More broadly, they allow us to discern ecological patterns based on generalizable rules rather than on potentially idiosyncratic species-specific findings (Lavorel and Garnier 2002, McGill et al. 2006). Such analyses depend on accurate assessments of organisms' trait values. While species-level traits from databases such as TRY (Kattge et al. 2011) are valuable in some contexts, analyses dependent on high levels of precision require that we consider intraspecific variation in trait values, which represents approximately 25% of trait variation within communities and 32% of variation between communities (Siefert et al. 2015).

In many contexts, intraspecific variation is itself an important phenomenon. Depending on its context or structure, for instance, intraspecific variability may facilitate (Uriarte and Menge 2018, Crawford et al. 2019) or inhibit (Barabás and D'Andrea 2016, Hart et al. 2016, Turcotte and Levine 2016) species coexistence. It can facilitate plastic adaptation of individuals to environmental conditions (Reich et al. 1996, Vitasse et al. 2010) and provides the raw material for population changes by natural selection. Failing to account for this variation, however, risks incorporating non-representative estimates of trait values into analyses (Bolnick et al. 2003, Funk et al. 2017, Chacón-Labella et al. 2023), or failing to detect environmental responses that occur through plasticity or selection rather than species turnover (La Pierre and Smith 2015). Species-level values may remain useful in the face of intraspecific change if species' relative positions along trait axes remain consistent (Westoby et al. 2002). Testing shows, however, that while such rankings appear stable in an approximate sense, non-negligible shifts do occur in many contexts as a result of intraspecific variation (Garnier et al. 2001, Albert et al. 2010, Kazakou et al. 2014).

An understudied component of intraspecific trait variation is variation over time. Repeated trait measurements within a single community are rare in the literature, likely owing to their labor demands. Those studies that do exist, however, have documented notable variation across years, albeit often with a limited number of study years (Garnier et al. 2001, Tatarko and Knops 2018, Chen et al. 2019) or a study design focused on community weighted, rather than intraspecific change (Tatarko and Knops 2018, Chen et al. 2019, Luo et al. 2019). This temporal variation may equal or exceed variation due to other drivers such as nutrient availability (Tatarko and Knops 2018, Wheeler et al. 2023).

Trait variation over time may, in many cases, represent a response to water availability. Under water-limited conditions, plants are expected to show resource-conservative trait values, such as low specific leaf area (SLA, the ratio of leaf area to dry mass) and leaf dry matter content (LDMC, the ratio a dry mass to fresh mass) (Wright et al. 2004, Poorter et al. 2009). This pattern does, however, appear to depend on the species, ecosystems and forms of variation considered. For instance, populations of the common C4 grasses *Andropogon gerardii* (Bachle et al. 2018) and *Bouteloua gracilis* (Bushey et al. 2023) do not differ in leaf traits across geographical precipitation gradients, but do show plastic responses to water availability (Griffin-Nolan et al. 2023). In California grasslands, species with conservative trait values are more abundant under dry conditions, but within-species variation follows an opposing pattern (Sandel and Low 2019), and resource-conservative European grassland species likewise tend towards reduced conservatism under drought (Rodríguez-Alarcón et al. 2022). Intraspecific trait responses of European species to moisture variability also appear to vary both between grasses and forbs and between temperate and Mediterranean systems (Wellstein et al. 2017).

While drought frequently contributes to intraspecific trait variation, the degree to which it explains temporal variation in these traits remains unclear. Other environmental factors may play important roles, and drought itself includes many components, with the potential for divergent effects on functional traits. In many cases, for instance the effects of vapor pressure deficit on plant hydrology exceed those of soil water (Novick et al. 2016). Experimental droughts may vary from observed droughts in parameters such as rainfall timing and relative humidity, so while their effects are consistent in some contexts, they may produce distinct patterns in others (Knapp et al. 2018). An understanding of drought effects requires that we consider the mechanisms behind observed effects (Knapp et al. 2023).

At our study site, Cedar Point Biological Station in western Nebraska, USA, annual variation in SLA and LDMC exceeds variation in response to long term nitrogen addition, with plants showing more conservative trait values in low-rainfall years (Wheeler et al. 2023). While this pattern is consistent with the hypothesis that annual trait variation at this site occurs as a response to water availability, its causal mechanism has yet to be experimentally confirmed. Through a rainfall shelter manipulation, we sought to experimentally evaluate whether variation in total growing season precipitation drives intraspecific trait variation and evaluate the degree to which such effects are able to explain annual variation in trait measurements.

Methods

Study Site

This study was conducted at Cedar Point Biological Station in western Nebraska, USA. This site's grassland areas contain a Nutrient Network site (Borer et al. 2014), where past research suggests an interactive effect of rainfall and nitrogen on productivity, with nitrogen increasing plant biomass in high rainfall years but producing no effect in low rainfall years (Wang et al. 2017). Trait sampling in these plots is also suggestive of a rainfall effect, with SLA, LDMC and chlorophyll showing more acquisitive values in wet years and more conservative values in dry years, with LDMC also showing an increased nitrogen response in wet years (Wheeler et al. 2023).

Experimental Design and Sampling

To determine the extent to which precipitation variation is indeed responsible for these patterns, we established a series of rainfall manipulation plots near the existing Nutrient Network plots. These plots were established following the protocols of the NPKD-Net collaboration and included six experimental blocks. Each block in turn contained six 2x2m treatment plots, with one 1x1m quadrant of each plot set aside for cover sampling and the remaining three quadrants available for biomass, functional trait and soil sampling.

Within each block, passive interception rainfall shelters (Yahdjian and Sala 2002) were established over two plots. These shelters have been shown to produce minimal non-drought effects on climate and plant physiology (Loik et al. 2019). Our shelters were designed to intercept 50% of rainfall, though due to logistical and engineering constraints this treatment could be maintained only during the growing season (approximately late May to early August). Rainfall excluded from these plots was collected and manually redistributed to two rainfall addition plots, while the remaining two plots received ambient rainfall. These rainfall manipulation treatments were crossed factorially with a nutrient addition treatment, with one plot per rainfall treatment in each block receiving a combined nitrogen, phosphorus, potassium and micronutrient treatment. Nitrogen, phosphorus and potassium were added annually as time-release urea, triple super phosphate and potassium sulfate, respectively, each at an annual rate of 10g elemental mass per square meter. Additionally, a micronutrient mixture (Micromax) was added in the first treatment year at 100g total mass per square meter. To confirm the efficacy of the rainfall manipulation treatments, we measured soil water gravimetrically in late June of each study year. Additionally, we measured biomass by clipping two 10x100cm strips in each plot, sorting by functional group, drying and weighing.

For functional trait measurements, we selected seven target species, representing the three graminoid species (*Bouteloua gracilis*, *Hesperostipa comata* and *Carex filifolia*) and three forb species (*Sphaeralcea coccinea*, *Artemisia frigida* and *Coreopsis tinctoria*) present in the greatest number of plots. A fourth forb species, *Lithospermum incisum* was initially included based on its abundance in 2021 and preceding years but ultimately excluded due to low abundance and extensive herbivore and drought damage in 2022 and 2023. All sampled species were native perennials. For each species, we measured plant height, SLA, LDMC and chlorophyll content in each of the three study years (2021, 2022 and 2023), with the seasonal timing of measurements varying from mid-June to early July based on species' phenology but kept consistent across study years. Height was measured for three individuals per plot as maximum vegetative height. Chlorophyll content was measured for three sample leaves using a CCM-300 Chlorophyll Content Meter (Opti-Science, Hudson, New Hampshire, USA), with these values similarly averaged at the plot level. For SLA and LDMC, we collected an aggregate sample of leaves for each plot and measured total fresh mass, dry mass and leaf area, dividing plot-level total dry mass by fresh mass to generate LDMC values and area by dry mass to generate SLA values. Leaf area was measured using an LI-3000A leaf area meter (LI-COR Lincoln, Nebraska, USA) for *Bouteloua gracilis, Hesperostipa comata, Lithospermum incisum* and *Sphaeralcea coccinea*. *Artemisia frigida, Carex filifolia* and *Coreopsis tinctoria* leaves were too narrow to be measured in this fashion and were instead imaged at 600DPI in a flat-bed scanner and analyzed using ImageJ software (NIH, Bethesda, Maryland, USA). In 2021 and 2022, we additionally measured individual-level SLA and LDMC using the same samples measured for chlorophyll content.

Statistical Analyses

Because of the complex nature of our data, including six study species, three treatment variables (two experimental and one observational), and four response traits, we evaluated our results through a nested series of analyses using R version 4.0.5 (R Core Team 2021). We first constructed an overall multivariate model with the four measured traits as responses to sampling year, rainfall treatment, nutrient addition, species identity and their interactions. We had originally planned to treat species identity as a random effect considering each species to be an instance of a broader community patterns. We found, however, that it was not possible to fully describe the responses to the treatment without viewing species as fixed effects. To account for differences in measurement units across the four response variables, functional traits were rescaled to a mean of zero and standard deviation of one for this analysis. This facilitates comparison across response variables in terms of magnitude relative to observed variation of each response variable. This model was constructed first using the MCMCGLMM (Hadfield 2010) function to allow for the inclusion of a block random effect in a multivariate model. As the block effect was very small relative to fixed effects and unexplained error, we fit a MANOVA model without this effect to allow hypothesis testing with available analytical tools.

Following the overall MANOVA test, we constructed trait-level ANOVA models, treating each of the four functional traits individually as a response to sampling year, rainfall treatment, nutrient treatment, species identity and their interactions. These models were constructed as linear mixed models using the lme4 package (Bates et al. 2015) which allowed for random effects of block. Interactive terms that were nonsignificant in the overall MANOVA were included in the trait-level analyses, but any significant responses to these interactions were noted as potentially spurious in light of multiple comparisons across four response traits.

While trait-level ANOVA results, with a single response variable, facilitated interpretation of the overall multivariate response quantified in the MANOVA, significant interactions with species still resulted in the need to interpret high-order interactions. To better understand species-specific patterns, for each species-trait combination we fit a species-level ANOVA of that trait’s response to the three treatment variables (rainfall treatment, nutrient treatment, and sampling year) and their interactions, again using the lme4 package to allow for block random effects. While all possible interactions among the treatment variables were included in these models, species-level interpretation centered on treatment variables and combinations thereof that showed significant interactions with the species term in the preceding MANOVA and trait-level ANOVA analyses.

Results

Our three study years differed substantially in their patterns of precipitation (Figure 4.1). 2021 featured approximately average total precipitation, though much of this was received as a single large event after soil water measurements and trait sampling for early season species (but before sampling of later season species and biomass). In contrast, 2022 was the driest growing season since the establishment of a weather station in 1997. Total rainfall in 2023 was similar to 2021, but with most of that rainfall falling in late April and May rather than late June. These rainfall differences were reflected in gravimetric soil water measurements, which averaged 3.2% in 2021, 2.3% in 2022 and 4.2% in 2023 (F = 65.8, p <0.001; Figure 4.2). The rainfall shelter treatment produced a smaller, but still detectable effect on soil water with an average soil water of 2.90% in drought plots, compared to 3.36% in ambient water plots (Tukey post-hoc, p=0.015) and 3.38% in water addition plots (p=0.007). Ambient and water addition plots did not differ significantly from one another (p=0.95).

Our MANOVA analysis of functional trait variation showed no evidence of a four-way interaction between the treatment variables and species in driving trait values (Pillai's Trace= 0.165, df=20, p =0.573). These trait values did, however, respond interactively to nutrient addition, sampling year and species identity (Pillai's Trace= 0.197, df= 10, p <0.001), and while the three-way interaction between rainfall treatment, nutrient addition and species did not produce a significant effect (Pillai's Trace= 0.016, df=10, p =0.779), rainfall manipulation did influence functional traits through two-way interactions with species (Pillai's Trace= 0.134, df=10, p =0.015) and nutrient addition

(Pillai's Trace= 0.034, df=2, *p* =0.049). There were no significant interactions between rainfall treatment and sampling year (Table 4.1).

Specific leaf area, in the trait-level ANOVA, responded to the three-way interaction between nutrient treatment, sampling year, and species identity (χ^2=31.5, df=10, *p*=0.0004), as well as to the interaction between rainfall treatment and species identity (χ^2=28.5, df=10, *p*=0.0015). Two species, *Artemisia frigida* and *Bouteloua gracilis*, showed decreasing, more conservative SLA values with experimentally reduced water availability, while *Coreopsis tinctoria* showed a more complex response to rainfall manipulation, and the remaining three species were not significantly affected by this treatment. Nutrient addition and sampling year produced interactive responses in four of six study species, all of which showed reduced SLA values in the driest sampling year, 2022, increased SLA with nitrogen addition, and increased nitrogen effects in wetter sampling years. Of the remaining species, *Coreopsis tinctoria* showed an additive response to nutrient addition and sampling year, while only *Bouteloua gracilis* lacked a nutrient response, and no species failed to respond to sampling year (Table 4.2, Figure 4.3). While the trait-level ANOVA also showed a highly significant four-way interaction between species and the three treatment variables (χ^2=44.5, df=20, *p*=0.0012), driven primarily by *Artemisia frigida*, this effect was not supported by the MANOVA analysis (F=0.963, df=20, *p* =0.57).

Leaf dry matter content likewise responded interactively to nutrient addition, sampling year and species (χ^2=36.3, df=20, *p*<0.001), and to rainfall treatment and species (χ^2=38.8, df=10, *p*<0.001), as well to rainfall treatment and nutrient treatment

(χ^2=6.76, df=2, p=0.034). Three species, *Artemisia filifolia*, *Bouteloua gracilis*, and *Carex filifolia* showed lower, more acquisitive LDMC values with experimentally increased water availability. The combination of rainfall addition and nutrient addition further reduced LDMC in the trait-level ANOVA, but this effect was small in magnitude and not apparent in the species-level analyses. Interactive responses to nutrient addition and sampling year occurred in three species, *Carex filifolia*, *Hesperostipa comata*, and *Sphaeralcea coccinea*, with nutrient addition reducing LDMC in 2023, the wettest sampling year, but producing smaller or even positive responses in drier years. *Bouteloua gracilis* and *Coreopsis tinctoria* instead showed additive LDMC declines in wet years and with nutrient addition (Table 4.3, Figure 4.4). As with SLA, *Artemisia frigida* showed a significant response to the three-way interaction between year, nutrient addition and rainfall treatment in the species-level analysis, but neither the MANOVA analysis (F=0.963, df=20, p =0.57) nor the trait-level LDMC analysis (χ^2=27.7, df=20, p=0.117) provided support for this effect.

Plant height responded interactively both to nutrient addition and rainfall treatment (χ^2=10.3, df=2, p=0.0049) and to nutrient addition and sampling year, with the latter effect, but not the former showing further interaction with species identity (χ^2=27.4, df=10, p=0.0022). The nutrient addition by rainfall treatment interaction, while significant in the overall trait-level analysis, could be detected in only two species-level analyses, with *Bouteloua gracilis* and *Carex filifolia* showing increased responses to nitrogen with experimentally increased rainfall availability. The remaining four species lacked either additive or interactive responses to rainfall treatment. Plant height increased with nutrient addition and in wetter sampling years, with these effects occurring interactively in *Bouteloua gracilis*, *Hesperostipa comata*, *Sphaeralcea coccinea* but additively in the remaining species (Table 4.4, Figure 4.5).

Chlorophyll content varied interactively with species across both sampling years (χ^2=190, df=10. *p*<0.001) and nutrient addition treatments (χ^2=54.7, df=5, *p*<0.001), but showed no response to water (χ^2=0.062, df=2, *p*=0.969) and only marginal evidence of interactive effects (Table 4.5). All species showed elevated chlorophyll content in nutrient addition plots and in 2023, the wettest sampling year. The magnitude of these differences was, however, contingent on species, as were patterns of variation between the 2021 and 2022 measurements (Figure 4.6).

Despite this variation in traits, total plant biomass did not respond to rainfall manipulation treatments (F=0.59, *p* = 0.56) or to the interaction of rainfall treatments with any other variable (Figure 4.7). This lack of effect occurred despite our continued observation of nitrogen by year interactions. Consistent with past observations of productivity in the associated Nutrient Network plots (Wang et al. 2017), biomass was elevated in nitrogen plots, with the highest biomass and largest nitrogen effects occurring in 2023 (the wettest year) and the lowest biomass and smallest nitrogen effects occurring in 2022 (the driest year).

Discussion

Despite our rainfall treatments' lack of effect on total biomass, we found that three of four functional traits responded to these treatments or to their interaction with nitrogen addition and species identity. Four of six species showed evidence of more acquisitive SLA and/or LDMC values with experimentally increased rainfall availability, while plant height showed increasing nutrient responses with water addition. Trait responses to rainfall manipulation were less consistent and smaller in magnitude than responses to sampling year, which occurred for all species and traits, frequently with interactive effects of nutrient addition. Both annual variation and variation across treatments show a pattern of increasingly acquisitive plant functional traits with water availability, with the effects of rainfall treatment providing evidence of a causal relationship, while the comparatively low frequency and magnitude of these effects suggest that factors beyond total precipitation also play important roles.

These results are largely consistent with those observed from 2017-2020 in nearby study plots (Wheeler et al. 2023), both in the general pattern of increasing trait acquisitiveness with water availability and in certain trait-specific patterns. LDMC and SLA, the study traits most closely associated with the leaf economics spectrum (Wright et al. 2004) showed the clearest associations with growing season precipitation in year-to-year comparisons and the clearest evidence of responses to experimental rainfall manipulation. In contrast, chlorophyll content showed clear variation across sampling years, but this variation did not consistently correspond to growing season precipitation, and chlorophyll showed no response to experimental rainfall manipulation, suggesting that its annual responses are largely driven by other forms of annual variation. Plant height, intriguingly, varied across study years in this study but not between 2017 and 2020. This distinction may stem from an expanding range of annual variation, with drought conditions more extreme in 2022 than in previous sampling years.

While this study shows evidence of a causal relationship between precipitation and functional trait values, particularly for SLA and LDMC, these responses are smaller and less consistent than might be expected if annual trait variation is fully explained by precipitation differences. This pattern may, in part, be explained by the relative magnitude of water variation under these treatments. Our rainfall manipulations were designed to exclude and add 50% of ambient rainfall, and treatment effectiveness may have been reduced by factors such as wind and runoff. In contrast, total growing season precipitation in 2022 was only one third of that occurring in 2021 or 2023. This difference in magnitude is reflected by our soil water measurements which, while limited in temporal scope, suggest treatment effects of rainfall manipulation on soil water slightly less than half the effects of annual variation. It is also possible that precipitation outside the April-August window of our rainfall manipulations also affects these traits or that the effects of rainfall variation on functional traits will scale in a nonlinear fashion. While rainfall manipulation in our system produced no detectable effects on biomass, a more extreme, year-round reduction in rainfall in a nearby *Bouteloua gracilis* dominated grassland produced a near total loss of biomass (Smith et al. 2023). Functional trait responses may be similarly contingent on the timing and magnitude of rainfall manipulation.

Even so, the relative consistency and strength of year and treatment effects suggests that factors beyond total growing season precipitation contribute to annual trait differences in this system. While factors unrelated to moisture may contribute, it is likely, given the consistent pattern of increased trait acquisitiveness in high rainfall years, that components of moisture availability beyond total precipitation play important roles. Such factors may include the timing and relative size of rainfall events. In Colorado shortgrass steppes, for instance, a small number of large precipitation events result in higher productivity than a large number of small events, even if total precipitation is held constant (Heisler-White et al. 2008), and eliminating variability in event size and timing can likewise increase productivity (Griffin-Nolan et al. 2021). Within our study, 2021 and 2023 featured similar total precipitation, but the former year's single large late-season rainfall event did not produce the same acquisitive trait values as the latter year's high early season precipitation.

Additionally, functional traits may respond to water stress associated with temperature and humidity. While rainfall shelter manipulations alter received precipitation and consequently soil water availability, they leave atmospheric properties largely unchanged. A rainfall exclusion treatment in a wet year, for instance, will receive reduced rainfall relative to an ambient rainfall treatment, but both plots will experience the increased humidity and reduced temperature that accompany that rain. Spatial comparisons at a global scale show that many traits, including SLA, correlate more strongly with temperature than precipitation, with the effects of temperature potentially including both direct effects and changes owing to increased evapotranspiration (Moles et al. 2014). The importance of atmospheric water demand relative to soil water is expected to increase further with climate change (Novick et al. 2016).

More broadly, it is noteworthy that rainfall manipulation studies often detect reduced or absent biomass effects relative to observational studies. While the Drought

Net collaboration shows a clear overall trend towards reduced biomass with experimental drought treatments, many of its individual sites lack detectable biomass changes, even when experimental drought is compounded by annual drought (Smith et al. 2024). Compared to observational studies of drought, experiments produce productivity effects averaging approximately 50% smaller , and while experiments are valuable as tests of causal mechanisms, observational studies may yield better estimates of effect size (Kröel-Dulay et al. 2022). Our results suggest that functional trait variation may be similar to biomass variation in this regard. Rainfall manipulation experiments have a valuable role to play in isolating the effects of precipitation per se on functional trait values, but observational studies may provide better estimates of responses to future droughts, which will include changes to event timing and size, and changes in atmospheric water demand, in addition to changes in total rainfall. As these effects can vary across species, trait-based analyses should consider the potential not only for functional traits to vary within species, but also the potential for variation in these species' responses.

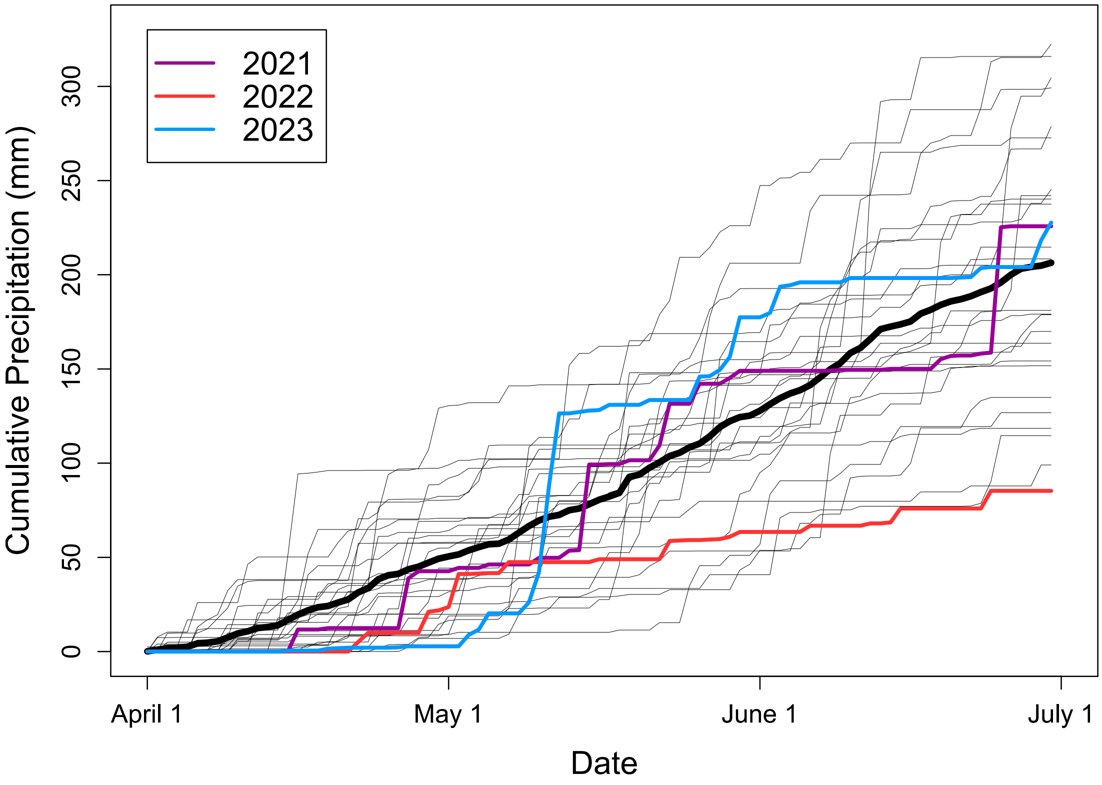

Figure 4.1. Cumulative precipitation: Narrow black lines indicate locally measured cumulative precipitation beginning April 1 for each year from 1997 to 2020, with the solid black line indicating mean cumulative precipitation. Colored lines indicate cumulative precipitation for each of this study's three sampling years.

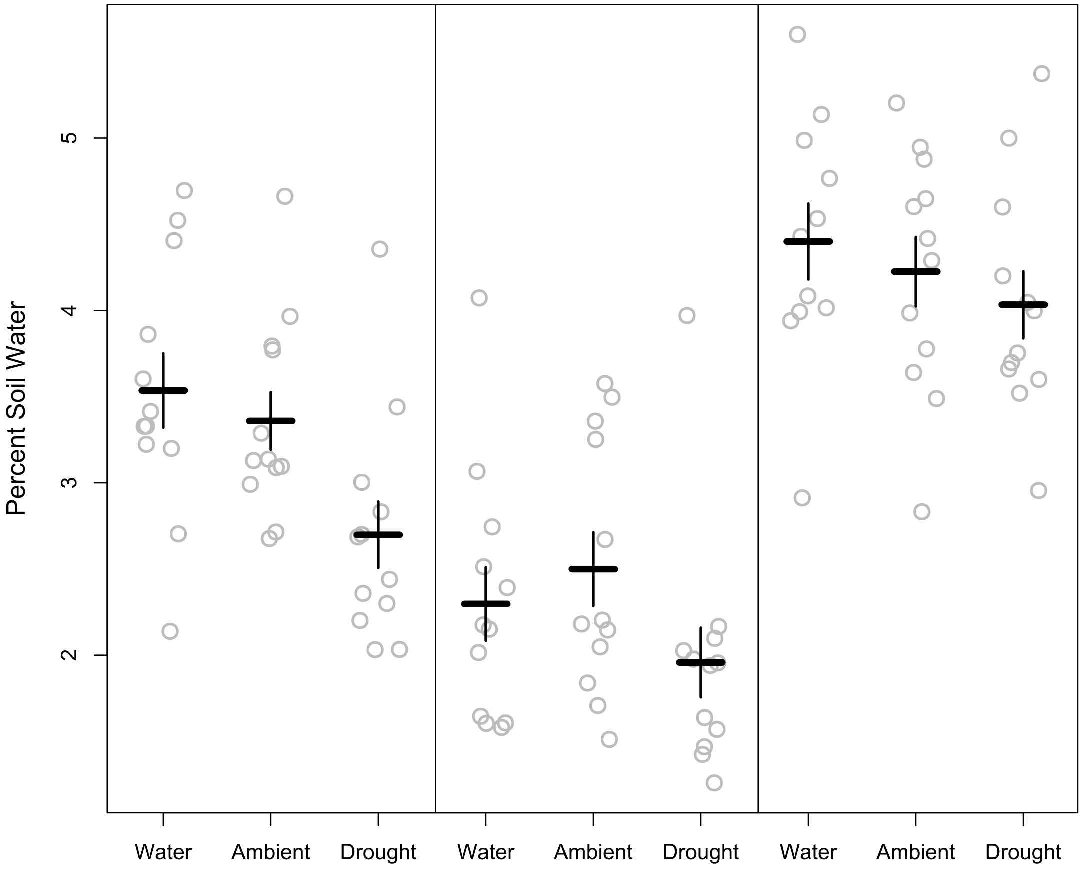

Figure 4.2. Soil water. Soil water was measured gravimetrically, using samples collected in late June. Data points represent plot-level measurements, horizontal lines represent mean values for each treatment-year combination, and vertical lines represent standard errors of those means. As nutrient addition did not affect soil water, nutrient treatments are aggregated within each rainfall treatment.

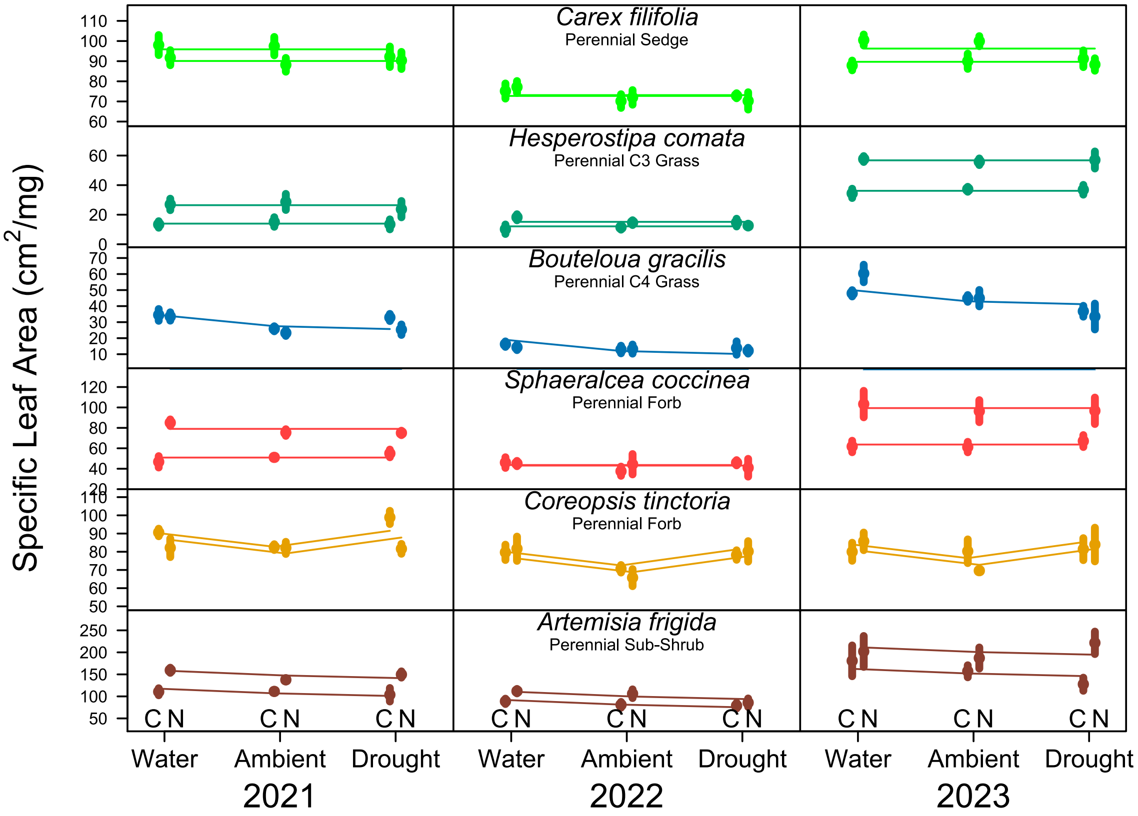

Figure 4.3. Specific Leaf Area. Points indicate mean measured SLA values for each species in the corresponding year and treatment, with vertical lines representing the standard errors of those means. Horizontal lines represent species-level modelled values, with non-significant effects omitted. These lines connect rainfall treatments within a given year and nutrient treatment. Thus, their slopes represent rainfall treatment effects, the vertical distances between lines in a single panel represent nutrient effects, and differences between panels represent year effects and interactions. Note that y-axis scaling is unique to each species.

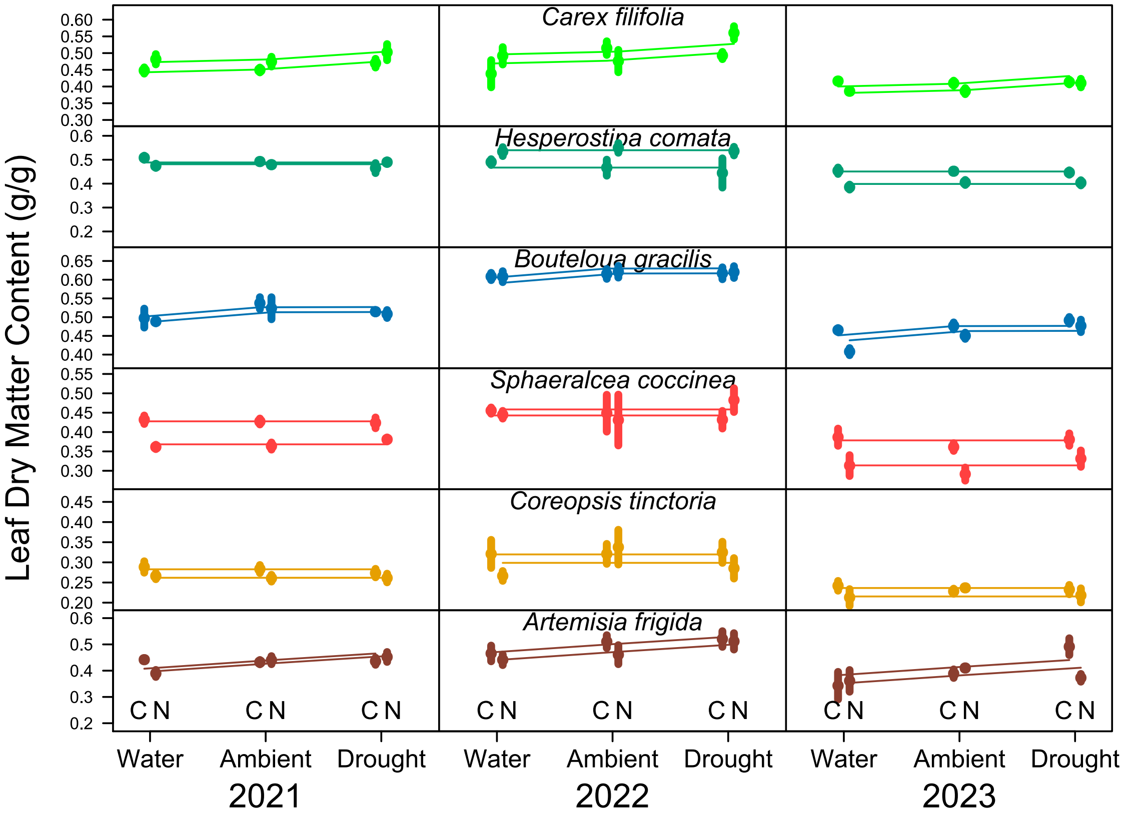

Figure 4.4. Leaf Dry Matter Content. Points indicate mean measured LDMC values, with representation of errors and modelled effects matching Figure 4.3.

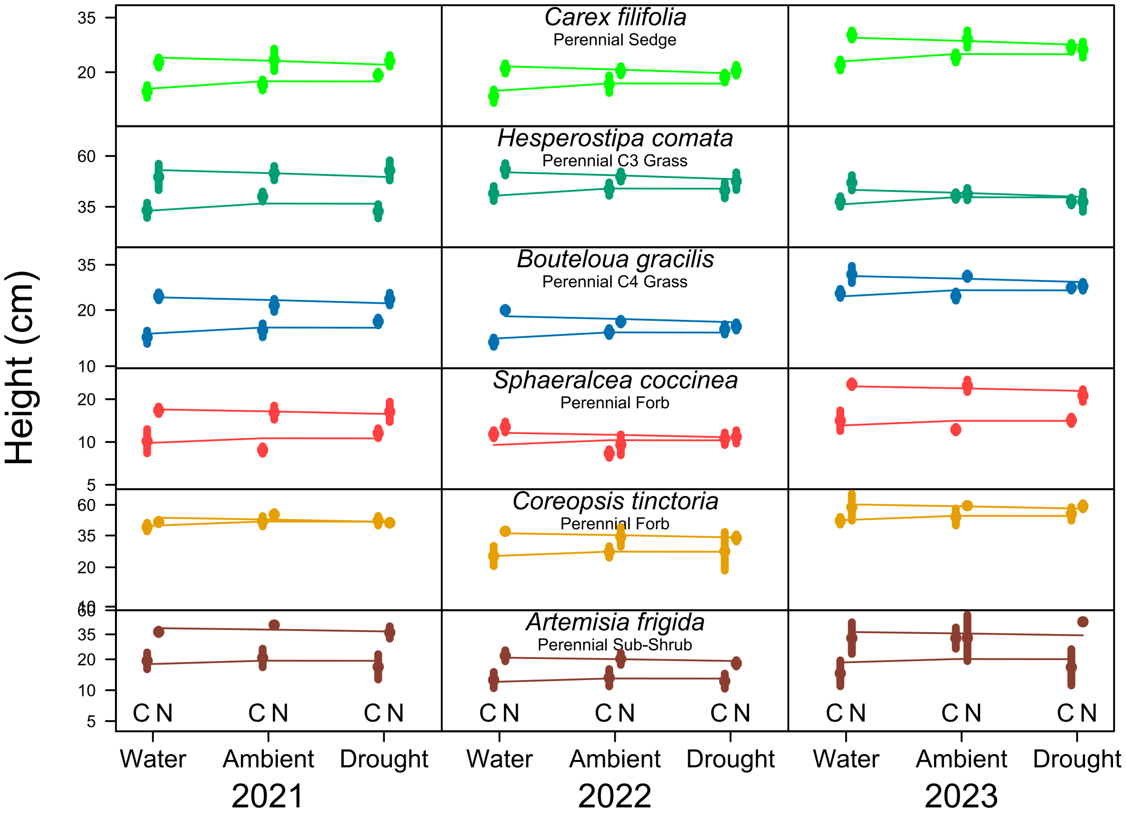

Figure 4.5. Plant Height. Points indicate mean measured height values, with representation of errors and modelled effects matching Figure 4.3, except that modeled effects are based on the trait-level model, due to the presence of a non-species-specific nutrient by rainfall treatment interaction

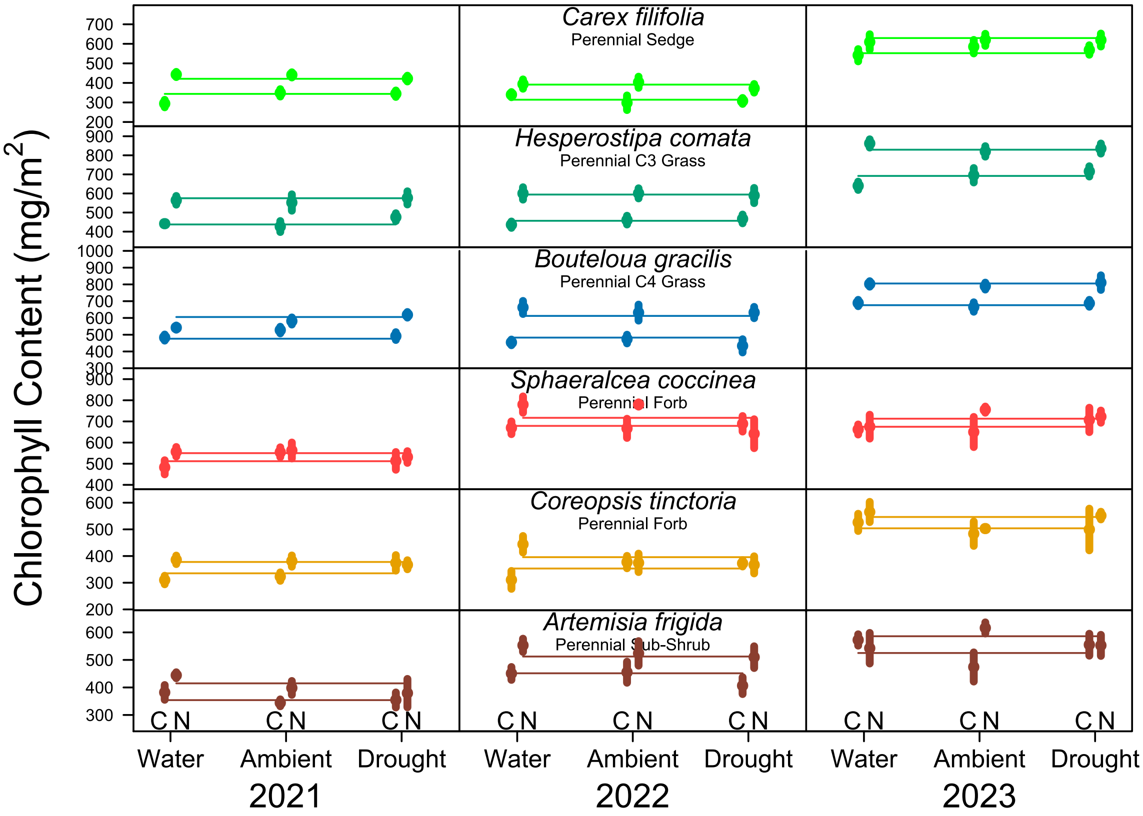

Figure 4.6. Chlorophyll Content. Points indicate mean measured chlorophyll values, with representation of errors and modelled effects matching Figure 4.3.

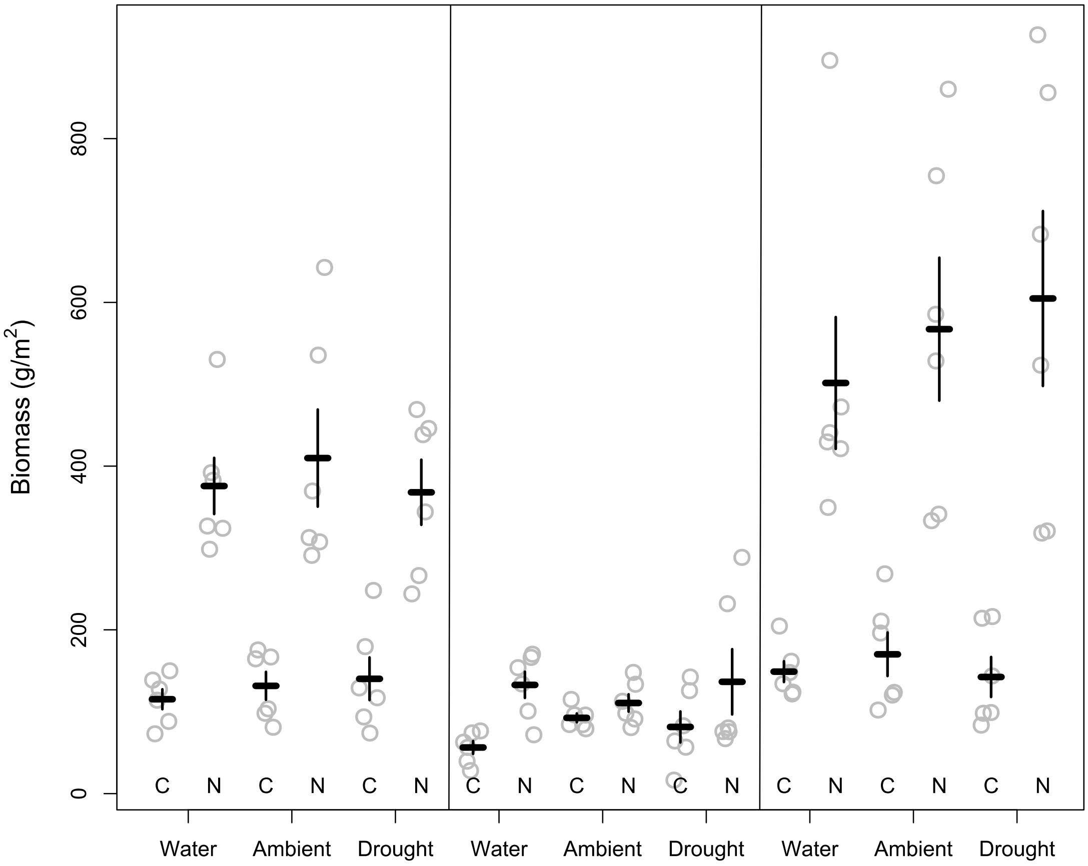

Figure 4.7. Aboveground biomass. Plant biomass was measured in July following Nutrient Network protocols. Results shown are summed across all functional groups. Data points represent plot-level measurements, with horizontal lines representing mean values for each treatment-year combination, and vertical lines representing standard errors of those means.

Table 4.1. MANOVA Results

	Df	Pillai's Trace	F	Numerator Df	Denominator DF	*p*
Nutrient	**1**	**0.453**	**92.0**	**4**	**445**	**<0.001**
Water (Rainfall Treatment)	**2**	**0.074**	**4.275**	**8**	**892**	**<0.001**
Year	**1**	**1.07**	**129**	**8**	**892**	**<0.001**
Species	**5**	**2.66**	**178**	**20**	**1792**	**<0.001**
Nutrient x Water	**2**	**0.034**	**1.96**	**8**	**892**	**0.049**
Nutrient x Year	**2**	**0.089**	**5.17**	**8**	**892**	**<0.001**
Water x Year	4	0.027	0.768	16	1792	0.723
Nutrient x Species	**5**	**0.395**	**9.82**	**20**	**1792**	**<0.001**
Water x Species	10	0.134	1.56	40	1792	0.014
Year x Species	**10**	**0.983**	**14.6**	**40**	**1792**	**<0.001**
Nutrient x Water x Year	4	0.016	0.453	16	1792	0.968
Nutrient x Water x Species	10	0.072	0.822	40	1792	0.779
Nutrient x Year x Species	**10**	**0.197**	**2.32**	**40**	**1792**	**<0.001**
Water x Year x Species	20	0.100	0.576	80	1792	0.999
Nutrient x Water x Year x Species	20	0.165	0.963	80	1792	0.572

Table 4.2a. SLA ANOVA Results

	χ^2	Df	*p*
Nutrient	**87.3**	**1**	**<0.001**
Water (Rainfall Treatment)	**20.4**	**2**	**<0.001**
Year	**681**	**2**	**<0.001**
Species	**4590**	**5**	**<0.001**
Nutrient x Water	3.24	2	0.198
Nutrient x Year	**29.9**	**2**	**<0.001**
Water x Year	3.95	4	0.413
Nutrient x Species	**157**	**5**	**<0.001**
Water x Species	**28.5**	**10**	**0.0015**
Year x Species	**326**	**10**	**<0.001**
Nutrient x Water x Year	2.91	4	0.573
Nutrient x Water x Species	8.88	10	0.544
Nutrient x Year x Species	**31.4**	**10**	**0.0004**
Water x Year x Species	13.8	20	0.839
Nutrient x Water x Year x Species	44.5	20	0.0012

Table 4.2b. SLA Responses by Species. Bolded values indicate significant results (p<0.05). Grey text indicates relationships not supported by the overall MANOVA analysis or the trait-level ANOVA

	Nutrient	Water (Rainfall Treatment)	Year	*Nutrient x Water*	Nutrient x Year	*Water x Year*	*Nutrient x Water x Year*
Artemisia frigida	**<0.001**	**0.019**	**<0.001**	0.506	**0.040**	0.714	0.015
Bouteloua gracilis	0.708	**<0.001**	**<0.001**	0.189	0.164	<0.001	0.386
Carex filifolia	0.799	0.090	**<0.001**	0.392	**0.006**	0.728	0.183
Coreopsis tinctoria	**0.046**	**<0.001**	**<0.001**	0.710	0.084	0.397	0.066
Hesperostipa comata	**<0.001**	0.893	**<0.001**	0.245	**<0.001**	0.822	0.803
Sphaeralcea coccinea	**<0.001**	0.597	**<0.001**	0.170	**<0.001**	0.999	0.853

Table 4.3a. LDMC ANOVA Results

	χ^2	Df	*p*
Nutrient	**8.98**	**1**	**0.0027**
Water (Rainfall Treatment)	**20.9**	**2**	**<0.001**
Year	**577**	**2**	**<0.001**
Species	**2130**	**5**	**<0.001**
Nutrient x Water	**6.76**	**2**	**0.0340**
Nutrient x Year	**35.2**	**2**	**<0.001**
Water x Year	4.96	4	0.291
Nutrient x Species	**29.0**	**5**	**<0.001**
Water x Species	**38.8**	**10**	**<0.001**
Year x Species	**66.4**	**10**	**<0.001**
Nutrient x Water x Year	4.66	4	0.324
Nutrient x Water x Species	14.0	10	0.175
Nutrient x Year x Species	**36.3**	**10**	**<0.001**
Water x Year x Species	19.9	20	0.462
Nutrient x Water x Year x Species	27.7	20	0.117

Table 4.3b. LDMC Responses by Species. Bolded values indicate significant results (p<0.05). Grey text indicates relationships not supported by the overall MANOVA analysis or the trait-level ANOVA

	Nutrient	Water (Rainfall Treatment)	Year	Nutrient x Water	Nutrient x Year	Water x Year	Nutrient x Water x Year
Artemisia frigida	**0.043**	**<0.001**	**<0.001**	0.851	0.660	0.113	0.0060
Bouteloua gracilis	**0.030**	**0.0006**	**<0.001**	0.534	0.546	0.111	0.775
Carex filifolia	0.083	**0.0020**	**<0.001**	0.042	**0.010**	0.190	0.100
Coreopsis tinctoria	**0.014**	0.531	**<0.001**	0.151	0.889	0.537	0.398
Hesperostipa comata	0.544	0.495	**<0.001**	0.098	**<0.001**	0.866	0.937
Sphaeralcea coccinea	**<0.001**	0.358	**<0.001**	0.125	**0.0006**	0.904	0.880

Table 4.4a. Height ANOVA Results

	χ^2	Df	*p*
Nutrient	**236**	**1**	**<0.001**
Water (Rainfall Treatment)	1.40	2	0.495
Year	**227**	**2**	**<0.001**
Species	**1660**	**5**	**<0.001**
Nutrient x Water	**10.6**	**2**	**0.0049**
Nutrient x Year	**7.58**	**2**	**0.022**
Water x Year	3.41	4	0.492
Nutrient x Species	**65.2**	**5**	**<0.001**
Water x Species	17.4	10	0.065
Year x Species	**172**	**10**	**<0.001**
Nutrient x Water x Year	1.86	4	0.761
Nutrient x Water x Species	10.8	10	0.375
Nutrient x Year x Species	**27.4**	**10**	**0.0022**
Water x Year x Species	13.5	20	0.855
Nutrient x Water x Year x Species	16.4	20	0.694

Table 4.4b. Height Responses by Species. Bolded values indicate significant results (p<0.05). Grey text indicates relationships not supported by the overall MANOVA analysis or the trait-level ANOVA

	Nutrient	Water (Rainfall Treatment)	Year	Nutrient x Water	Nutrient x Year	Water x Year	Nutrient x Water x Year
Artemisia frigida	**<0.001**	0.198	**<0.001**	0.612	0.304	0.562	0.403
Bouteloua gracilis	**<0.001**	0.713	**<0.001**	0.0002	**0.0040**	0.520	0.424
Carex filifolia	**<0.001**	0.128	**<0.001**	0.0015	0.431	0.876	0.761
Coreopsis tinctoria	**<0.001**	0.694	**<0.001**	0.487	0.081	0.893	0.940
Hesperostipa comata	**<0.001**	0.360	**0.0017**	0.244	**0.0013**	0.666	0.599
Sphaeralcea coccinea	**<0.001**	0.597	**<0.001**	0.170	**<0.001**	0.999	0.854

Table 4.5a. Chlorophyll ANOVA Results

	χ^2	Df	*p*
Nutrient	**276**	**1**	**<0.001**
Water (Rainfall Treatment)	0.062	2	0.970
Year	**1070**	**2**	**<0.001**
Species	**1000**	**5**	**<0.001**
Nutrient x Water	5.86	2	0.0535
Nutrient x Year	5.99	2	0.0501
Water x Year	8.03	4	0.0904
Nutrient x Species	**54.7**	**5**	**<0.001**
Water x Species	11.3	10	0.336
Year x Species	**190**	**10**	**<0.001**
Nutrient x Water x Year	1.78	4	0.776
0.0Nutrient x Water x Species	12.4	10	0.261
Nutrient x Year x Species	17.3	10	0.067
Water x Year x Species	12.6	20	0.894
Nutrient x Water x Year x Species	25.2	20	0.194

Table 4.5b. Chlorophyll Responses by Species. Bolded values indicate significant results (p<0.05). Grey text indicates relationships not supported by the overall MANOVA analysis or the trait-level ANOVA

	Nutrient	Water (Rainfall Treatment)	Year	Nutrient x Water	Nutrient x Year	Water x Year	Nutrient x Water x Year
Artemisia frigida	**<0.001**	0.119	**<0.001**	0.746	0.223	0.673	0.207
Bouteloua gracilis	**<0.001**	0.840	**<0.001**	0.363	0.0003	0.205	0.671
Carex filifolia	**<0.001**	0.556	**<0.001**	0.583	0.087	0.464	0.392
Coreopsis tinctoria	**0.0010**	0.821	**<0.001**	0.016	0.976	0.449	0.223
Hesperostipa comata	**<0.001**	0.252	**<0.001**	0.155	0.398	.0789	0.691
Sphaeralcea coccinea	**0.0031**	0.426	**<0.001**	0.0025	0.919	0.167	0.102

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