Understanding Spatiotemporal Variation in Plant Form-Function Relationships

by

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DISSERTATION ABSTRACT

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Title: Understanding Spatiotemporal Variation in Plant Form-Function Relationships

Leaf traits represent the wide variety of leaf forms plants have evolved to maximize carbon gains over their lifetimes. Although extensive research supports the use of these physiological metrics, much variation in leaf traits remains unexplained. In this thesis, I address three influences on leaf trait variation: leaf ontogeny, environmental stress, and cross-kingdom interactions.

In the first study, I use two evergreen dwarf shrubs as case studies to demonstrate that leaf traits vary depending on cohort (current year's growth or previous year's growth) in *Vaccinium vitis-idaea* and *Empetrum nigrum*. Despite this difference, researchers often do not specify which cohort they measured or differentiate between the cohorts.

In the second study, I test how plant form and function hold in herbaceous prairie species under experimental drought conditions in a U.S. Pacific Northwest grassland. I found that plant form-function relationships were robust against drought, and leaf traits remained unchanged.

In the third study, I used stable isotope tracers to determine if common mycorrhizal networks favor fungal resource acquisition at the expense of plant resource demands, or if they are passive channels through which plants regulate resource fluxes. I found that plant functional type and tissue stoichiometry were the most important predictors of interspecific resource transfer.

This dissertation includes previously published and unpublished coauthored material.

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The data used for analyses in Chapter 3 are available at the Dryad digital data repository via https://doi.org/10.5061/dryad.vmcvdncr7 with a CC0 1.0 license. The data used for analyses in Chapter 4 will be forthcoming on the Dryad digital data repository following publication at https://doi.org/10.5061/dryad.7pvmcvdxt with a CC0 1.0 license.

I dedicate this work to the women natural historians in my family, especially those who pursued their curiosity-driven questions without academic training or acknowledgement.

TABLE OF CONTENTS

Chapter Page

Chapter Page

3. LEAF TRAITS PREDICT WATER-USE EFFICIENCY IN U.S. PACIFIC NORTHWEST GRASSLANDS UNDER RAIN EXCLUSION TREATMENT ... 43

LIST OF FIGURES

Figure Page

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

Table Page

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 1:

INTRODUCTION

Plants evolved a variety of leaf forms to maximize lifetime net carbon gain, forms which can be measured as traits that represent economic tradeoffs that optimize carbon gain (Wright et al., 2004). Despite the clear link between metabolism, carbon, and plant phenotypic expression, we struggle to accurately scale plant form and function from organisms to sites to regions (Van Der Plas et al., 2020). Large error may come from how we handle variation (Chacón-Labella et al., 2023); however, we must also understand that plants are contextual (Hagan et al., 2023). They interact with other organisms and their environment.

Metabolic scaling theory (MST) proposes that we can predict organismal carbon fluxes by scaling by mass and incorporating environmental and physiological properties such as traits (Enquist et al., 2003). At the core of MST are two scaling variables. Mass scaled by ¾ ($M^{3/4}$) is instantaneous carbon gained and is constrained by optimal resource transport (West et al., 1997). Mass scaled by ¼ ($M^{1/4}$) is lifetime carbon gained and represents an organism's carbon efficiency (West et al., 2001). This scale is reasonably consistent in plants around the world. *Leaf optimal lifespan theory* (LOLT) proposes a similarly carbon-based prediction: natural selection favors leaves that gain more carbon than they use over their lifetime. This explains why thick leaves long-lived and thin leaves are short-lived–there is a tradeoff between carbon investment and durability, and, when the environment allows, a plant favors younger leaves with highest instantaneous carbon gain (Castorena et al., 2022). By combining MST and LOLT, G_{max} *theory* predicts that a plant's carbon gain (plant mass) is scaled by time (lifespan) and space (plant size). When all factors are accounted for, total lifetime carbon gain is independent of size.

Leaf carbon economy is the primary constraint for variation among most, if not all traits. This leads to a generalizable correlation between traits, a relationship known as the leaf economic spectrum (LES; Wright et al., 2004). Plants tend to be either “fast” and resource-acquisitive or “slow” and resource-conservative, trait strategies that polarize the LES and represent one of the two primary axes of global trait variation (Díaz et al., 2016). The other primary axis is total plant height, the space component of the G_{max} theory. However, these relationships were originally described under “ideal” conditions on mature leaves. In Chapter 2, I test the effect of evergreen leaf ontogeny on leaf traits to determine how leaves grown in the current or previous growing season differ. In Chapter 3, I test the effect of experimental drought conditions on allometric structure-function relationships like those of the LES.

Plant traits can also provide valuable metrics for understanding community dynamics when combined with environmental factors, biotic interactions, and performance metrics (McGill, 2006). This can extend beyond plant-plant interactions into quantifying and predicting plant-heterotroph interactions. For example, in Chapter 4 I used ^{15}N-enriched ammonia and ^{13}C-enriched carbon dioxide to trace nutrient transfer between plants and mycorrhizal fungi. My results suggest that the primary driver of this interaction is leaf nitrogen content. Annuals had lower leaf nitrogen than perennials, demonstrating how community structure can alter inter-taxa interactions and community function.

Despite the clear link between metabolism, carbon, and plant phenotypic expression, we struggle to accurately scale plant form and function from organisms to sites to regions. While large error may come from how we handle variation (Chacón-Labella et al., 2023), we must also understand that plants interact with other organisms and their environment. Specifically, most plants rely on soil and soil microbes for structure, water, and nutrients. In turn, plants can modify their environment to be more favorable for their survival, including altering soil properties, creating microclimates, or, in the case of large forests, modifying the climate itself.

Metabolic and allometric scaling theory postulates that intrinsic physiological processes, particularly those based on carbon, can be measured and predicted from plant structure (Enquist, 2002). Inclusive fitness theory goes further, proposing that trait success (such as any regulating carbon production or ability to participate in mycorrhizal carbon-nutrient exchanges) is determined by community composition and relatedness (Hamilton, 1964). When applied to plant-soil interactions, and related carbon functions, certain host-specific fungi are expected to have advantages in belowground carbon, water, and nutrient "markets", which are tied to performance of the whole ecosystem (Noë & Kiers, 2018).

My dissertation research was incorporated into and expanded upon two existing large collaborative projects: The Underappreciated Roles of Dwarf-Shrubs in Responding to and Influencing Global Climate Change (DURIN) led by the Between the Fjords Lab at the University of Bergen, Norway, and the Heating of Prairie Systems (HOPS) experiment, a collaboration of several labs at the Institute of Ecology and Evolution in the University of Oregon, USA.

DURIN is a climate gradient experiment that pairs inland and coastal sites in northern and southern Norway to parameterize dwarf shrubs for the CLM-FATES model. Forty plots were established at each of the four core sites—twenty in a forested area and twenty in a nearby open area. Each plot is dominated by a focal dwarf shrub species that is commonly found throughout Norway (*Vaccinium vitis-idaea*, or lingonberry/tytebær; *Vaccinium myrtillus*, or bilberry/blåbærlyng; *Empetrum nigrum*, or crowberry/krekling; and *Calluna vulgaris*, or heather/lyng; n = 5 plots per species per environment per site). In summer 2023, I co-led a leaf traits campaign that covered this gradient. Chapter 2 uses a subset of the resulting leaf traits dataset collected for the DURIN experiment as a whole. I analyzed data from the two focal evergreen dwarf shrubs (*E. nigrum* and *V. vitis-idaea*) at Sogndal, the inland southern site. This chapter is titled "Evergreen Leaf Cohort Alters Plant Function: A Case-Study of Two Evergreen Dwarf Shrubs" and was a collaboration between myself, Sonya R. Geange, Akuonani Phiri, Julia Schlick-Steiner, Dagmar Egelkraut, Lucas C.R. Silva, Aud Halbritter, and Vigdis Vandvik. I found that leaf age cohort affected leaf traits regardless of environmental variation. I compared my dataset to existing data available from regional and global databases and found that my data had a similar or greater range than what was available from the databases. However, the databases did not report which leaf cohort was sampled and the range of leaf trait values suggest that both were included indiscriminately. In a broad survey of the literature, I found that many studies also did not report which leaf age cohort was sampled, and that current year leaves were overrepresented. Only approximately 15% of studies measured both cohorts separately. Using biomass estimates of the proportion of each cohort on the plants I measured, I demonstrated that it is important to sample both cohorts deliberately when using evergreen leaf trait data at a regional or greater scale. My results will inform a subsequent manuscript that describes the leaf traits across the entire gradient.

HOPS was an active warming and artificial drought experiment located on a climate gradient spanning 520 km from southern Oregon to central Washington west of the Cascade Mountains. Each site had thirty plots that represented five replicates of four treatments (warming [n = 5], drought [n = 10], warming + drought [n = 5], control [n = 10]). The drought and control plots also had a restoration treatment, where half the plots (n = 5 control, n = 5 drought) were heavily disturbed to remove existing vegetation and reseeded with a native seed mix multiple years in a row. This restoration treatment was contrasted with the existing introduced pasture grass community (n = 5 control, n = 5 drought). In my thesis, I only focused on the drought and control plots, both restored and pasture, without any analyses of the warming treatments.

Chapter 3 is called “Leaf Traits Predict Water-Use Efficiency in US Pacific Northwest Grasslands Under Rain Exclusion Treatment” and is published in Journal of Geophysical Research: Biogeosciences in 2022. It is co-authored by myself, Toby M. Maxwell, Paul B. Reed, Scott D. Bridgham, and Lucas C. R. Silva. We asked, ‘Does drought stress in temperate grasslands alter the relationship between plant structure and function?’ We report data from the HOPS experiment using a total of 139 individual plants of 12 species (11 genera and four families) across all three sites. We found rain exclusion did not alter the interspecific relationship between foliar traits and stoichiometry or intrinsic water-use efficiency (iWUE). Rain exclusion reduced iWUE in grasses, an effect was primarily species-specific, although leaf morphology, life history strategy, and phylogenetic distance predicted iWUE for all 12 species when analyzed together. Variation in specific leaf area explained most of the variation in iWUE between different functional groups, with annual forbs and annual grasses at opposite ends of the resource-use spectrum. Our findings are consistent with expected trait-driven tradeoffs between productivity and resource-use efficiency and provide insight into strategies for the sustainable use and conservation of temperate grasslands.

Chapter 4 is called “Plant functional types and tissue stoichiometry explain nutrient transfer in common arbuscular mycorrhizal networks of temperate grasslands” and was co-authored by myself, Katherine L. Shek, Toby M. Maxwell, Paul B. Reed, Barbara Bomfim, Scott D. Bridgham, Brendan Bohannan, and Lucas C. R. Silva. We asked, ‘Do common mycorrhizal networks (CMNs) favor fungal resource acquisition at the expense of plant resource demands (a fungi-centric view), or are they passive channels through which plants regulate resource fluxes (a plant-centric view)?' In the HOPS experiment, we used stable isotope tracers ($^{13}CO_2$ and $^{15}NH_3$), plant traits, and mycorrhizal DNA to quantify above- and belowground carbon and nitrogen. Plant functional type and tissue stoichiometry were the most important predictors of interspecific resource transfer. Of "donor" plants, 98% were ^{13}C-enriched, but we detected transfer in only 2% of "receiver" plants. However, all donors were ^{15}N-enriched, and we detected transfer in 81% of receivers. Nitrogen was preferentially transferred to annuals (0.26 ± 0.50 mg N per g leaf mass) compared to perennials (0.13 ± 0.30 mg N per g leaf mass). This corresponded with tissue stoichiometry differences. Our findings suggest that plants and fungi that are located closer together in space and with stronger demand for resources over time are more likely to receive larger amounts of those limiting resources.

Chapter 5 summarizes the results from Chapters 2-4 in the context of the literature introduced in Chapter 1.

CHAPTER 2:

LEAF AGE COHORT ALTERS PLANT FUNCTION: A CASE-STUDY OF TWO EVERGREEN DWARF SHRUBS

Contributions

Hilary Rose Dawson, Sonya Geange, and Vigdis Vandvik designed the research; Hilary Rose Dawson led the data collection with assistance from Akuonani Phiri, Julia Schlick-Steiner, Vigdis Vandvik, and Aud Halbritter; Hilary Rose Dawson analyzed the data and wrote the paper, Dagmar Egelkraut provided logistical support, Vigdis Vandvik, Sonya Geange, Aud Halbritter, and Dagmar Egelkraut funded the project, Lucas C. R. Silva funded Hilary Rose Dawson's participation in the project, Hilary Rose Dawson led revisions to the paper with assistance from Sonya Geange, Vigdis Vandvik, and Lucas C. R. Silva.

Introduction

Leaf traits vary significantly, even within species and individual plants. Given that leaves are responsible for primary biomass production in natural systems, it is important to consider the natural history of the plant studied when selecting which leaves to measure (Barrows et al. 2016). However, trait sampling protocols generally limit their guidance on which leaves should be sampled to fully grown, healthy, and sun-lit (Pérez-Harguindeguy et al. 2013) while some studies suggest that randomly selected leaves may be more representative of plant performance (Maitner et al. 2023). This one-size-fits-all advice raises important sampling concerns for plants with different life histories. For example, evergreen plants different leaf year cohorts experience a variety of conditions over their lifespan that may alter their physiology. Which leaves should be sampled in these plants with multiple leaf cohorts? This question of which leaves to sample is broadly applicable to studies around the world. Evergreen plants dominate biomes as diverse as the tropics, Mediterranean scrublands, northern and southern temperate forests, and boreal heathlands (Givnish 2002). We use two evergreen dwarf shrub species (Empetrum nigrum and Vaccinium vitis-idaea) with broad boreal/northern temperate distributions to demonstrate the meaningful difference in leaf traits between current and previous year leaves. We compare data on current and previous year leaves collected in the field to data sourced from the literature and trait databases. We also analyze standard practices in the literature and demonstrate that although not all studies need to incorporate both leaf year cohorts, it is important to consider the natural history of the plant studied when selecting which leaves to measure.

Leaf longevity is a key plant functional trait that represents the balance between the resources necessary to make a leaf and the carbon gained from that leaf over its lifespan (leaf optimum lifespan theory; Castorena et al. 2022). Evergreenness requires a greater carbon investment in leaf structure to withstand seasonal extremes and longer exposure to threats such as herbivory, but with the tradeoff of multiple years of carbon capture from that leaf (Smith et al. 2019). However, there is an ontogenetic effect between leaves on the same plant (Havrilla et al. 2021), and evergreen leaves can have altered physiology depending on whether they are expanding in their first year or already expanded in subsequent years. For example, when adjusted for mass, one-year-old Vaccinium vitis-idaea leaves had the highest photosynthetic rate compared to current and two-year-old leaves which had 80% of the photosynthetic rate of one-year-old leaves (Karlsson 1985). An additional consideration is that leaves of different ages occupy different positions in the canopy, with more recent leaves shading previously developed leaf cohorts. Trait relationships are based on the assumption of full-light conditions, reducing the accuracy of models of understory species that more commonly grow in the shade but potentially not representative of a plant's physiology (Keenan and Niinemets 2016, Petruzzellis et al. 2017). This is further exacerbated in models for evergreen species where leaves may experience higher light conditions in their first year than in subsequent years and adapt accordingly even in open habitats (Wright et al. 2006).

By incorporating different leaf qualities based on demography and ontogeny, Wu et al. (2016) demonstrated that a shift driven by producing new leaves drives an ecosystem-scale seasonal increase in photosynthesis of Amazonian evergreen broadleaf forests. This 'leaf demography-ontogeny model' (LDOM) emphasizes the importance of understanding leaf trait variation in different phenostages and could improve parameterization of tropical forests in dynamic global vegetation models, particularly if we incorporate continuous ontogenetic trait changes (Menezes et al. 2022). Although the LDOM has occasionally been applied to temperate systems (e.g., Luo et al. 2022), it is used less frequently than in the tropics. We investigated how leaf demography and ontogeny affect the physiology of E. nigrum and V. vitis-idaea, two evergreen dwarf shrubs that are dominant in Norwegian heathlands and we propose the differences found between current and previous year leaves in these species suggest that LDOMs could be useful in temperate and boreal systems in a way that is not currently utilized in studies based on leaf traits.

Materials and Methods

We developed a case-study in two parts: first, field measurements of leaf trait variation from the same branches between current and previous year leaf cohorts of two evergreen dwarf shrubs; and second, a systematic literature review documenting how the existing literature handles leaf sampling in these dwarf shrubs. We compare the variation in our dataset with that found in large databases (TRY, LEDA, Tundra Traits) and the literature with leaf trait ontogenetic variation that we measured in two Norwegian dwarf shrubs, *Vaccinium vitis-idaea* L. (Ericaceae) and *Empetrum nigrum* L. (Ericaceae). These two evergreen dwarf shrub species were chosen as they exhibit contrasting leaf forms, leaf lifespans, and cultural importance. Both species are true evergreens as defined by Chabot and Hicks (1982), maintaining multiple leaf cohorts in a given season. *Vaccinium vitis-idaea* (lingonberry) has broad leaves with a leaf longevity of 2-3 years. *E. nigrum* has narrow, needle-like leaves with a leaf longevity of 3-4 years. Both species are widespread in Norwegian heathlands and forest understories, habitats that cover >58% of the land area in Norway (Bryn et al. 2018). Both species have a wide distribution across the subarctic and subalpine to temperate regions of the Northern Hemisphere.

Field-collected data

Site description

We collected *E. nigrum* and *V. vitis-idaea* leaves from contrasting habitats at Kaupanger in Sogndal, Vestland, Norway (61.16° N, 7.15° E, 460 m.a.s.l) in June 2023 (Fig. S2.1a). We established 2 x 2m plots with continuous pine canopy (forested habitat, n = 10) and located away from trees (open environment, n = 10). Each plot had at least 25% coverage by the focal species (n = 5 plots per species per environment). The overstory of the forest was monodominant, *Pinus*. The understory was continuous coverage dominated by ericaceous dwarf shrubs (primarily *E. nigrum, V. vitis-idaea,* and *V. myrtillus*). The open plots were placed in clearings between scattered *Pinus* trees and had near continuous dwarf shrub coverage, predominantly of ericaceous dwarf shrubs (*E. nigrum, V. vitis-idaea*, *V. myrtillus,* and *Calluna vulgaris*).

Leaf trait measurements

We collected one shoot from three individual shrubs of the focal species in each plot between the 26th to 28th June 2023. To avoid sampling ramets of a single shrub, we sampled from different portions of the plot where possible. We selected shoots with at least three leaves without herbivory that represented the current size and characteristics for the plot. We selected shoots with at least three leaves grown this year ("current" year leaves) and at least three leaves grown last year ("previous" year leaves) and avoided shoots that only had a single leaf age cohort. Current year leaves were fully expanded at time of collection. Shoots were stored in a sealed plastic bag with a moist paper towel in the field and during transport back to the lab, where the bagged shoots were stored at 4º C until processing.

In the lab, we removed six leaves (three current, three previous) from each shoot. We differentiated the two leaf cohorts by locating the leaf bud scars on an individual shoot. This point was often marked by smaller leaves directly above and below it (Fig. S1b). Current year leaves were visibly lighter green than previous year leaves in both species. Each leaf was processed separately for all traits. The previous year leaves of three *E. nigrum* plants growing in the open habitat all had rust fungus infections. We measured the current year leaves of these plants, but resampled new plants for the previous year leaves. Because of the small leaf size of *E. nigrum*, we counted five individual leaves as a single bulk leaf for trait measurements. These were later averaged into individual leaf measurements during analyses. Leaves were stored in a sealed plastic bag with a moist paper towel until fresh matter traits were measured. Leaves were kept at 4º C when not actively being measured.

We measured six traits using a modified protocol from Pérez-Harguindeguy et al. (2013): field moisture mass, leaf area, leaf thickness, dry mass, specific leaf area, and leaf dry matter content. Field moisture mass was measured on a precision balance accurate to 0.0001 g. Leaf area was measured using a flatbed scanner and a semi-automated protocol based on an external Raspberry Pi computer (Raspberry Pi Foundation, Cambridge, England) to standardize measurements between different users. Leaf area was calculated using a modified version of the 'LeafArea' R package (Katabuchi 2015). Leaf thickness was measured at three points on each leaf (or on three different leaves per bulk leaf for *E. nigrum*) using micrometers accurate to 0.001 mm. Leaves were then dried for 72 hrs at 65º C before dry mass measurements (following the same protocol as field moisture mass on a balance accurate to 0.00001 g. Specific leaf area (SLA) was calculated as the area (cm^2) divided by the dry mass (g) of each leaf. Leaf dry matter content (LDMC) was calculated as the dry mass (mg) divided by the field moisture mass (g).

Literature survey

Systematic literature review

To determine the relevant methods used when measuring evergreen leaves, we downloaded bibliographies of the search term *ALL=((trait* OR "leaf" OR "leaves") AND (("Vaccinium vitis-idaea" OR "vitis-idaea" OR ("lingon*" OR puolukka OR "mountain cranberr*" OR "partridgeberr*" OR "cowberr*")) OR (("Empetrum" AND ("hermaphroditum" OR "nigrum")) OR "E hermaphroditum" OR "E nigrum" OR ("crowberr*" OR kragebær OR sortebær OR krekebær OR krøkebær OR kråkebær OR kråkbär OR kråkris OR Variksenmarja OR mustavariksenmarja)) OR "dwarf shrub*" OR ((ericoid* OR ericac*) AND shrub*)))* from Web of Science (WoS) on 10 April 2024 using a University of Oregon subscription. WoS searched all available metadata for these terms, including titles, abstracts, and keywords and returned 1,005 results. All languages were included as long as they were registered with WoS. The initial search included articles in Russian, German, Polish, Spanish, French, Portuguese, and Turkish. Often the abstracts were written in English and the subsequent articles in another language. In this case, they were translated by using an optical character reader (OCR) in Adobe Acrobat and then copied into Google Translate.

We determined the relevance of each article based on first, whether it addressed one of our focal species and second, whether the authors measured fresh leaves of any leaf age cohort. For *E. nigrum,* we included subspecies such as *E. nigrum nigrum* which is synonymous with *E. hermaphroditum* Hagerup. We excluded studies that only studied bulk biomass or phenology. The former is a whole-plant metric more than a leaf-metric and the latter is constrained to fresh leaves or old leaves only (depending on whether the authors study spring/emergent or fall/senescent phenology). We also excluded studies that only studied bulk shoot samples, unless the tip of the shoot was studied as a "bulk leaf" in the case of physiological measurements of small leaves. We discarded seven articles that we could not obtain the full text for. Of the resulting 148 articles relevant for our analysis, 19 had measured leaves of both species, 32 only measured *E. nigrum*, and 94 only measured *V. vitis-idaea*. Using locations and first and senior authors, we identified studies that measured the same samples and combined their metrics to avoid double-counting (n = 16 duplicate studies discarded). We coded the included articles for whether they specified leaf cohort collected; the justification they gave for the cohort collected; if they collected separate cohorts, whether they displayed their data in separate cohorts or lumped in a single cohort; the country they collected leaves from; the month they collected leaves; the category of trait they collected; and the biological season they collected leaves (see Table S2.1 for a complete description of all metadata collected).

Within the articles included above, we narrowed our focus to articles that measured morphological leaf traits because these traits are easily and commonly measured and have a wide variety of applications. We also expanded our search to the discarded articles to find those that pulled leaf morphology data from a database (n = 8). Of the resulting 40 articles relevant to our analysis, 11 had data for both species, 21 had data for only *E. nigrum*, and 19 had data for only

V. vitis-idaea. We coded these articles for the same metadata as above, as well as whether data were collected or pulled from a database; if downloaded, which database(s) were consulted; which traits were measured; whether leaves were collected from open or forested habitats; and if data could be extracted from the article to be used in our analyses and what form those data were in.

Traits extracted from the literature

We extracted morphological trait data from the papers we identified in our literature review as having measured one of four traits (SLA/LMA, LDMC, leaf area, dry leaf mass) from fresh leaves. Fifteen papers presented relevant data in usable form (means with errors or points on a correlation graph with a linear scale). Four papers provided their full dataset as a supplementary file or deposited in a data repository. Papers that reported their results in log scale ($n = 1$), without reporting units ($n = 1$), with units that could not be standardized ($n = 1$), or as a median instead of mean were excluded ($n = 1$), as was one paper with unreasonably high values (likely due to misreported units). One paper had some usable traits, but the LDMC were all outliers. In total, we extracted 2,454 usable trait values for *V. vitis-idaea* and 42 trait values for *E. nigrum* (Table S2.2). We standardized units across all datasets with R code. LMA was converted to SLA (1/LMA) to standardize it.

Traits extracted from multi-national databases

In our literature review, we identified TRY, LEDA, BIEN, and the Tundra Traits Team database (TTT) as widely used multi-national databases (Kleyer et al. 2008, Bjorkman et al. 2018, Maitner et al. 2018, Kattge et al. 2020). We downloaded TRY data on 16 October 2023 for both species and four traits (leaf dry mass, leaf dry matter content, leaf area, and specific leaf area), removed all values with an error risk >= 4, removed values that duplicate TTT or LEDA, and used rtry::rtry_remove_dup to remove known duplicate values (Lam et al. 2024). We downloaded TTT v.1 from the GitHub repository and removed values with an error risk >= 4. We downloaded LEDA on 30 Aug 2023 and retained only raw (actual measurement) values. From all datasets, we removed studies already extracted from our literature search (2 from TTT, 1 from LEDA, as well as TTT and LEDA values from TRY). We downloaded BIEN data for both species on 16 Oct 2023. All of the observations of relevant traits in BIEN were determined to be from another, already cited, dataset (either TRY or LEDA). In total, we extracted 484 trait values from TRY, 1,782 trait values from TTT, and 86 trait values from LEDA (Table S2.3).

Data Analysis

All analyses were performed in R code ver. 4.3.1 (R Core Team 2022) using a tidyverse framework (Wickham et al. 2019). Plots were constructed in ggplot2 using the viridis palette and patchwork framework (Garnier et al. 2023, Pedersen 2024).

Field study

Leaves of different cohorts were collected from the same shoot of each individual plant. To create current/previous leaf pairs out of the three replicates per shoot, we assigned randomized numbers to each leaf. We grouped the leaves by their plant and sorted by the randomized number before assigning a sequential number (1, 2, 3) to form the pairing. In the case of leaf thickness, which was measured three times per leaf (or bulk leaf), we assigned the pairings to each measurement within each leaf (1, 2,...6). The same method was used to randomly pair plants within plots.

Literature survey

For the systematic literature review, all metadata were coded into Zotero tags and extracted and processed in R code with tidyverse (Corporation for Digital Scholarship 2024).

We removed one *E. nigrum* LDMC outlier from the dataset collected in this paper. One *E. nigrum* leaf is missing dry mass measurements which also impacted SLA and LDMC.

For each species and trait combination, we tested the significance of leaf year cohort and habitat with a linear mixed model with the Satterthwaite method (afex::mixed; Singmann et al. 2024). Individual plants were treated as a grouping (random) factor with leaf age as a within factor.

Results

Paired leaf trait ontogenetic measurements

Across both species, current year leaves had a significantly higher SLA and lower dry mass, LDMC, and leaf thickness compared to previous year leaves (linear mixed model; Fig. 2.1, Table 1). There was an interaction between leaf age and habitat for SLA in E. nigrum. Greatest difference between leaf year cohort traits was in SLA, which was 74% (V. vitis-idaea) to 78% (E. nigrum) higher, on average, in current year leaves compared to previous year leaves. The trait with the least (but still significant) difference between years was E. nigrum leaf area, where current year leaves were, on average, 9% smaller. Leaf area did not differ between leaf year cohorts for V. vitis-idaea.

Traits in the two species were differently affected by tree cover. In V. vitis-idaea, only leaf area and dry mass were significantly different, where leaves were twice as large and heavy in the forest compared to the open. All five traits were significantly different for E. nigrum leaves growing in different habitats but had less change (by percent shift) than V. vitis-idaea leaves. Leaf thickness and SLA had the greatest difference at, on average, 27% thicker and 25% higher in the forest compared to the open for each species respectively. Leaf area had the least difference, where leaves from the forest were, on average, 10% larger than leaves from the open for both species.

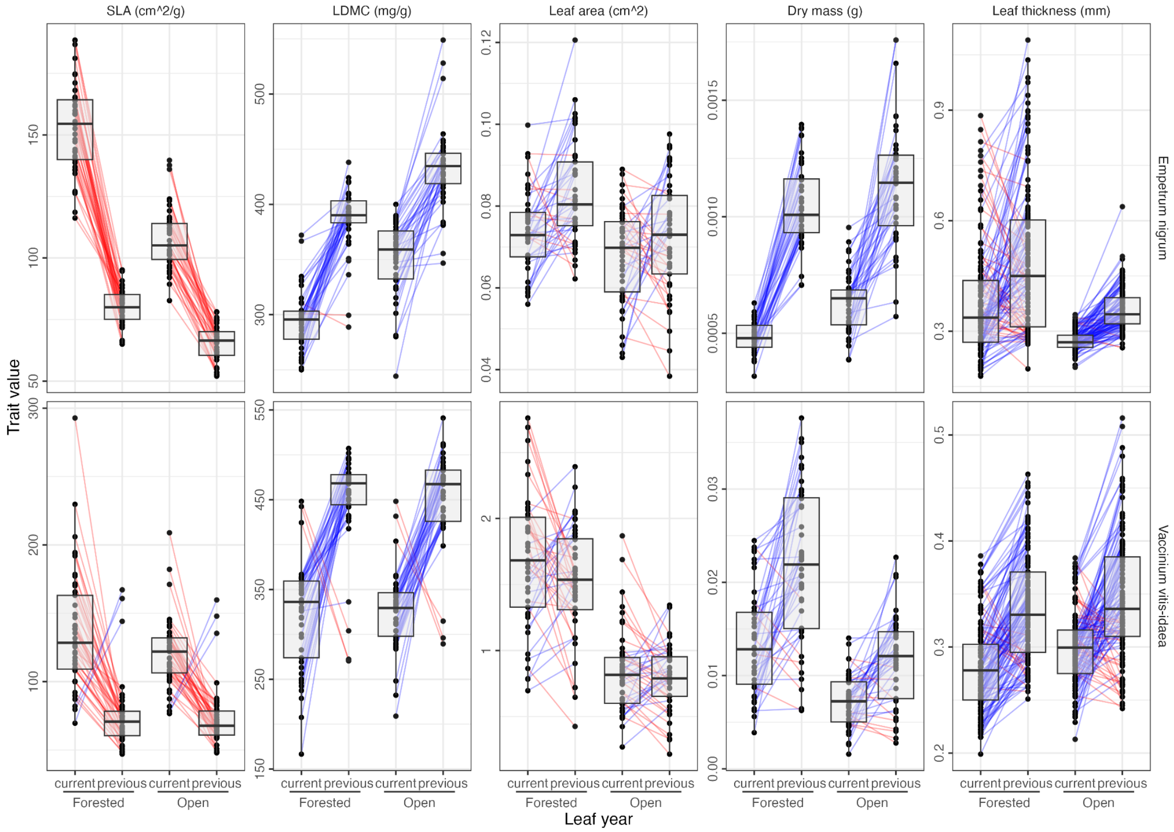


Figure 2.1: Trait variation between leaf cohorts within habitat for *Empetrum nigrum* and *Vaccinium vitis-idaea*. Lines connect leaves of different cohorts from a single shoot. Red indicates a negative relationship (decline in trait value), and blue indicates a positive relationship (increase in trait value) from current to previous year leaves. Boxplots show medians and interquartile ranges, lines show the largest (or smallest) value no further than 1.5x IQR from the hinge.

Table 2.1: Linear mixed model results for leaf trait differences based on leaf age and habitat in *Empetrum nigrum* and *Vaccinium vitis-idaea*. Each species x trait combination was tested with a separate linear mixed model. Plants were treated as a grouping factor with leaf age as a within factor.

Empetrum nigrum															
	SLA			**Leaf area**			**Dry mass**			**LDMC**			**Thickness**		
	df	**t**	**P**	**df**	**t**	**P**	**df**	**t**	**P**	**df**	**t**	**P**	**df**	**t**	**P**
Habitat	29.379	10.385	**<0.001**	29.721	2.992	**0.006**	30.751	-2.962	**0.006**	47.715	-8.171	**<0.001**	31.895	3.762	**0.001**
Leaf age	29.057	-23.106	**<0.001**	29.742	2.827	**0.008**	31.250	14.801	**<0.001**	32.442	10.363	**<0.001**	31.939	4.133	**<0.001**
Habitat: Leaf age	29.057	-5.771	**<0.001**	29.742	0.918	0.366	31.250	0.856	0.398	32.442	0.910	0.370	31.939	0.295	0.770

Vaccinium vitis-idaea															
	SLA			**Leaf area**			**Dry mass**			**LDMC**			**Thickness**		
	df	**t**	**P**	**df**	**t**	**P**	**df**	**t**	**P**	**df**	**t**	**P**	**df**	**t**	**P**
Habitat	28.013	1.311	0.200	28.045	7.623	**<0.001**	28.018	5.431	**<0.001**	28.028	-0.253	0.802	27.997	-0.975	0.338
Leaf age	28.015	-6.516	**<0.001**	28.047	-1.595	0.122	28.042	5.406	**<0.001**	28.010	7.849	**<0.001**	28.004	5.690	**<0.001**
Habitat: Leaf age	28.015	-0.922	0.364	28.047	-0.858	0.398	28.042	1.745	*0.092*	28.010	0.120	0.905	28.004	0.612	0.545

Leaf age cohort and habitat affect the trait distribution, particularly for common allometric traits such as SLA and LDMC (Fig. 2.2). For example, current year leaves have nearly double the mean SLA as previous year leaves in the forested habitat. For traits scaled by mass (SLA, LDMC), there was less interspecific variation than intraspecific variation between cohorts. For example, *E. nigrum* current year leaves had a mean SLA of 129.7 cm^2/g ± 26.9 SD and *V. vitis-idaea* current year leaves mean SLA was 129.4 cm^2/g ± 36.2 SD, while the previous year cohort mean SLA measured 72.8 cm^2/g ± 10.3 SD and 74.0 cm^2/g ± 24.8 SD respectively.

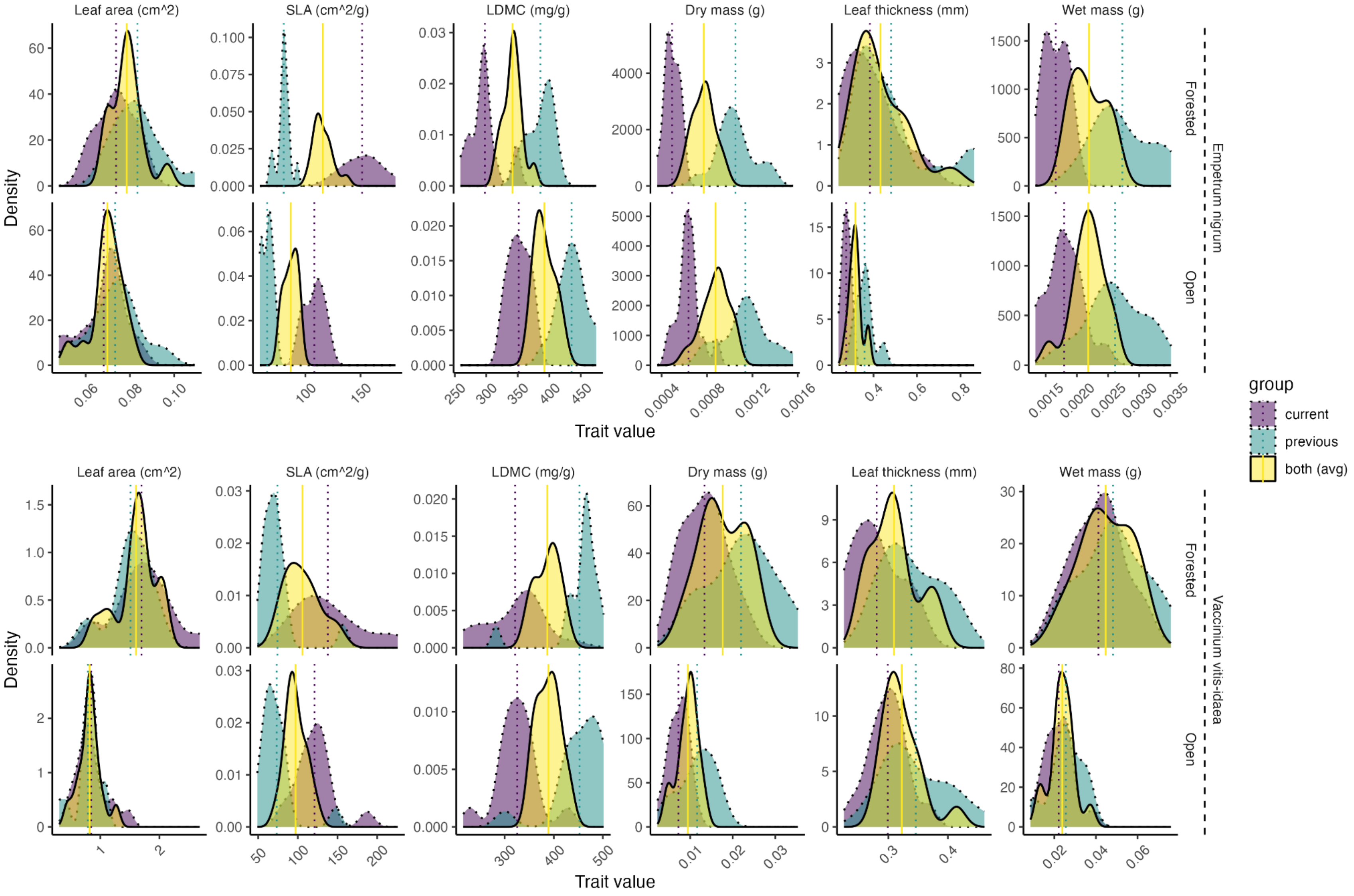


Fig 2.2: Density plot of per plant trait means comparing each cohort compared to both cohorts together weighted equally. Vertical lines indicate the mean of each group.

Leaf traits in the literature

All studies were conducted in the Northern Hemisphere, and all the *E. nigrum* studies were conducted in Eurasia or Greenland (Fig. S2.3). Sweden was the country where most studies were located, with half of *E. nigrum* studies and nearly 20% of *V. vitis-idaea* studies taking place there. Most of these were conducted at or near Abisko Scientific Research Station.

Approximately 20% of studies did not mention the month that they sampled leaves in (Fig. S2.2). July and August were the most common months to sample both *E. nigrum* and *V. vitis-idaea.* The former was also commonly sampled in September. *V. vitis-idaea* was sampled in all twelve months. *E. nigrum* was sampled in all months except November through January. At least half of the studies did not mention the season of when they sampled leaves, a time which gives context to the leaf developmental stage. For those that did specify a season, growing season or parts of the growing season were common sampling times; however, some studies sampled during winter, before new leaves were produced, or at maximum snow depth (Fig. S2.2).

Of the 52 studies that measured *E. nigrum* leaves, 48% did not specify which leaf cohort they measured; this was 58% for the 113 *V. vitis-idaea* leaf studies (Fig. 2.3). For the studies that specified which leaf cohort they measured, current year leaves were the most common to measure for both species. Out of all studies included in this analysis, only 15% of all *E. nigrum* leaf studies and only 13% of the *V. vitis-idaea* studies measured both leaf cohorts separately; about half of these displayed data for the cohorts separately (Fig. S2.2). The most common traits to measure were leaf morphology, leaf stoichiometry, and chemical compounds within the leaf (Fig. S2.2).

Of all the studies included, approximately three-quarters for each species did not provide a reason why they sampled the leaf age cohort they did or why they did not specify which cohort they sampled (Fig. 2.3). Approximately 10% of studies were specifically testing leaf physiology by cohort or leaf age and one *E. nigrum* study sampled both cohorts because it was representative of the leaves the plants had (Ogren 2001). A related reason was given in 14% of *E. nigrum* studies and 21% of *V. vitis-idaea* studies: both cohorts were sampled because they both happened to be present; however, this was often implied in the methods rather than stated intentionally (e.g., Monni et al. 2001, Gerdol et al. 2004, Walker et al. 2010 sampled all leaves on the plant and separated the leaf cohorts).

Comparing measured leaf traits and data extracted from the literature

The leaf trait data we collected was generally representative of the data available from published sources (Fig. 2.4). However, the extracted data (most of which did not include metadata regarding which leaf cohort was collected; Table S2.2) and database data (none of which included leaf cohort metadata) spanned both cohorts for most traits. The database medians did not consistently represent either leaf age cohort.

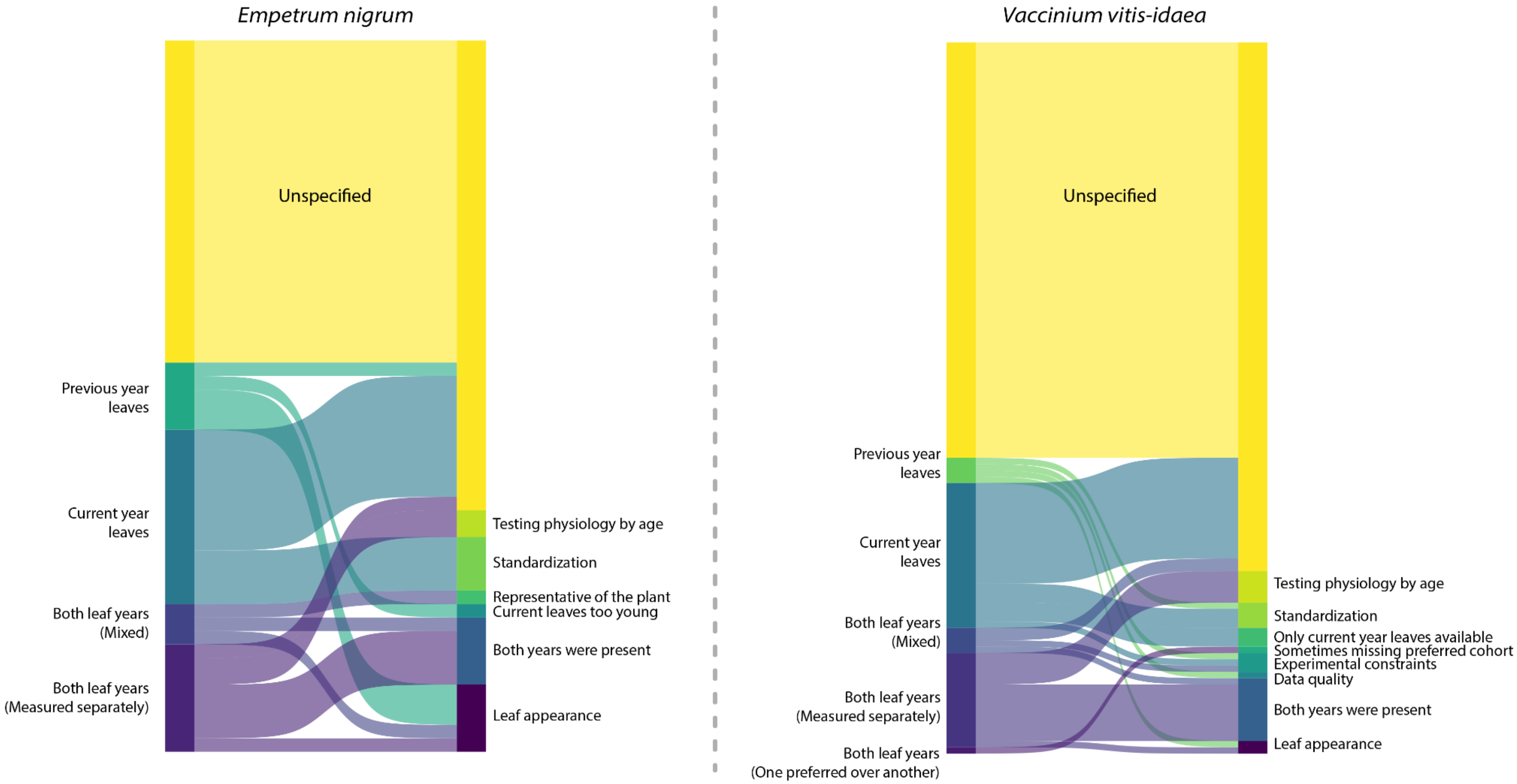


Figure 2.3: Alluvial Sankey plot of how leaves were measured in the literature of *Empetrum nigrum* and *Vaccinium vitis-idaea*. Explanations of each variable are in Table S2.1. Additional variables extracted from the literature are in Fig. S2 and S3.

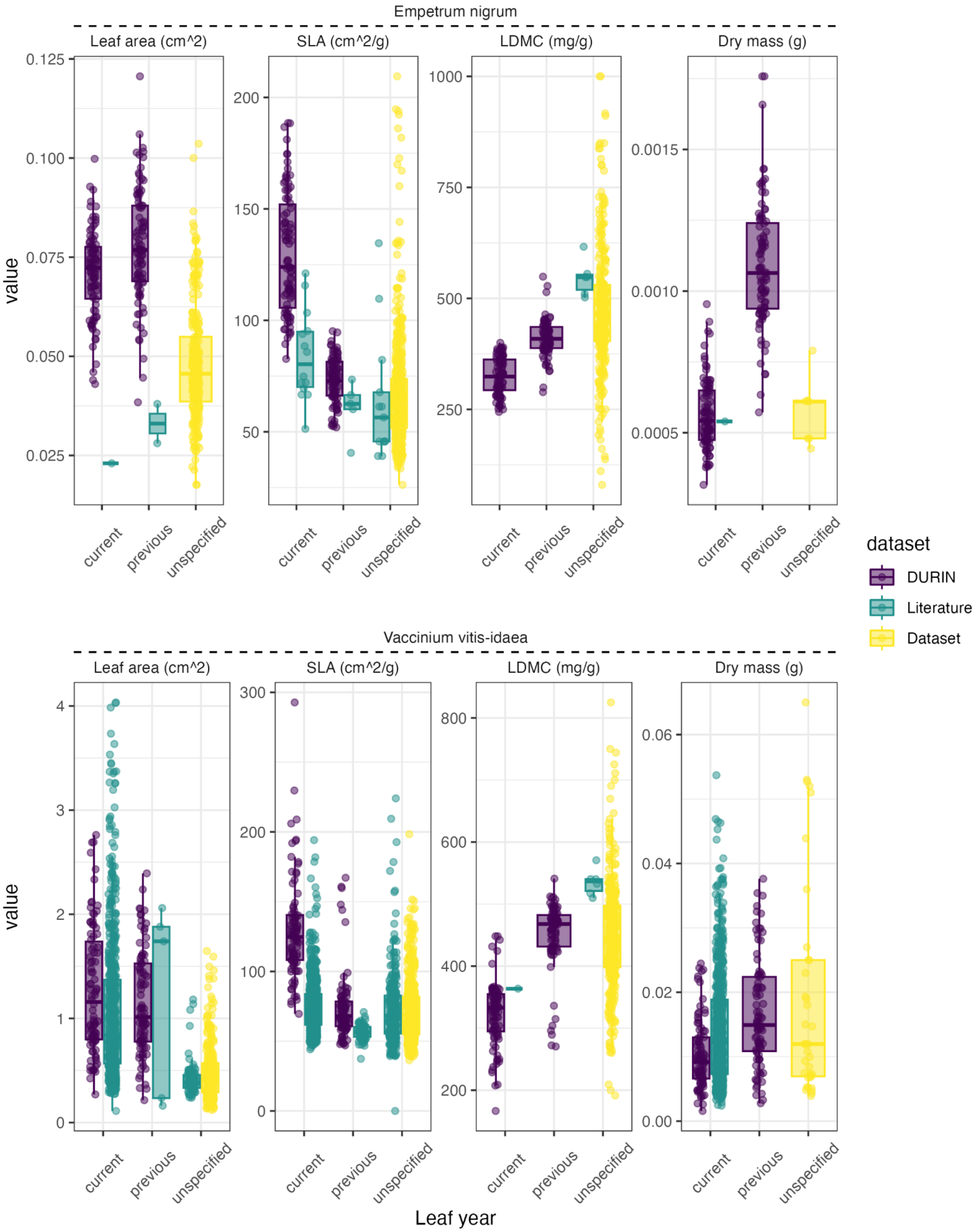


Figure 2.4: Trait variation between leaf cohorts and species as compared between different sources. Data point counts are available in Table S2.3.

Discussion

In comparing current and previous leaf cohorts of the evergreen shrubs *E. nigrum* and *V. vitis-idaea* in the middle of their growing season at a single site in Norway, we found significant differences between commonly measured leaf traits. This difference was found in both open and forested habitats. However, the literature suggests that only half of study authors considered the importance of leaf year cohort when measuring these species for a wide range of leaf traits, from morphology to chemical composition to functions such as photosynthesis.

Variation in field-collected data

In the dataset that we collected in western Norway, leaf year cohort altered five commonly measured morphological leaf traits (SLA, LDMC, dry mass, leaf area, and leaf thickness) in both evergreen dwarf shrub species. Current year leaves had a more resource-acquisitive strategy compared to previous year leaves on the same shoot, with higher SLA and lower leaf thickness. Although the needle-like leaves of *E. nigrum* and broad leaves of *V. vitis-idaea* are visually quite different, the mean scaled traits of SLA and LDMC by leaf year cohorts were more similar to each other than between cohorts within a species (i.e., within cohort interspecific variation was less than between cohort intraspecific variation). This brings into question studies such as Iturrate-Garcia et al. (2020) where leaf traits were compared across species without consideration of which leaf year cohort was measured for the evergreen species, as well as studies such as Tonin et al (2020) where intraspecific variation was analyzed without controlling for which leaf year cohort was measured.

Comparison to the literature

Studies from our systematic literature review that focused on comparing cohorts examined functional or chemical compound traits, rather than the more commonly measured morphological traits. However, these studies consistently showed differences between current and previous year leaves, summarized here. Previous year leaves had a higher intrinsic water-use efficiency compared to current year leaves, and this difference was greater between cohorts than when *V. vitis-idaea* was compared to the deciduous shrub *V. myrtillus* (Gerdol et al. 2000). Previous year cohorts of *V. vitis-idaea* absorbed more foliar N, although there was no difference in relative N enrichment between cohorts (Chester and Oechel 1986). This could be because previous year leaves serve as a N reserve and their N can be remobilized to new growth (Gerdol et al. 2000, Grelet et al. 2001). Gerdol et al. (2000) found consistently higher N and P in current year leaves compared to previous year leaves. *V. vitis-idaea* leaves reached full photosynthetic capacity either two weeks after snow melt (previous year cohort) or two weeks after emergence (current year cohort), a temporal difference of more than a month. One-year-old leaves had the highest photosynthetic rate, while current and two-year-old leaves had a rate roughly 80% that of the one-year-old leaves at mid-growing season (Karlsson 1985). Over time, previous year leaves had a constant dry-weight scaled CO2 exchange rate (A_{weight}) while current year leaves decreased during the growing season to match previous year leaves (Gerdol et al. 2000). Phenolic compounds varied between cohorts in *E. nigrum* (Nilsson 1994, Wallstedt et al. 1997) and anthocyanin concentrations were higher in *V. vitis-idaea* current year leaves (Solanki et al. 2019).

Scale and context

Given that leaf structure and seasonality are two of the most important characteristics to incorporate into dynamic vegetation models (Wullschleger et al. 2014), parameterizing evergreen shrubs with measurements of both leaf year cohorts and their representation within each species could improve model outputs. This may be particularly important in the tropics, where the development of new leaves in evergreen species produces a noticeable effect in ecosystem-scale photosynthesis (Wu et al. 2016). Our analyses found variation between open and forested habitats but were limited to leaves we measured at a single site. Although here we find leaf year cohort to have significant variation–indeed, leaves of the same cohort were more similar than leaves of different cohorts and the same species–we did not test the importance of this variation when comparing leaves across sites and across environmental gradients. Habitat affected the two species included in our case study to different degrees. *V. vitis-idaea* leaves were substantially larger and heavier under the forest canopy, while *E. nigrum* leaves were heavier without as great a leaf area expansion. In the literature review, 82% of studies reported the habitat of their sample plants, while only half reported which leaf year cohort they measured. This may reflect the perceived greater influence of habitat on leaf growth compared to intraspecific variation. However, our results found otherwise, and we suggest that further analysis is needed to confirm this holds true across sites.

Implications

Although it is standard practice to measure leaves when they are at peak maturity (Pérez-Harguindeguy et al. 2013), this is not always feasible given the constraints of fieldwork and may not reflect the plant's biological reality. For instance, we found a significant difference in leaf area between current and previous year leaf cohorts, implying that the current year leaves may not have been fully expanded. However, we measured the leaves in the middle of the growing season, at a point most representative of the plants' physiology as they photosynthesized throughout the snow-free season. To understand how these plants photosynthesize and optimize carbon and water uptake, it is important that we measure the traits that these plants express in their leaves. Many studies that we reviewed understood this reasoning and measured leaf trait during the growing season. However, some emphasized that they measured leaves later in the growing season at an estimated maximum current year leaf expansion or in the autumn after current year leaves bulked up and were more similar to previous year leaves (e.g., Shevtsova et al. 2005, Lagerström et al. 2013). Although this can be important for standardizing data collection, it is worth considering whether the traits measured at these peaks of maturity reflect the physiology experienced by plants throughout the growing season.

BRIDGE

Recent hypotheses for unexplained variation in traits include poorly selected traits, faulty analyses, lack of mechanistic understanding, and community complexity (Chacón-Labella et al., 2023; Hagan et al., 2023). Another source of unexplained variance could be driven by developmental change, such as the differences in evergreen leaf cohorts that is explored in Chapter 2. In Chapter 3, I explore the effects of the environment on leaf trait variability by applying experimental drought to herbaceous plants in three Pacific Northwest grasslands. Although leaf traits are not easily predicted by the environment (Anderegg, 2023) nor do they predict ecosystem properties (van der Plas et al. 2020, but see Hagan et al. 2023), it is expected that they change in response to environmental stressors. In particular, Chapter 2 tests whether the expected relationship between morphology and function (Wright et al. 2004 Science) holds under experimental drought stress.

CHAPTER 3:

LEAF TRAITS PREDICT WATER-USE EFFICIENCY IN U.S. PACIFIC NORTHWEST GRASSLANDS UNDER RAIN EXCLUSION TREATMENT

From Dawson, H. R., Maxwell, T. M., Reed, P. B., Bridgham, S. D., & L. C. R. Silva. (2022). Leaf traits predict water-use efficiency in U.S. Pacific Northwest grasslands under rain exclusion treatment. *Journal of Geophysical Research: Biogeosciences*. 127(10), e2022JG007060.

Contributions

Hilary Rose Dawson, Toby M. Maxwell, Paul B. Reed, Scott D. Bridgham, and Lucas C. R. Silva designed the research; Hilary Rose Dawson collected the data; Hilary Rose Dawson analyzed the data and wrote the paper; Lucas C. R. Silva and Scott D. Bridgham funded the project, all authors edited the paper and helped interpret the data.

Introduction

Grasslands in the western Pacific Northwest face an increased risk of drought stress due to rising temperatures, decreasing summer precipitation, and increasing evaporative demand (Dalton & Fleishman, 2021; Jung & Chang, 2012). Recent research suggests that drought stress has the potential to change grasslands, their species composition, and their forage production function (Mackie et al., 2019). Biodiverse communities may mitigate these negative changes, as functional differences between species, and related resource-use complementarity, provide a significant degree of resistance and resilience to drought stress (Hallett et al., 2014; Li et al., 2019). To better understand the mechanisms behind the stability documented in biodiverse grassland communities, and perhaps harness its benefits for conservation and management, we must consider the degree to which plants can morphologically and functionally adjust to persistent drought.

The relationship between plant functional traits and associated strategies for water and nutrient use provides a basis for interpreting the limits of stress response from individuals to communities and ecosystems (Enquist et al., 2015, 2017). For example, foliar morphology is related to primary productivity and nutrient cycling through a trait-dependent tradeoff termed the leaf economic spectrum (Wright et al., 2004). Notably, the same relationship that delineates the leaf economic spectrum, ranging from short-lived to long-lived leaves, with intrinsically high and low photosynthetic capacity respectively, makes morphological traits a proxy for potential foliar function such as the amount of carbon gained per unit of nutrient or water used for plant growth (Kröber et al., 2015). Indeed, the intrinsic water-use efficiency (iWUE; the ratio of net assimilation, A, to stomatal conductance, g_s) and nutrient-use efficiency (e.g., reflected in leaf carbon to nitrogen ratios) of key functional groups tend to be correlated with plant growth form, life span, and leaf area per mass (i.e., specific leaf area, SLA). Although iWUE is not typically included in the leaf economic spectrum, it has been linked multiple times to traits that comprise the spectrum (Gouveia & Freitas, 2009; Soh et al., 2019; Temme et al., 2017). We can use these form-function relationships, measured at the level of individuals or species, to infer how stress responses might scale up to the level of communities and ecosystems, as in landscapes where variation in the relative cover of dominant species allows for leaf-to-canopy upscaling of water and nutrient costs of photosynthesis (Silva & Lambers, 2021). Click or tap here to enter text.

Here, we examined whether rain exclusion affects the foliar morphology, nitrogen and carbon to nitrogen ratios, and iWUE of 12 common grassland species at three sites spanning a 520 km latitudinal gradient in the Pacific Northwest. We examined how different functional groups responded to rain exclusion based on their growth strategies and trait plasticity. We focused of relationships that reflect differences in species life history strategy (annual or perennial) and functional group (grass or forb), both of which are expected to affect species reproduction and survival during drought stress (Tilman & El Haddi, 1992). Closely related species tend to resemble each other in form and function, a phenomenon known as "phylogenetic signal" (Blomberg & Garland, 2002). Because phylogenetic signal is not random across a subset of species, it is important to include phylogeny as a variable when testing patterns of drought stress acclimation to control for the "relatedness" of species of interest (Avise & Wollenberg, 2010). To study form-function relationships we also focused on phylogenetic differences between species influenced how traits varied in response to drought. Specifically, we used a climate gradient, from cool and wet to warm and dry, to test the hypothesis that differences in species' sensitivity to drought would be explained by their physiology (leaf traits, life history strategies, and functional group) and the phylogenetic distance between them. Our experiments provide insight to the physiological and environmental mechanisms that link species form and function, which could help improve temperate grassland management and conservation.

Materials and Methods:

Site descriptions

We conducted replicated experiments at three different sites along a 520 km latitudinal gradient in the Pacific Northwest (Fig. 3.1, Table 3.1). The study areas share a Mediterranean climate with increasingly hotter and drier summers from north to south, where we see an earlier onset of summer senescence, despite the southern site having the highest mean annual precipitation (Table 3.1). Each experimental site contained 20 plots: 10 had their species composition manipulated as part of separate phenology and demography experiments (Reed, Bridgham, et al., 2021; Reed et al., 2019), and 10 had their species composition unmanipulated and consisted primarily of the already established pasture grasses that dominated at each site prior to the experiment. The manipulated plots were mowed, raked, received herbicide, and seeded with a mix of 29 native prairie grass and forb species between 2014-2015, followed by repeated seeding with 14 native grasses and forbs in fall 2015, 2016, and 2017 (Reed et al., 2019), a process that is analogous to typical restoration efforts in the region.

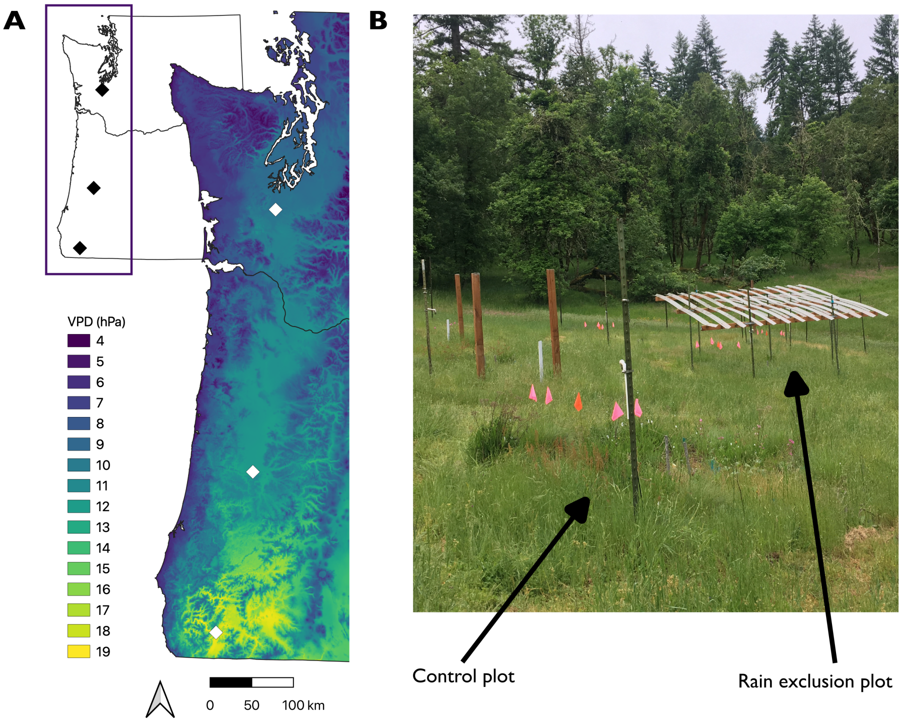


Figure 3.1 a. Site locations with maximum vapor-pressure deficit (VPD) expressed as hectopascal (hPa), or 100 x 1 pascal, pressure units which are equal to millibar pressure unit, and b. example of experimental set up depicting a rain exclusion shelter. VPD data is from PRISM for 1991 to 2020 (http://www.prism.oregonstate.edu/normals/). Points show site locations in Washington and Oregon.

Table 3.1 Characteristics of the three experimental site locations.

	Experimental Site		
	Southern	**Central**	**Northern**
	Selma, Oregon	**Eugene, Oregon**	**Tenino, Washington**
Latitude; longitude	42.27811; -123.642278	44.02615; -123.182171	46.86415; -122.958918
Elevation (m)	394	165	79
Mean air T (ºC)	10.26 ± 6.80	10.70 ± 6.49	8.68 ± 5.52
Min air T (ºC)	2.11 ± 4.56	5.49 ± 4.89	2.43 ± 4.69
Max air T (ºC)	20.10 ± 9.76	17.63 ± 8.88	15.25 ± 7.61
Mean annual precipitation (mm)	1325 ± 327	894 ± 207	1300 ± 247
Soil taxonomy	Loamy-skeletal, mixed, superactive, mesic Entic Ultic Haploxerolls	Very-fine, smectitic, mesic Vertic Haploxerolls	Medial, mixed, mesic Typic Haploxerands

Notes: Climate data calculated from site specific dataloggers in 2017 and 2018. Minimum and maximum calculated as annual means of daily averages. Errors represent standard deviation. Elevation and soil taxonomy from Reed et al. (2019).

For both the manipulated and unmanipulated plots, five were randomly assigned to a rain exclusion treatment while the remaining five were assigned as controls. We estimate to have experimentally imposed reduced annual rainfall by ~40% using a fixed rainout shelter design blocking no more than ~8% light transmittance (Yahdjian & Sala, 2002). Rainout shelters were erected in February 2016 for the manipulated plots and February-March 2017 for the unmanipulated plots and maintained until after our sampling in summer 2019 (see Appendix B: Fig. S3.1 and S3.2 for the effects of the rainout shelters). The vegetation plots under the shelters were circular with 1 m diameter. Each plot was nested within a larger 3 m diameter plot. Rainout shelters were 3.7 m x 3.7 m squares and stood 1.5 m above the vegetation plots, sloped to 1 m above the plot on the far side to promote drainage, providing ~30 cm buffer around the vegetation plots. Phenology and demography information from these experimental plots have been previously published in (Peterson et al., 2021; Reed, Bridgham, et al., 2021; Reed et al., 2019; Reed, Pfeifer-Meister, et al., 2021).

Species and phylogenetic data

For this study, we selected 12 species that were abundant in at least one site (Fig. 3.2, Appendix B: Table S3.1). The species studied represented a total of 139 individuals spanning 11 genera and four families. Two species (*Bromus hordeaceus* and *Sidalcea malviflora* ssp. *virgata*) were present across all three sites; the former was present in both manipulated and unmanipulated plots across all sites. We collected up to three leaves for each species in each plot. Because not all species were present at all sites or all plots, our sampling was uneven; however, this unevenness was distributed throughout the relevant groups and should have biased our analyses (Appendix B: Table S2.2). Leaves were sampled on 8 May 2019 (southern site), 29 May 2019 (central site), and 6 June 2019 (northern site) at approximately peak growing season in each site. For each species at each plot, leaves were taken from multiple mature individuals where available and were stored in envelopes in paper bags which were dried for at least 48 hours at 50ºC before being stored at ambient conditions. All species (including all grasses) included in this study are described as possessing cool-season (C_3) metabolism (Jackson et al., 2010; Osborne et al., 2014).

We determined plant functional group based on family where members of Poaceae were designated ‘grasses’ and all other plants (all non-graminoid) were designated ‘forbs’. We used the USDA PLANTS database to establish life history strategy (USDA NRCS 2019). This database designated three species (*Bromus diandrus, Daucus carota,* and *Geranium dissectum*) as either annual/biennial or biennial. We treated these species as annuals, reflecting their shorter lifespan compared to ‘true perennials’.

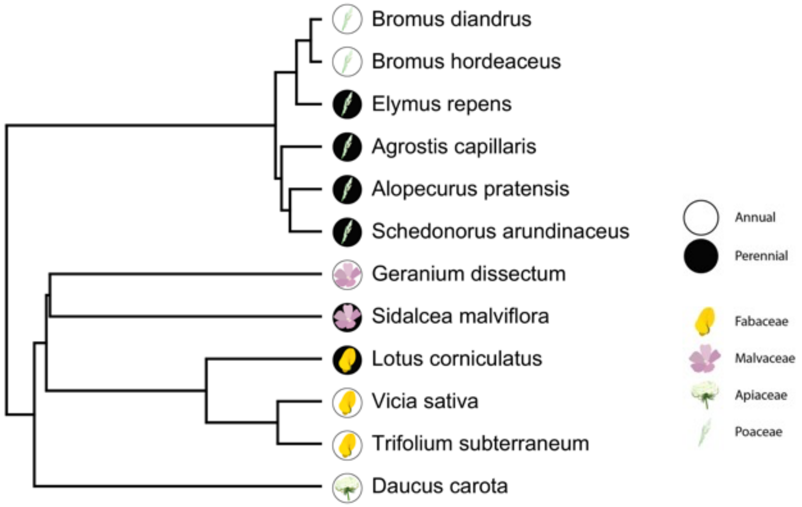


Figure 3.2: Phylogeny of the twelve species from four families that were examined in our data. Phylogenetic data and tree structure is pruned from the mega-tree in Jin and Qian (2019). Symbols represent family membership with backgrounds that show life history.

All analyses were conducted in R v.3.6.1 (R Development Core Team, 2017). To make it easy to reproduce our analyses, we use the format of R package::function, followed by the appropriate citation for each package mentioned. Phylogenetic data, including tree structure and branch lengths, were derived from a mega-tree compiled by Jin and Qian (2019) using two well-established, recent mega-trees based on molecular data and the Angiosperm Phylogeny Group 2016 (Chase et al., 2016). The tree was pruned with V.PhyloMaker::phylo.maker and the resulting tree was both ultrametric and binary. To integrate phylogenetic distance into our traits dataset, we calculated mean phylogenetic distance (also known as mean pairwise distance) using a method modified from Ness et al. (2011). Using this method, we created a cophenetic table using stats::cophenetic. From this table we calculated mean phylogenetic distance per species, a metric which we defined as the mean of the phylogenetic distance between a set species and every other species in a cophenetic table.

Leaf trait data

To measure leaf area, we scanned one leaf sample per individual at a standardized cropping area at 600 dpi (see Appendix B: Table S3.1 for leaves per species). We prepared images with the magick package (Ooms, 2019) and analyzed leaf area with LeafArea::run.ij (Katabuchi & Masatoshi, 2015; Rueden et al., 2017). We weighed each sample on a Sartorius CP2P-F microbalance (Göttingen, Germany) and calculated specific leaf area (SLA) as area divided by mass. We used SLA from dried material to test relationships with nutrient content and iWUE, inferred from stable carbon isotope ratios, assuming that SLA from dried material scales to SLA measured with fresh material (Perez & Heberling, 2020).

Stable isotope analysis was conducted by the Stable Isotope Facility at the University of California, Davis, on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). We calculated iWUE according to Werner et al. (2012). The isotopic discrimination compared to the atmosphere ($\Delta^{13}C$) was calculated in Eq. 1 using the $\delta^{13}C$ values of the air ($\delta^{13}C_a$) derived from Keeling et al. (2010) and the measured plant ($\delta^{13}C_p$):

Equation 1

$$\Delta^{13}C = {(\delta^{13}C_a - \delta^{13}C_p)} \Big/ \left(1 + \frac{\delta^{13}C_p}{1{,}000}\right)$$

We used the isotopic discrimination in Eq. 1 and the diffusive and photosynthetic fractionation constants (a: 4.4‰ and b: 28‰) to calculate the ratio of CO_2 partial pressures inside the leaf (C_i) and in the atmosphere (C_a).

Equation 2

$$\Delta^{13}C = a + (b - a)\frac{C_i}{C_a}$$

From this, we solved for iWUE, scaling to 1.53 (the ratio of water vapor to CO_2 diffusivity).

Equation 3

$$iWUE = \frac{A}{gs} = \frac{C_a\left(1 - \frac{C_i}{C_a}\right)}{1.53}$$

Leaf nitrogen (N) was collected as part of the stable isotope analysis. Leaf carbon to nitrogen ratio (C:N) was calculated as

Equation 4

$$\frac{C_{leaf}}{N_{leaf}}$$

Site data

Since the three experimental sites have unique soil characteristics, we calculated soil matric potentials as a comparable metric of plant moisture availability. Daily matric potentials were calculated from daily volumetric water contents and site-level measurements of percent sand, clay, and soil carbon using methods described in Saxton & Rawls (2006). We aggregated data to daily values before calculating growing season values, and we defined a growing season as October 1 to June 30, beginning with the return of fall rains and ending with the onset of summer drought (Fig 3.3; Reed et al., 2019). We collected six soil cores from each site, dried the soil for 48 hours at 60°C and sieved it to 2 mm. We then determined percent sand from the weight after sieving to 53 µm, percent clay using the hydrometer method (Gee & Bauder, 1986), and total soil carbon using a Costech Analytical Technologies 4010 elemental combustion analyzer (Valencia, CA, USA). To correct the left-skewed data, we log-transformed for a more normal distribution. We used -log(1-SMP) units to be consistent with raw values where negative values indicate drier conditions. Soil temperatures were measured in situ and continuously logged at all three sites along with daily volumetric water content.

Statistical analyses

We tested the relationships between functional and structural traits using linear correlations and mixed-effects models. The measured traits (SLA, iWUE, C:N, and N) were not normally distributed, so we log-transformed the data prior to performing statistical analysis. We determined plant growth strategy, site, and treatment effects on each trait using Type II analysis of variance (ANOVA), including interaction terms. We calculated post-hoc significance using Tukey-adjusted P-values. Response ratios were calculated as

Equation 4

$$\%\, Change\ of\ trait = \frac{mean\ experimental - mean\ control}{mean\ control} * 100$$

by species in each manipulation treatment at each site (Myers et al., 2014).

We ran ANOVAs for each growing year to determine if the rain exclusion treatment had a significant effect on soil matric potential. The interaction between site and drought treatment (term site x treatment) was significant, so we ran t-tests within each site to see where rain exclusion was significant.

At each site, we calculated means and standard errors of each trait for each species and used these data to determine phylogenetic signal (i.e. the strength of phylogeny on species' traits) using phytools::phylosig, using Pagel's λ. We used mean pairwise distance to test the phylogenetic signal across our entire dataset, and we used that signal as one of the predictive variables in a mixed effect model, effectively including it as a constraint on species responses to drought (De Vienne et al., 2011).

We then constructed a principal component analysis (PCA; Appendix B: Fig. S3.3) of the four continuous trait datasets (iWUE, SLA, C:N, and N) using stats::prcomp. We hypothesized seven biologically sound mixed effect models and determined the strongest with Akaike's information criteria with small sample bias adjustment (AICc) using AICcmodavg::aictab (Table S3.3; Mazerolle, 2006, 2020). Some degree of multicollinearity was expected in the mixed-effects models due to associations between structural and functional leaf traits, which are inherently correlated. However, the PCA analysis shows a near orthogonal (i.e. perpendicular) trait continuums: iWUE-SLA and C:N-N which suggests independent contribution of those variables to mixed-effects models. All models excluded the functional group effects shown in the response ratio analysis because functional groups represent categorical variables that summarizes phylogenetic differences included in the mixed models as a continuous variable (i.e., phylogenetic distances). Errors are shown as standard deviation unless specified otherwise.

Results

Overall effects of rain exclusion

Rainout shelters reduced soil matric potential in the central site in all years but had a limited effect in the other two sites (Appendix B: Table S3.4, Fig. S3.1). Rainout shelters also increased soil temperatures in the northern site in all three years and in the central site in 2018 (Appendix B: Table S3.5, Fig. S3.2).

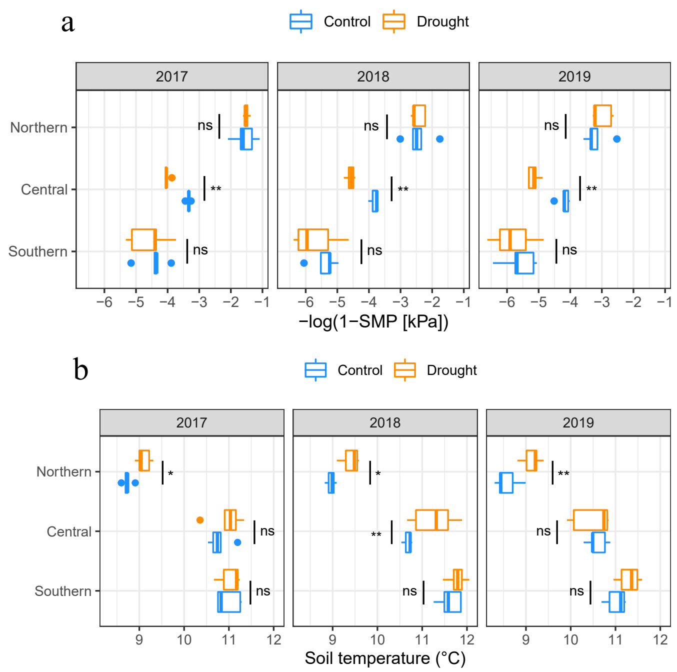


Figure 3.3. Effects of rain exclusion treatment on a) soil matric potential and b) soil temperature at each site for each growing season. Growing season is defined as 1 Oct to 30 Jun and each season is labeled by the spring months of the growing year (e.g. 2018 is 1 Oct 2017 to 30 Jun 2018). Asterisks indicate significant differences tested by ANOVA (ns = not significant, ** = 0.001, *** < 0.001, see Table S3.5 for results and Table S3.7 for summary statistics and Figs. S3.1 and S3.2 for daily variation). Median values are indicated by the central vertical line, with vertical lines to the left and right indicating the interquartile range. Horizontal lines indicate the interquartile minimum and maximum. Dots indicate outliers.

Response ratios of rain exclusion compared to control plots showed that rain exclusion had a significant effect on only annual and perennial grass iWUE at 95% confidence interval at all sites (Table 3.2). Specifically, the rain exclusion treatment significantly reduced grass iWUE compared to the control but had no significant effects on the other measured functional forbs or group or any other grass traits (Table 3.2, Fig. 3.4). Site and the interaction term between site and rain exclusion treatment were not significant for all variables except SLA (Table 3.2), which was highest in the north (0.154 ±0 .05 cm^2/mg) and lowest in the south (0.130 ± 0.04 cm^2/mg).

Table 3.2: Statistical summary of four full factorial mixed effect Type II ANOVAs testing the effects of rain exclusion treatment and sites on functional species traits.

Predictor		**log(iWUE)**		**log(SLA)**		**log(C:N)**		**log(N)**	
Fixed effects	*df*	c^2	*P*	c^2	*P*	c^2	*P*	c^2	*P*
Treatment	1	4.019	**0.045**	0.266	0.61	0.590	0.442	0.840	0.359
Functional group	1	55.834	**<0.001**	38.660	**<0.001**	17.698	**<0.001**	13.466	**<0.001**
Life history strategy	1	41.405	**<0.001**	179.915	**<0.001**	6.794	**0.009**	4.582	**0.032**
Site	2	1.605	0.448	35.625	**<0.001**	2.277	0.320	1.183	0.553
Treatment:Functional group	1	0.295	0.587	0.717	0.40	0.341	0.559	0.393	0.531
Treatment:Life history strategy	1	0.374	0.541	2.071	0.15	2.851	*0.091*	2.468	0.116
Life history strategy:Functional group	1	7.940	**0.005**	12.885	**<0.001**	2.967	*0.085*	2.144	0.143
Treatment:Life history strategy:Functional group	2	0.852	0.653	0.754	0.69	1.766	0.414	2.083	0.353
Treatment:Site	1	0.002	0.968	0.206	0.65	0.547	0.460	0.687	0.407
Random effect									
Plot	1		1		0.839		0.248		0.236

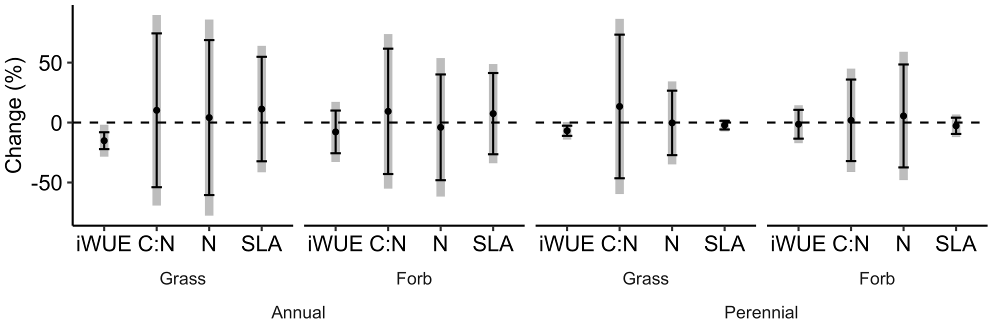


Figure 3.4: Response ratios measured as percentage change in traits at 40% reduced rainfall relative to unaltered rainfall. There was a significant interaction between life history strategy and functional group for iWUE and SLA (Table 3.2). Error bars represent 95% confidence intervals. Grey bars represent 90% confidence intervals. Traits include intrinsic water-use efficiency (iWUE), carbon to nitrogen ratio (C:N), nitrogen content (N), and specific leaf area (SLA).

Predicting intrinsic water-use efficiency

The strongest statistical model to predict iWUE from all measured species traits and environmental variables included SLA, mean phylogenetic distance, C:N, N, life history strategy, and rain exclusion treatment (Fig. 3.5, Appendix B: Table S3.3). Variation in SLA explained most of the variation in iWUE between different functional groups, with annual forbs and annual grasses at opposite ends of the resource-use spectrum. Notably, functional group and life history strategy significantly affected iWUE, SLA, C:N, and N content (Table 3.2). However, each variable divided into two nearly orthogonal trait continuums: iWUE-SLA and C:N-N, continuums which had a nearly orthogonal relationship that explained 86.6% of variation across species and sites (Appendix B: Fig. S3.3). Life history strategy was divided along the iWUE-SLA continuum, where annuals were associated with higher SLA and perennials with higher iWUE. Nutrient status assessed as leaf C:N and total N content had the least effect of the factors in the strongest mixed-effects model.

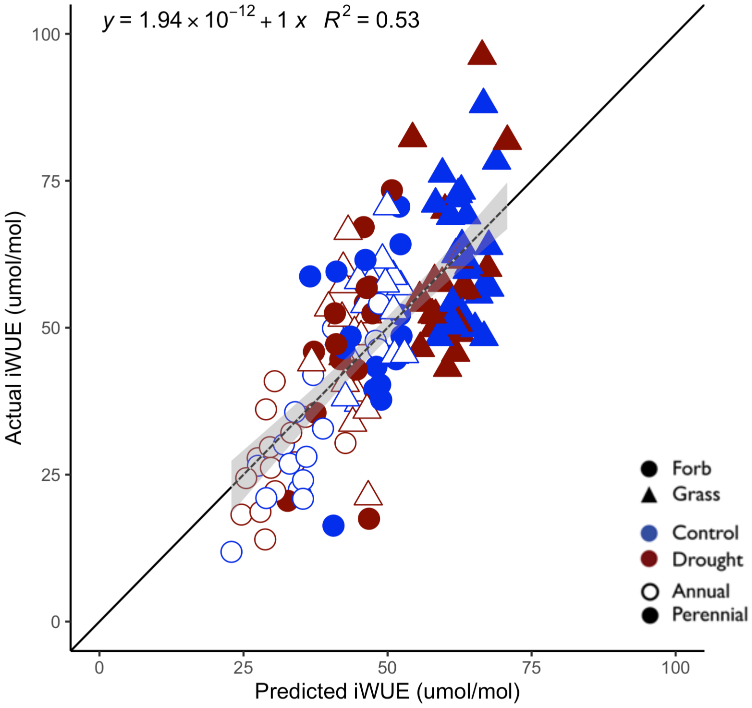


Figure 3.5 Statistical model of intrinsic water-use efficiency (iWUE) observed versus predicted by species traits and environmental variables. Black line indicates 1:1 relationship. Grey line indicates linear relationship described by equation in top left. Light grey indicates standard error. There was a significant interaction between life history strategy and functional group (Table 3.2). The significance of the model is detailed in Table S3.8.

Model variables and interactions

There was a strong inverse relationship between iWUE and SLA (Fig. 3.6, $R^2 = 0.67$, $P < 0.001$), but SLA had no significant effect with C:N or N (Appendix B: Fig. S3.5). The structure-function relationship follow expectations for differences between major functional groups, as reflected in significant effects of life history strategy (annual or perennial) and functional group (grass or forb) on iWUE and SLA but not for C:N or N. Beyond functional categories, iWUE had a significant association with continuous phylogenetic distances between species (Pagel's λ

= 0.78, P = 0.038) but SLA (λ = 0.18, P = 1), C:N (λ = 0.14, P = 0.78), and N (λ < 0.30, P = 1) did not.

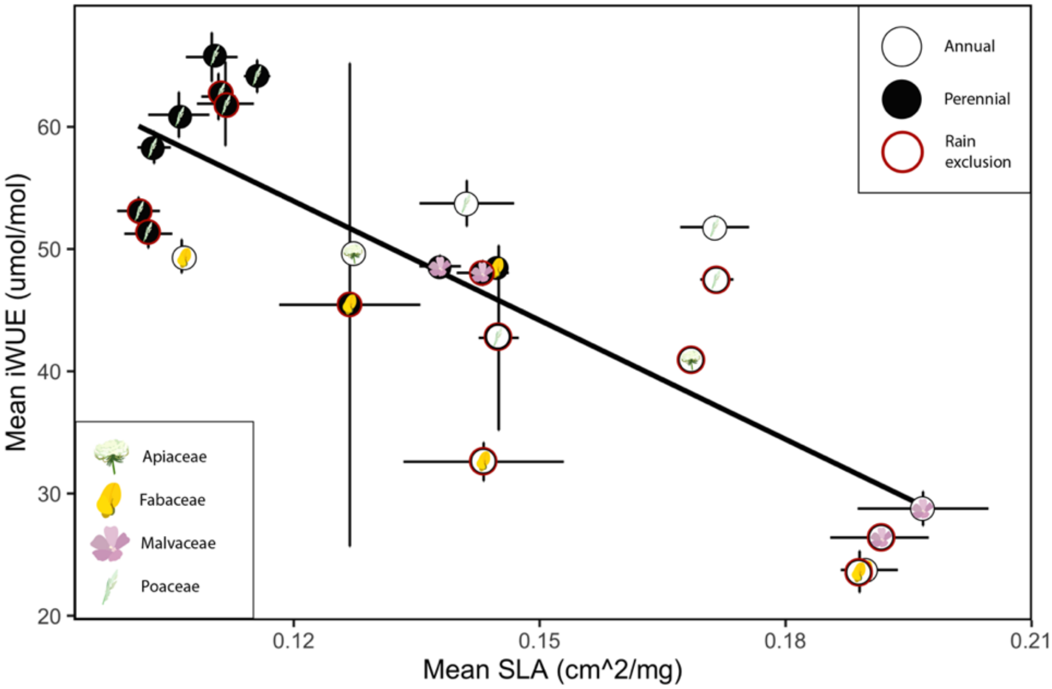


Figure 3.6 Linear correlation plot of mean intrinsic water-use efficiency (iWUE) with mean specific leaf area (SLA). Each point represents the mean value by species and rain exclusion treatment. There was a significant interaction between life history strategy and functional group; Table 3.2). Plot of all data points is available in Fig. S3.4. Symbols represent different families. Symbol fill represents life history strategy (black fill = perennial, white fill = annual). Red outline indicates the rain exclusion treatment. Fitted line is significant ($R^2 = 0.67$, $P < 0.0001$, $y = -324.71x + 92.79$). Errors indicate standard deviation.

Despite a significant interaction between life history strategy and functional group, on average, perennials had lower SLA and higher iWUE compared to annuals (Tables 3.2 and 3.3). In particular, perennial grasses had the lowest observed SLA (0.11cm/mg^2 ± 0.24 SD) while annuals (both grasses and forbs) had the highest SLA (0.16 cm/mg^2 ± 0.04 SD and 0.18 cm/mg^2 ± 0.05 SD respectively; Tables 3.2 and 3.3). In most cases, differences in SLA translated into differences in iWUE, with perennial grasses showing the highest iWUE (60.38 μmol/mol ± 11.93 SD) while annual forbs had the lowest iWUE (29.82 μmol/mol ± 10.41 SD). Life history strategies and growth forms were not significantly divided across the continuum of plant N content. However, perennial forbs had significantly lower C:N than other functional groups and forbs had higher leaf N concentration than grasses but not significantly so.

Table 3.3 Summary of mean trait values by life history strategy and functional group.

	Annual		**Perennial**	
Trait	**Grass**	**Forb**	**Grass**	**Forb**
SLA (cm/mg^2)	0.16 ± 0.03 ***a***	0.18 ± 0.04 ***a***	0.11 ± 0.02 ***c***	0.14 ± 0.04 ***b***
iWUE (μmol/mol)	49.57 ± 10.60 ***b***	29.82 ± 10.41 ***c***	60.38 ± 11.93 ***a***	48.17 ± 13.94 ***b***
C:N	24.05 ± 6.47 ***a***	22.73 ± 11.82 ***a***	23.90 ± 10.91 ***a***	16.33 ± 4.87 ***b***
N (%)	1.87 ± 0.49	2.30 ± 1.05	2.01 ± 0.70	2.70 ± 0.72

Notes: Letters indicate post-hoc (Tukey's honestly significant difference) significant differences for each trait among the four life history strategy and functional group combinations that were statistically different (see Table 3.2). Traits include intrinsic water-use efficiency (iWUE), carbon to nitrogen ratio (C:N), nitrogen content (N), and specific leaf area (SLA). Errors indicate standard deviation.

Discussion

Our data show that leaf traits and stoichiometry predicted iWUE across a range of species under rain exclusion and control plots along a broad latitudinal Mediterranean-climate gradient. Our rain exclusion treatment impacted iWUE in grasses (the only trait and functional group affected by treatment; Table 3.2), although in an unexpected direction. Previous studies using similar rain shelters have found iWUE increases under drought conditions (e.g., Beer et al., 2009; Ocheltree et al., 2020); however, in our study the grasses (particularly the annual grasses) had significantly reduced iWUE in the rain exclusion treatment compared to controls (Fig. 3.4). This could be an effect of phenology where flowering plants have greater iWUE than plants in which flowering is delayed due to reduced rainfall (Franks, 2011); however, a different study previously conducted at the same experimental sites did not show any significant changes in phenology in the rain exclusion treatment (Reed et al., 2019).

Most annual grasses in this system (and indeed, those in our study) are nonnative, winter-growing species that are increasing in abundance with warming (Reed, Pfeifer-Meister, et al., 2021). These species seem to have an avoidance mechanism to drought by their winter-growing, early maturing strategy and are able to outcompete other functional groups, perhaps explaining a reduced iWUE, as inferred from the isotopic signal which captures the integrated effect of environmental conditions (e.g. VPD, soil water, and air temperature) representative of the whole leaf lifespan (Maxwell et al., 2018). Change in phenology could therefore lead to unexpected shifts in iWUE by reflecting the less stressful period of leaf development for species that grow fast and early in the season to avoid drought stress, such as nonnative grasses. Additionally, the relative cover of such drought-adapted species likely affected the performance of the individuals measured here without changing the general trend between form and function across species (Figs. 3.4 and 3.5). For example, the rain exclusion treatment only had an effect in the shoulder fall and spring seasons because the Mediterranean climate contrasts very wet winters with dry summers, as shown in soil matric potential across the sites and treatments (Appendix B: Fig. S3.1). Further, the seasonality and absolute amount of available soil water, as represented by a natural climate gradient and a rain exclusion treatment, had relatively minor effects on iWUE in a Mediterranean-type climate.

Soil type and site-specific microclimate are also important controls of plant physiological performance, and thus water and nutrient use, and yet we see a consistent and predictable relationship between form and function across species at all sites and experimental treatments.

Indeed, despite differences in soil type and microclimate, we found a strong relationship between iWUE and SLA (Fig. 3.6). Although the leaf economic spectrum does not typically include iWUE, this structure-function relationship holds across species with a range of nitrogen-use strategies, but is not consistent within related taxa, such as Poaceae.

Stoichiometric relationships

Plant growth strategies (annual vs. perennial, grasses vs. forbs) had statistically significant effects on leaf functional traits (Table 3.3). On average, perennials were associated with increased iWUE, and decreased SLA compared to annuals. However, there was a significant interaction term between functional group and life history group, whereas life history strategies and growth forms did not significantly correlate with plant N content. The relationships between iWUE-SLA and C:N-N were expected to be co-linear, but they formed two quasi orthogonal axes (Appendix B: Fig. S3.3). Evidently, C:N and N were strong covariates (Appendix B: Fig. S3.4), but we included both in the statistical model because there is evidence that each may respond differently to environmental stressors (Li et al., 2015), and thus represent potentially distinct ecological processes. In particular, our results concur with Li et al.'s (2015) findings that iWUE is not correlated with N or C:N. Excluding either N or C:N marginally raised the power of the PCA to 87% (data not shown) but this did not adequately show the distribution of the data because C:N had a weaker relationship with SLA compared to iWUE (Appendix B: Fig. S3.4). We suspect that this is due to differences in symbiotic and competitive interactions that occur belowground and that shape the differential investments in leaf structure and resource use within and across functional groups, as discussed above for nonnative grasses. Although all of our focal species participate in arbuscular mycorrhizal interactions (Appendix B: Table S3.1), only three species (all members of Fabaceae) have the capacity to fix N. The large variation around the mean within and across species could represent differences in phenology as well as variation in root development and common mycorrhizal networks, which were not measured in this study. This variation would eventually be reflected in the integrated measure of ecophysiological performance and should be a research priority going forward. In all but one instance, nitrogen-fixing species displayed lower than average iWUE relative to the general trend expected from variation in leaf area (Fig. 3.5). This could be explained by the greater evaporative loss observed in other Fabaceae species (e.g. alfalfa) which make them outliers in terms of evaporative enrichment. For example, we have previously observed this phenomenon in oxygen isotope signatures of cellulose and bulk lipid extracts in field experiments where the isotopic signatures of the source soil water could be compared with the leaf water evaporative enrichment across multiple collocated functional groups (Silva et al., 2015).

Rain exclusion and water availability

Our sites span ~520 kilometers in latitude with a strong drought gradient where drought has an earlier spring onset at the southern sites; however, site did not affect iWUE (Table 3.2). Our data show that changing the amount of precipitation in this Mediterranean climate gradient had a small effect on iWUE, an effect that is unexpected due to the known link between iWUE and drought stress (e.g., Farquhar et al. 1989) and deserves further investigation in future studies. The effects of the rain exclusion treatment were species-specific and less detectable when analyzing species by common groupings (Fig. 3.4), although leaf traits varied according to life history strategy and functional group (Table 3.3). Clark et al. (2012) found that plant traits predicted bunchgrass prairie restoration success in the Pacific Northwest equally well as individual species status. However, they analyzed a suite of traits with greater breadth and depth, and they found that the effect varied significantly between sites. While measuring iWUE typically requires access to expensive equipment, we found that it can be reliably inferred from SLA, mean phylogenetic distance, and life history strategy (Fig. 3.5, Appendix B: Table S3.8). These are all metrics that can be measured with simple equipment (SLA) or determined using widely available information (mean phylogenetic distance, life history strategy).

Phylogenetic relationships

Overall, the generalizable leaf trait relationships that we found in our data allowed us to identify tradeoffs in plant resource-use for different growth forms. These relationships can act as a new lens for land managers who can use these relationships to target species when managing Pacific Northwest grasslands. This could manifest as a tool to aid in designing seed mixes for functional restoration in a particular site or region. New perspectives such as this are critical for adaptive management that may also mitigate the impacts of climate change. Structure-function relationships can be used to improve on the relatively coarse restoration paradigm that biodiversity alone is the key to resistance and resilience to future climates. For example, we found that species relatedness measured as phylogenetic distance improved the strength of our model when used with functional traits (Appendix B: Table S3.3). Phylogenetic data are now widely available and accessible with open source software such as the V.PhyloMaker R package (Jin & Qian, 2019). Although there are multiple functions that can incorporate and analyze single trait values for each leaf of a phylogenetic tree, mean phylogenetic distance is different in that it can be used as a standalone trait in statistical models. It has been successfully adapted to measure the relatedness of native and exotic organisms, testing correlation between an organism's relatedness to native species and predicting species introduction success (Ness et al., 2011; Van Wilgen & Richardson, 2011). However, it is worth noting that key morphological traits such as SLA was predicted by the categorical grass/forb designation but not the continuous phylogenetic distance variable. Although our study only includes twelve species, our results suggest that phylogenetic analysis when coupled with trait-based analysis could become valuable in ecological predictions.

Experimental limitations

There are caveats to our findings. We only installed plot-specific temperature and soil moisture dataloggers in manipulated plots, so our localized climate data were limited (Appendix B: Table S3.1). Additionally, as explained above, the rain exclusion treatment only had a significant effect on soil water content across the entire growing season at the central site. During the dry summer months, there is negligible precipitation, so a 40% reduction is not biologically meaningful. As a result, at least at the southern and northern sites, there may only be short shoulder seasons during the early fall and late spring when a reduction in rainfall has a measurable effect on soil water content. To the extent that the shelters had an effect, they would reduce evapotranspiration, which would warm the soil (Appendix B: Fig. S3.2). This suggests that the shelters were more effective than shown by the soil moisture data. Also, previous studies at the same site have found that soil moisture and plant community composition affect each other, potentially obscuring the effects of the rainout shelters (Reed, Pfeifer-Meister, et al., 2021). The results show that iWUE varies primarily with functional group (grass vs. forb) and life history (annual vs. perennial), with marked differences between nitrogen-fixer (i.e. Fabaceae) consistent falling below the average iWUE as predicted by leaf structure, as explained above (Fig. 3.5).

The weak effects of the rain shelters, combined with the relatively low number of sampled individuals for each of the 12 species, makes it difficult to assert whether variation in precipitation would alter intraspecific traits as expected. Additionally, we calculated iWUE from

δ^{13}C values, a proxy that is well-established but can be blurred by compounding factors unrelated to actual water use. One of the most notable of these is variation in phenology (i.e. leaf age) on mesophyll conductance which can act on ^{13}C conductance without impacting iWUE. The equation for iWUE can be scaled to reduce this error (Ma et al., 2020), but a more accurate estimation of water-use efficiency would include direct measurements of gas exchange and mesophyll conductance. Indeed, our observations are consistent with previous experiments in which the timing of leaf emergence, under low and high water availability, shifted both δ^{13}C ratios and actual A/g_s to a greater degree than the drought treatment (Franks, 2011). Finally, not all species were present at all sites (Appendix B: Table S3.1), and therefore species identity may be confounded with environmental conditions. To address this, we looked at the effects of control and drought treatments which are paired at each site. All functional groups were represented at all sites, which allowed for a cross-site comparison of structure-function relationships. The best model was a global one (Appendix B: Table S3.3), which included all functional groups across sites. Our analysis incorporates species, treatment, and site effects, whose relative predictive power can be parsed statistically (Table 3.2) using plot random effects and MPD to evaluate covariation between plots. Our data were plant-centric and focused on aboveground traits. However, belowground traits and microbial interactions could further improve models to predict form-function relationships at community and ecosystem levels (Silva & Lambers, 2021).

Conclusion

We found that the experimental drought stress did not affect leaf trait and stochiometric relationships across species. We used a mixed-effects model to infer variation in iWUE within and across 12 different species as a function of structural (SLA, life history strategy) and mean phylogenetic distance across species and sites. Our analyses included all relevant functional groups found in managed and natural Pacific Northwest grasslands and the results were consistent along a climate gradient from central Washington to southern Oregon. We found that species-specific responses were important across functional groups, a factor that should be taken into consideration for future management and restoration decisions. Going forward, our findings could inform trait-based approaches to predict water and nutrient use in ecosystems restoration and climate change mitigation and adaptation projects in the Pacific Northwest and other temperate grassland systems.

BRIDGE

Experimental drought stress did not affect leaf trait and stoichiometric relationships in three Pacific Northwest grasslands. Species-specific responses were important across functional groups, a factor that should be taken into consideration for future management and restoration decisions. However, species-specific error bars were not insignificant. I proposed that this range of error could be caused by interactions with other organisms, particularly mycorrhizal fungi. Plants and mycorrhizal fungi form close mutualistic relationships that affect the structure and function of ecosystems. Common mycorrhizal networks (many plants associated with the same fungus) can facilitate preferential transfer of carbon and limiting nutrients, but the drivers of these interactions are rarely tested in ecological settings. In Chapter 4, I used stable isotopic tracers to follow carbon and nitrogen transfer through the grassland system described in Chapter 3, and tested how leaf traits can inform our understanding of this transfer.

CHAPTER 4:

PLANT FUNCTIONAL TYPES AND TISSUE STOICHIOMETRY EXPLAIN NUTRIENT TRANSFER IN COMMON ARBUSCULAR MYCORRHIZAL NETWORKS OF TEMPERATE GRASSLANDS

Contributions

Hilary Rose Dawson, Toby M. Maxwell, Paul B. Reed, Scott D. Bridgham, and Lucas C. R. Silva designed the research; Hilary Rose Dawson and Toby M. Maxwell collected the leaf data, Katherine L. Shek and Hilary Rose Dawson collected the fungal DNA data, and Barbara Bomfim collected the soil data; Hilary Rose Dawson analyzed the data with assistance from Katherine L. Shek; Hilary Rose Dawson wrote the paper; all authors edited the paper and helped interpret the data; Lucas C. R. Silva, Scott D. Bridgham, and Brendan Bohannan funded the project.

Introduction

Plant-mycorrhizal associations are thought to have emerged as rudimentary root systems over 400 million years ago, facilitating the expansion of terrestrial life that followed (Kenrick & Strullu-Derrien, 2014). The transformative power of early fungal symbioses is still evident today in all major plant lineages, from bryophytes to angiosperms (Heijden et al., 2015). Over 85% of all contemporary flowering plant species form symbioses with fungi, with arbuscular mycorrhizal (AM) associations being the most common (Brundrett, 2009). The relationships between plants and AM fungi dominates both managed and unmanaged landscapes and are estimated to be responsible for up to 80% of global primary productivity (Heijden et al., 2015). Fungi can form symbioses with more than one individual plant, particularly AM fungi which have low host specificity (Selosse et al., 2006). By extension, it has been widely hypothesized that the multi-plant-fungal relationships form “common mycorrhizal networks” (CMNs) which facilitate carbon and nutrient transfer between organisms, beyond the immediate plant-fungus mutualism formed by individuals. Although CMNs are traditionally defined by strict criteria that are difficult to test experimentally (Karst et al., 2008), the concept is ecologically relevant and useful in designing new experiments that may bring insight into CMNs structure and function. Here, we use 'CMN' under the proposed new definition of Rillig et al (2024) "where at least one mycorrhizal fungal genet interacts (connecting and colonizing or growing in close proximity) with the roots of a minimum of two plants of the same or different species." As in previous studies of this kind, the CNM relationships are often simplified to the mutual exchange of carbon-based photosynthates for soil nutrients that are more readily bioavailable to fungi (Smith & Read, 2008), but exist along a continuum from parasitic to mutualistic (Johnson et al., 1997; Karst et al., 2008; Luo et al., 2023).

The literature holds myriad and often complimentary, but sometimes contradictory, hypotheses that could explain the CMN mutualism as a key structural and functional component of ecosystems. For example, the "economics" hypothesis (Kiers et al., 2011) proposes that plants and fungi engage in "trades" of nutrients mined by fungi in exchange for plant photosynthates (Averill et al., 2019; Fellbaum et al., 2014; Werner & Dubbert, 2016). In the economics hypothesis, the terms of trade between plant and fungi are mediated by supply and demand for limiting resources, which could create a dynamic market emerging from interactions between environmental, biochemical, and biophysical variables. The "Wood Wide Web" hypothesis emerged from the analysis of isotopically labeled carbon transferred between plants, presumably through fungal mycorrhizae. Simard et al. (1997) hypothesized that plants that allocate carbon to sustain common fungal symbionts also benefit from shared nutrients, while plants associating with mycorrhizal fungi outside that network cannot. Complementing the analogy, the "kinship" hypothesis proposes that plants of the same species preferentially receive more resources in CMNs (Pickles et al., 2017; Tedersoo et al., 2020).

The past two decades have seen extensive but inconclusive research on these hypotheses and how they relate to empirical measurements of CMN structure and function. On the one hand, economic analogies suggest that the reciprocally regulated exchange of resources between plants and fungi in CMNs should favor the most beneficial cooperative partnerships (Fellbaum et al., 2014; Kiers et al., 2011). On the other hand, reciprocal transfer is only found in a subset of symbionts under specific conditions, while increased competition in CMNs is a more common observation (Walder & Van Der Heijden, 2015; Weremijewicz et al., 2016). At the core of this controversy is whether CMNs actively support fungal resource acquisition at the expense of plant resource demands (i.e., a fungi-centric view) or function as passive channels through which plants regulate resource fluxes (i.e., a plant-centric view). If plant-centric, we expect to find that the structure and functioning of CMNs give rise to consistent spatiotemporal patterns of resource allocation similar to those predicted by the kinship hypothesis. If fungi-centric, we expect to find that spatiotemporal patterns of resource allocation reflect the composition and functioning of the fungal community regardless of the connecting plant nodes in CMNs. Other perspectives emphasize that CMNs are experimentally under-documented and that this is an area that warrants further research (Henriksson et al., 2023; Karst et al., 2023; Rillig et al., 2024; Robinson et al., 2024). Given that data exist to support multiple, sometimes opposing views (Figueiredo et al., 2021; Silva & Lambers, 2021); we posit that CMNs are neither plant- nor fungi-centric.

In this study, we ask if interactions among biophysical and biogeochemical processes could explain resource transfer in CMNs with more accuracy than previous plant- or fungi-centric analogies. We use a grassland system where fungal ASVs are frequently found within the roots of multiple plants in a small area, a system that meets the broader CNM definition given by Rillig et al (2024). In our study, we focus on dynamics in a system that has a high probability of connectivity. We quantify interspecific carbon and nitrogen transfer focusing on plant traits that are known to regulate physiological performance (Dawson et al., 2022) , rather than aiming to prove that CMNs are the only explanation. By measuring plant traits and environmental variables that affect resource-use efficiencies across different species we describe how the transfer of carbon and nitrogen occurs in paired experiments designed to affect soil water and nutrient mass flow. We labeled perennial plants central to each plot (hereafter, 'donors') with stable isotopically enriched gases and monitored leaf ^{15}N and ^{13}C for the surrounding plants ('receivers'), monitored from immediately after labeling to to 21 days after. We replicated our paired experimental setting at three different locations, with study sites distributed across a 520 km latitudinal gradient. We also sequenced strain-level variation in root fungal DNA, plant functional types, and leaf stoichiometric traits to test if relatedness (same or different species as the donor) explained difference in resource transfer. We interpret our data considering that the diversity of plant growth forms included in our study can affect CMN composition and function (Davison et al., 2020). Both plants and fungi have economic spectra characterized by contrasting traits and nutrient strategies which together form an interacting continuum potentially driven by resource use and availability (Ward et al., 2022). It is unclear to what extent plant or fungi characteristics drive these plant-fungal interactions. Therefore, we focused on quantifying how plant-fungal interactions influence the structure and functioning of CMNs across environmental gradients and resource constraints.

Materials and Methods

We conducted our experiment at three sites situated on a 520 km latitudinal transect that spans three Mediterranean climates: cool, moist (northern site; Tenino, WA) to warm, moist (central site; Eugene, OR) to warm, dry (southern site; Selma, OR). Each circular plot was 3 m in diameter. Half of our plots were restored prairie systems (n = 10 per site) while the other half of the plots had introduced pasture grasses established prior to restoration (n = 10 per site). Restored prairie plots were mowed, raked, received herbicide, and seeded in 2014-2015, followed by seeding in fall 2015, 2016, and 2017 (Reed et al., 2019). We erected rainout shelters that excluded 40% of the rainfall on half the plots at each site (n = 10 rain exclusion, 10 control per site; Fig. 4.1). Due to the climatically driven differences in communities across sites, not all species were present at all sites (Appendix C: Table S4.1); however, all functional groups were present at all sites and most species were present at more than one site. Our experimental design was nested in a multi-year experiment where data loggers were used to continuously measure temperature and moisture in all the manipulated plots.

We recognize that there are many challenges for establishing field experiments of CMN effects, such as treatments for severed versus intact connections (Karst et al., 2023). The plants we experimented on grow in a shared plot where nutrients could transfer via soil, water, bacteria, other fungal guilds, or other non-CMN mechanisms. This does not exclude the possibility that fungi play a large role in these interactions, especially in a system where all plant species have the potential to engage in the most common form of a mycorrhizal connection (Appendix C: Table S4.1; Heijden et al., 2015). As Rillig et al. (2024) point out, maintaining a strict definition for CMN limits research from exploring meaningful ecological interactions. We have approached this experiment under their proposed broader definition of CMN, an approach which allows us to advance our understanding of how CMNs may function in natural ecosystems.

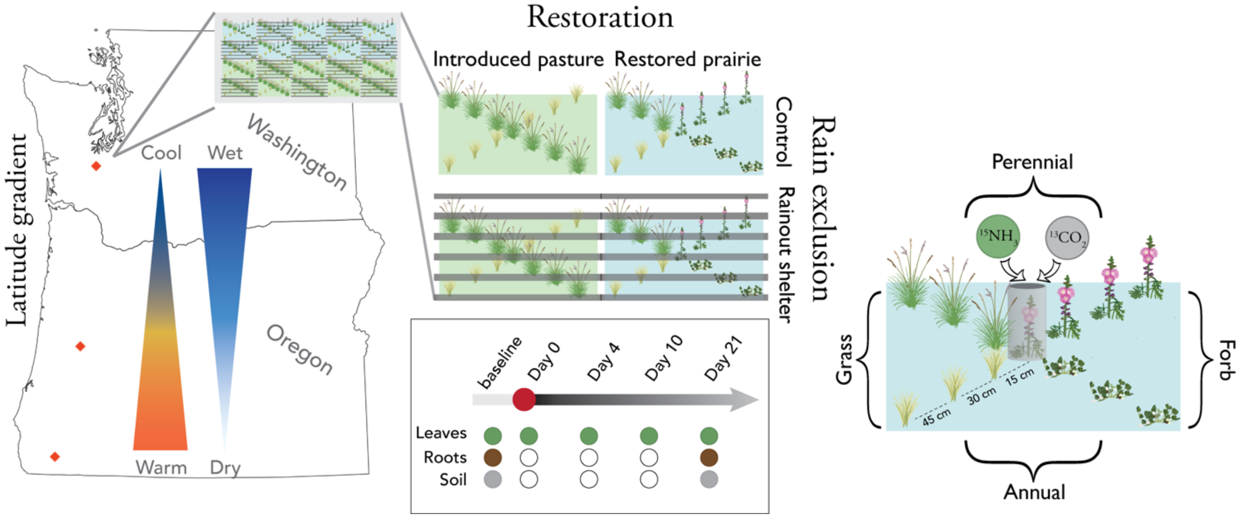


Figure 4.1: Schema of experimental set up, sampling, and effects of rainout shelters. Figure shows the three locations of the sites (orange points on map) in relation to the climatic gradient (cool to warm, wet to dry). Each plot had a central perennial plant that was labeled with isotopically enriched gas (shown with a grey cylinder here) and we sampled plants of each functional type at three distances from the central labelled plant. Inset shows the temporal sampling scheme of leaves, roots and soil.

Previous work demonstrated that the rainout shelters had minimal effects on aboveground community structure or function (Dawson et al., 2022), possibly due to the shoulder season effect of the Mediterranean rain seasonality. This network of experimental sites was established in 2010 and has been extensively studied since then (Brambila et al., 2023; DeMarche et al., 2021; Reed, Bridgham, et al., 2021; Reed et al., 2019, 2023; Reed, Peterson, et al., 2021; Reed, Pfeifer-Meister, et al., 2021), including work on mycorrhizal fungi (Vandegrift et al., 2015; Wilson et al., 2016). Treatments had marginal effects on the soil water potential (especially during the early growing season). Despite those differences, we did not find significant changes in the plant community composition or productivity under rain exclusion, which also did not affect morphological and functional traits (i.e., specific leaf area, iWUE, and C:N ratios) of the functional groups we selected for this experiment (Dawson et al., 2022; Reed, Pfeifer-Meister, et al., 2021).

Isotopic labelling

At each site, we selected a healthy perennial forb (*Sidalcea malviflora* ssp. *virgata* in restored prairie plots [except in one plot where we used *Eriophyllum lanatum* due to a lack of *S. malviflora ssp. virgata*]), or a perennial grass (*Alopecurus pratensis, Schedonorus arundinaceus,* or *Agrostis capillaris*) in pasture plots at the center of each plot to receive the isotopic labels. On sunny days between 11AM and 3PM, we applied isotopically enriched carbon (^{13}C) and nitrogen (^{15}N) as a pulse of carbon dioxide (CO_2) and ammonia (NH_3) to the leaves of target "donor" species common across experimental sites. Although gases are not the primary source of nitrogen for most plants, applying gaseous nitrogen allowed us to limit the amount leaked into the soil compared to applying nitrogen directly to the soil (Silva et al., 2015). Plant leaves are known to uptake ammonia (Farquhar et al., 1980; Sutton et al., 2008). We performed the labeling experiment using custom-made clear chambers with internal fans, following established protocols (e.g., Earles et al., 2016; Silva et al., 2015; Sperling et al., 2017). Before performing the experiment, we tested our approach in the field to optimize gas exposure and labeling amounts, which included checking for leaks and contamination outside of the chamber. We covered the donor plant with a clear plastic cylindrical chamber and injected gas in sequence at 20 minute intervals. For $^{13}CO_2$, we made three injections of 2 mL pure CO_2 (98 atm % ^{13}C) to double the amount of CO_2 in the chamber each time. For NH_3, we made two injections of 10 mL pure NH_3 (98 atm% ^{15}N). The dates of application were based on peak productivity estimated from Normalized Different Vegetation Index (NDVI) at each site (see Reed et al., 2019 for details). We sampled leaves from each donor plant immediately after labeling (time point 0) as well as from all plants approximately 4 days (time point 1), 10 days (time point 2), and 21 days (time point 3) post-labelling (Fig. 4.1, Appendix C: Table S4.2). Time points were chosen to balance the potentially rapid transfer of nutrients through the system with the logistical difficulties of a single team sampling along a 520 km gradient. We also collected leaves at time points 1, 2, and 3 from up to twelve plants in each plot representing three replicates of grass/forb structural groups and annual/perennial life history strategies (Appendix C: Table S4.1). The number of plants and groups depended on which plants were growing in each plot.

At the end of the experiment, we harvested entire plants and the soil surrounding the roots at time point 3 and kept them in cool conditions until processing. We separated the roots and rhizosphere soils and selected approximately ten ~3 cm fine root fragments per sample (i.e., third order or finer, where available) for DNA extraction and identification. All roots and rhizosphere soils were stored at -80º C until processing.

Baseline and Resource Transfer Calculations

Before isotopic labeling, we collected soil, leaves, and roots from each site. We collected soils in late spring and early summer 2019 to 20 cm depth in each plot. From these soil samples, we removed root fragments that represented the typical roots seen in each plot. We collected leaves for each species in each plot; however, these leaves were contaminated with ^{15}N during transport. To replace contaminated samples, we separately sampled leaves from biomass samplings collected in late spring and early summer 2019, ensuring that annual and perennial grasses and forbs were represented at each site.

We oven-dried all samples at 65° C to constant mass and encapsulated them for stable isotope analysis. All stable isotope analysis was done at UC Davis Stable Isotope Facilities. We calculated the amount of carbon and nitrogen in each plant compartment (leaves and roots) using standard label recovery equations (Silva et al., 2015), using baseline values measured before application of the labelled gases to capture background variations in isotopic composition of unenriched leaves, roots, and soil samples.

We designated all samples with greater than two standard deviations above baseline samples as "enriched" in a particular isotope. Baseline values were calculated on a site by rain exclusion treatment basis by plant functional type basis (Appendix C: Table S4.3). Site-specific baseline soil and root isotope ratios represent the whole community because of interconnected rhizospheres where is it was not possible to identify specific species. In all cases, baseline values fell within the expected range for our region (Appendix C: Fig. S4.1). For each enriched sample, we calculated isotope excess as

Equation 1

$$atm\%_{excess} = atm\%_{post\ label} - atm\%_{baseline}$$

For enriched donor plants, we calculated % derived from label immediately following label application as

Equation 2

$$\%DFL_{donor} = \frac{atm\%_{excess}}{atm\%_{labelling\ gas} - atm\%_{baseline}} * 100$$

For enriched receiver plants, we calculated % derived from label (%NDFL and %CDFL, respectively) for each relevant point in space and time as

Equation 3

$$\%DFL_{receiver} = \frac{atm\%_{receiver\ excess}}{atm\%_{\ donor\ excess}} * 100$$

When we calculated %DFL in roots, we used donor leaves as the source (atm% donor excess). We then calculated the amount derived from label on a per mass basis as

Equation 4

$$DFL\ (\mathrm{mg\ N\ /\ g\ leaf}) = 1000 * 1g * \left(\frac{N\ or\ C\%}{100}\right) * \left(\frac{DFL\%}{100}\right)$$

We calculated intrinsic water-use efficiency following Farquhar and Richards (1984) using the baseline ^{13}C values from the original samples. Because of sampling discrepancies, we did not have intrinsic water-use efficiency data for 32 plants.

We selected a subset of rhizosphere soils that represented six donor plants at each site divided equally between restored prairie and pasture plots and selected the three most highly ^{15}N-enriched interspecific receivers in each plot across the sites. In addition, we sampled three most highly enriched interspecific receivers at each site and restored prairie-introduced pasture combination. We sampled the top three enriched intraspecific receivers at each site and treatment. In total, this came to 48 post-labelling soil samples in 29 plots.

Fungal DNA analysis

We extracted DNA from roots of 450 plants harvested at time point 3 (21 days post-label) using Qiagen DNeasy Powersoil HTP kits (Qiagen, Hilden, Germany). We only analyzed DNA from roots, not from the soils collected from each plant's rhizosphere. We characterized each sample's AM fungal composition with a two-step PCR protocol that amplified a ~550bp fragment of the SSU rRNA gene (the most well-supported region for AM fungal taxonomic resolution (Dumbrell et al., 2011). We used WANDA (5'- CAGCCGCGGTAATTCCAGCT- 3') and AML2 (5'- GAACCCAAACACTTTGGTTTCC-3') primers (Langmead & Salzberg, 2012; Lee et al., 2008). We used primers with unique indices so we could multiplex several projects on a single run. We quantified successful PCR amplicons with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Waltham, MA, USA) on a SpectraMax M5E Microplate Reader (Molecular Devices, San Jose, CA, USA) before purifying with QIAquick PCR Purification kits (Qiagen). We sequenced the purified pools on the Illumina MiSeq platform (paired-end 300bp, Illumina Inc., San Diego, CA, USA) at the University of Oregon Genomics and Cell Characterization Core Facility (Eugene, OR, USA). Reads were deduplicated with UMI-tools using unique molecular identifiers (UMIs) inserted during PCR (Smith et al., 2017).

We assigned amplicon sequence variants (ASVs) using the dada2 pipeline (version 1.18.0) with standard quality filtering and denoising parameters (Callahan et al., 2016). The dada2 pipeline maintains strain-level diversity at the scale of individual sequence variants rather than clustering sequences into OTUs. This fine-scale measure of fungal sequence diversity was particularly important for our analyses to maintain the greatest chance of detecting a single AM fungal 'individual' in multiple plant root samples. Taxonomy was assigned to ASVs using the MaarjAM database (2019 release) (Öpik et al., 2010). We used a Bayesian mixture model in the DESeq2 package (Love et al., 2014) to scale ASV counts within and across samples to avoid artificial taxon abundance biases (Anders & Huber, 2010).

Replication statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Individuals	Individual donors and receivers	1,441 leaves, 450 root DNA samples, and 364 root stable isotope samples from 60 donor and 502 receiver plants
Species	Functional groups	18 species with at least 3 individual plant replicates each (details in Table S1) Grouped by 61 annual forb plants, 131 annual grass plants, 81 perennial forb plants, 161 perennial grass plants
Community	Pooled by site and	3 sites (northern, central, and southern latitude sites spanning ~520 km; 20 plots per site; 60 total)
	by climate and restoration treatment	Rain exclusion (10 plots per site; 30 total) vs ambient (10 control plot per site; 30 total); Restored prairie (10 plots per site; 30 total) vs pasture (10 plots per site; 30 total) treatments;

Data analysis

We performed all analyses in R ver. 4.0.4 (R Core Team, 2022). Graphs were made in ggplot2 (Wickham, 2016). We removed one outlier plant with a ^{15}N atm% more than twice as high as the next highest measurement. We also removed five mislabeled samples. To meet statistical assumptions, we only included data from enriched plants with successful root fungal DNA extraction in our analyses and figures. We limited receivers to those for which we also had sufficient root fungal DNA. In total, from 1441 leaves measured for isotopic content and with successfully recovered fungal DNA, we analyzed data from 353 unique plants: 54 donors and 353 receivers. We excluded two plots (one central rain exclusion restored plot and one central rain exclusion pasture plot) because either no donor or no receiver leaves were recovered.

We tested the relationship between receiver leaf %DFL and plant traits (grass/forb, annual/perennial, iWUE, C:N, degrees of connectivity, interaction term between grass/forb and annual/perennial) and site conditions (position on latitude gradient, pasture/restored, rain exclusion treatment, distance from donor, time from labelling) with a mixed-effect ANOVA (plot nested within site as random effect; Table 4.1). We used a Tukey post-hoc test for differences within groups shown in the following figures and tables. We constructed a phyloseq object using the ASV table with normalized counts (McMurdie & Holmes, 2013), and used iGraph, metagMisc, and RCy3 (Gustavsen et al., 2019; Mikryukov, 2017; Nepusz & Csardi, 2006) to create networks for each plot. In each network, nodes represented individual plants and edges between nodes represent plants sharing at least one fungal DNA sequence variant. The weighted edges are based on how many fungal ASVs were shared among plants. We calculated degrees of connectivity with tidygraph (Petersen, 2022) to examine how many plants each individual plant was 'connected' to (by means of shared fungal ASVs) in each plot (Appendix C: Fig. S4.2). We also calculated whether each receiver plant shared fungal ASVs with the central donor plant in each plot. We visualized shifts in AM fungal community composition using non-metric multi-dimensional scaling (NMDS) in the vegan package, demonstrating the AM fungal community similarity across plants (Oksanen, et al., 2022).

Results

All donors were ^{15}N-enriched in their leaves at time of labelling. Two donors were not ^{13}C-enriched in their leaves at time of labelling. We recovered DNA data from the roots of 88.3% of donors. We sampled 1,444 leaves from 434 receiver plants at three time points. Of these leaves, 81.0% were ^{15}N-enriched and 2.4% were ^{13}C-enriched. We recovered DNA from the roots of 77.9% of the receivers. At time point 3 (~21 days post-labelling), we collected roots from 46 of the initial 60 donors and 306 of the initial 434 receivers. Of the roots of the collected donors, 97.8% were still 15N-enriched and 23.9% were still ^{13}C-enriched at time point 3. Of the roots of the collected receivers, 33.0% were still ^{15}N-enriched and 10.5% were still ^{13}C-enriched at time point 3.

Assimilation of isotopic tracers was similar between labeled "donor" plants with no significant differences on average between sites or experimental treatments within sites, including rainfall exclusion or restored status (Appendix C: Fig. S4.3). At all sites, foliar assimilation of ^{15}N and ^{13}C by donor plants led to enrichment levels ranging from approximately 5-10 fold higher than baselines. Foliar enrichment levels decreased consistently at all sites and treatments over the 21 day sampling period. Annual forbs had the greatest enrichment level and perennial forbs the lowest enrichment level (Fig. 4.2). We found significant spatial and temporal differences in foliar and root isotope ratios in donors and receivers resulting from interspecific transfer of carbon and nitrogen (Table 4.1; Appendix C: Fig. S4.4). Receiver foliar enrichment levels did not correlate with donor foliar enrichment levels within the same plot (Appendix C: Fig. S4.5).

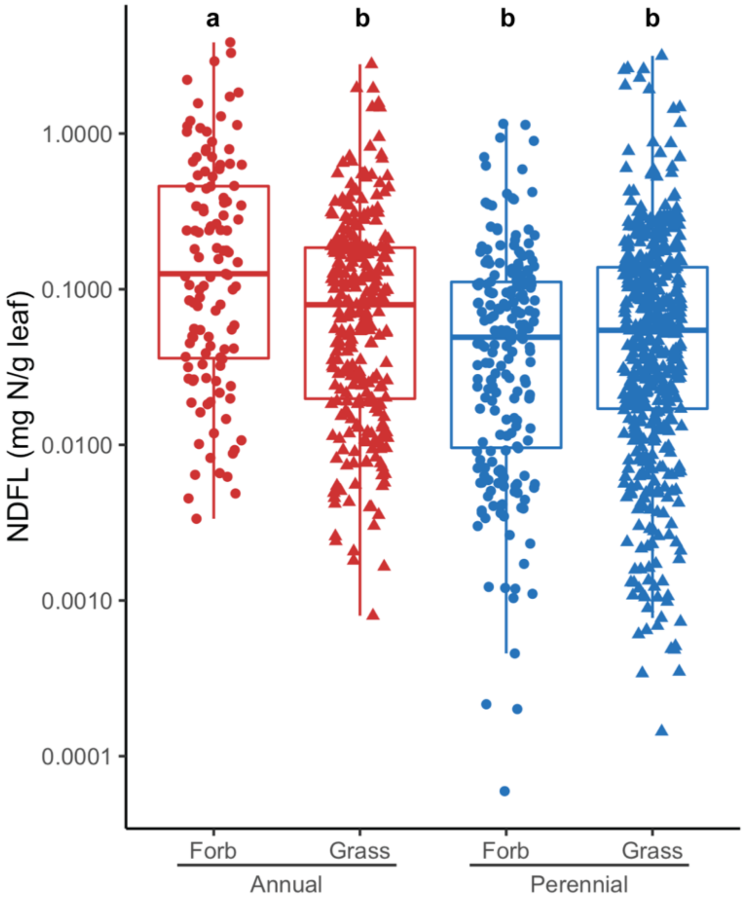


Figure 4.2: Annuals had greater leaf nitrogen derived from label (NDFL) scaled by mass than perennials. Points represent enriched individual receiver plants with associated DNA data at one time point of sampling. Y-axis is log10 scale with raw value datapoints. Boxplots show medians and interquartile ranges, lines show the largest (or smallest) value no further than 1.5x IQR from the hinge. Letters above the boxplots indicate significant differences.

Table 4.1. Mixed-effects ANOVA results effects on leaf nitrogen derived from label (%NDFL). Random effect is plot nested within site. Only enriched receiver leaves with associated DNA data were included in the analysis (n = 1094). Leaf %CDFL results available in Table S4.

	F-statistic	DF	P-value
Annual/perennial	9.81	**1**	**<0.001**
Grass/forb	11.0	**1**	**<0.001**
Same species as donor	0.47	1	0.463
Degree of connectivity	0.02	1	0.68
iWUE	0.03	1	0.81
C:N	35.51	**1**	**<0.001**
Site	0.40	2	0.71
Drought treatment	1.09	1	0.48
Restoration treatment	0.85	1	0.34
Distance from donor	9.61	**1**	**0.002**
Time from labelling	83.59	**1**	**<0.001**
Annual/perennial: Grass/forb interaction	7.75	**1**	**0.01**

Allocation and transfer varied significantly between functional groups due to their intrinsic differences in tissue stoichiometry (Table 4.1). We selected 18 common annual/perennial and grass/forb species of receiver plants, which revealed significant differences between functional types for NDFL (Fig. 4.2) but little detectable CDFL relative to baseline (Appendix C: Fig. S4.4, S4.6). Rain exclusion treatment, restoration treatment, and site did not affect interspecific transfer of nitrogen (Fig. S4.7; ANOVA, $P > 0.05$, Table 4.1). We observed very low carbon enrichment, but of the 2.4% of leaves that were enriched in carbon, plant functional type and site affected carbon transfer (Appendix C: Table S4.4). We did, however, observe significant differences in nitrogen transfer by plant functional type (Table 4.1), mirroring intrinsic differences in tissue stoichiometry and iWUE (Fig. 4.3), despite no significant enrichment in soils collected from the rhizosphere of those same plants (Appendix C: Fig. S4.8). We also found that C:N affected NDFL, although not in a simple linear manner and with no apparent correlation between NDFL and iWUE (Fig. 4.3). We did detect a low level of soil enrichment in 1 out of 30 receiver soil samples (0.377 atm% ^{15}N) and 5 out of 18 donor soil samples (ranging from 0.376 to 0.479 atm% ^{15}N; Appendix C: Fig. S4.8).

Annuals had greater ^{15}N foliar enrichment compared to perennials (ANOVA, $P < 0.001$, Tables 1 and 2). Foliar enrichment decreased over both time and space (ANOVA $P < 0.001$; Appendix C: Fig. S4.3, Fig. S4.4). On average, annuals had a lower leaf nitrogen content and higher C:N than perennials (Table 4.2, Appendix C: Fig. 4.3). Forbs had higher NDFL than grasses (ANOVA, $P < 0.001$, Table 1) as well as a lower C:N. There was a significant interaction between annual/perennial and grass/forb form (ANOVA, $P = 0.003$, Table 4.1). There was no correlation in ^{15}N-enrichment and whether the donors and receivers were the same species (Table 4.1).

Table 4.2. Leaf nitrogen derived from label (NDFL) and leaf tissue nitrogen (N%) four days after labeling.

			%NDFL				**NDFL (mg N/g leaf)**				**N (%)**			
		n	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*
Annual	**Forb**	54	6.528	9.769	0.101	56.005	0.749	0.819	0.041	3.858	0.749	0.819	0.041	3.858
	Grass	92	4.847	9.032	0.098	63.884	0.34	0.463	0.011	2.79	0.340	0.463	0.011	2.790
Perennial	**Forb**	67	0.952	1.069	0.090	5.582	0.214	0.228	0.021	1.156	0.214	0.228	0.021	1.156
	Grass	148	1.632	3.066	0.088	22.434	0.252	0.484	0.013	2.948	0.252	0.484	0.013	2.948

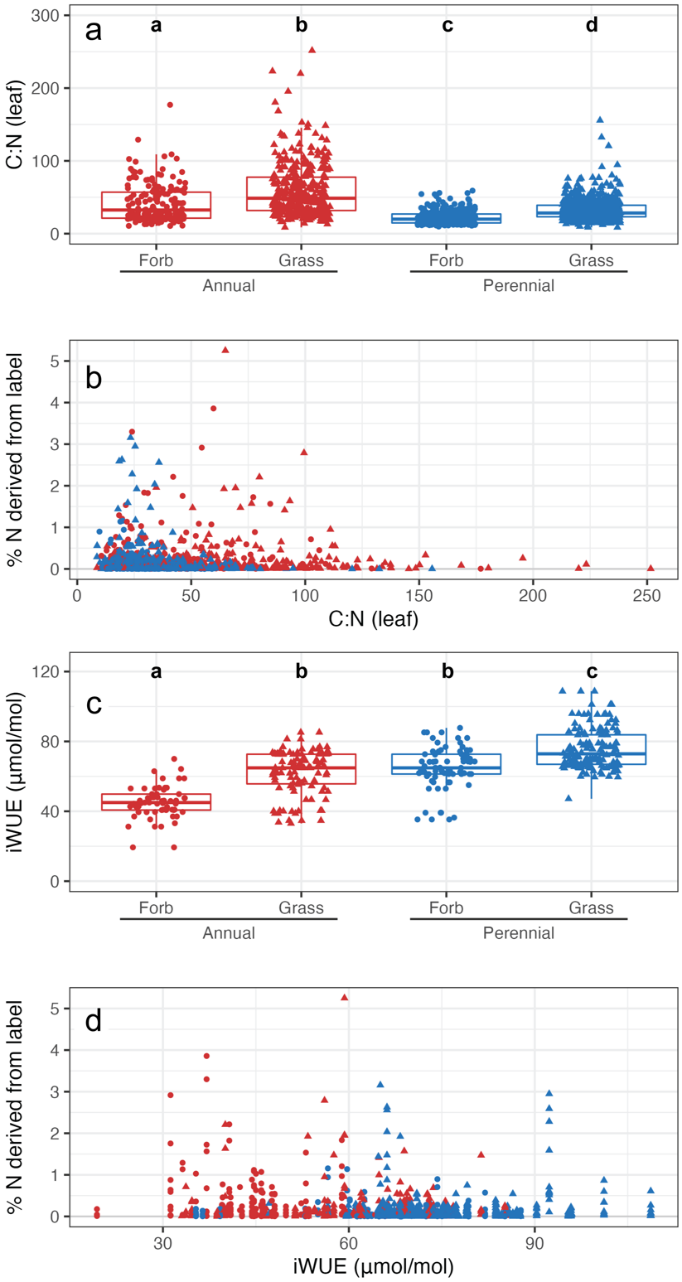


Figure 4.3: Stoichiometric and functional traits compared to nitrogen derived from transfer. A) Leaf C:N by annual/perennial. B) Percent ^{15}N derived from label (DFL) compared to C:N in leaves. C) iWUE by annual/perennial as measured before labelling. D) Percent ^{15}NDFL compared to iWUE in leaves at all time points. Boxplots show medians and interquartile ranges, lines show the largest (or smallest) value no further than 1.5x IQR from the hinge. Letters above boxplots indicate significant differences.

Fungal community composition demonstrated a high degree of connectivity between plants of different species but no obvious pattern of connectedness that could explain preferential nutrient transfer by plant functional groups. We found that 97.25% ± 8.01 (SD) of all plant roots within each experimental plot shared at least one fungal DNA sequence variant (ASV) with another plant of the same plot. Fungal community composition was similar across plant functional groups (Fig. 4.4, PERMANOVA pseudo-F statistic = 2.269, $R^2 = 0.005$, $p = 0.001$). Annual plants shared fungi with more plants in the same plot (4.74 plants ± 2.62 SD) compared to perennials (4.04 plants ± 2.49 SD ; t-test, $P = 0.019$; Appendix C: Table S4.5), but degrees of connectivity did not predict nitrogen transfer (Table 4.1). Seventy-three percent of plants were colonized by four or fewer fungi and shared fungi with five or fewer other plants in the plot, making it difficult to determine if strength of connectivity altered nitrogen transfer (Appendix C: Fig. S4.9).

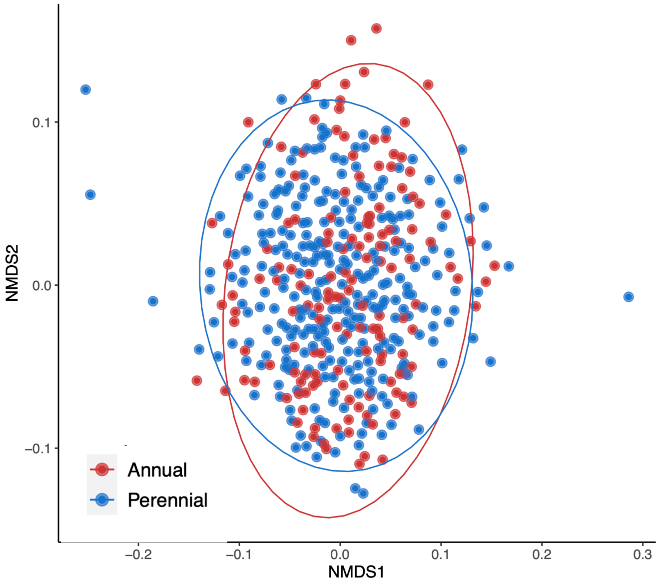


Figure 4.4. NMDS of fungal communities. Each point represents an individual plant ordinated by the Bray Curtis dissimilarities for AM fungal composition. PERMANOVA pseudo-F statistic = 2.269, $R^2 = 0.005$, $p = 0.001$.

Discussion

In a system where fungal DNA sequence variants were shared between ~97% of plants, we found that the transfer of nitrogen is regulated by plant functional traits that are known to influence resource use and allocation in plant communities. Using a recently proposed definition of CMNs that prioritizes ecological understanding (Rillig et al., 2024), our data suggest that the assimilation and allocation of limiting resources in CMNs was neither plant- nor fungi-centric. In our study, the rates and direction of resource transfer in our potential CMNs, inferred from pulse labeling and recovery of ^{15}N in leaves and rhizospheres, could be predicted from leaf C:N and distance from donor species (Table 4.1). Across all sites and treatments, we observed a stronger sink for ^{15}N in annual plants (Table 4.2), indicating preferential transfer of limiting resources to that functional group of plants. We applied the ^{15}N enriched label to perennial species in all plots, so this greater enrichment in annual plants precludes a preference for receiver plants of the same species as would be expected under the kinship hypothesis (Table 4.1). Although nearly all plants shared fungal ASVs in their roots (Fig. 4.4), connectivity did not predict ^{15}N transfer (Table 4.1). Our data suggest that rates and direction of resource transfer in CMNs reflect plant nutrient requirements and spatial proximity.

We conducted repeated spatiotemporal sampling of isotopic-enrichment levels at increasing distances from donor species, days to weeks after labelling, and in well-established communities exposed to multiple years of experimental treatments, expecting to find evidence of kinship (i.e., greatest resource transfer in plants of the same species), driven by CMN economics (i.e., ^{15}N transfer rates coinciding with ^{13}C investment in root and fungal mass). Annuals received, on average, an order of magnitude higher enrichment than perennials even though our donor plants were perennials. We also did not find evidence of a relationship between ^{15}N in leaves and ^{13}C in roots because we did detect ^{15}N-enrichment in leaves but no ^{13}C-enrichment in roots. We did, however, find significant ^{13}C-enrichment in the donor plants. Therefore, our data do not support either hypothesis, and instead suggest AM fungi form CMNs where the rates and direction of resource transfer ultimately reflects a sink-source strength effect, consistent with previous observations of stoichiometric source-sink manipulations of carbon and nitrogen within plants (Cai et al., 2021; Ruiz-Vera et al., 2017; Tegeder & Masclaux-Daubresse, 2018), but in our case observed at the community scale.

Nitrogen enrichment levels remained high in leaves and many roots at the end of the experiment, allowing us to measure NDFL across the community and infer the main predictors of N transfer. However, carbon enrichment levels faded before plants were harvested approximately 21 days post-labelling. After controlling for variation in assimilation rates by calculating NDFL, we found that annual plants received greater ^{15}N-enrichment than perennial plants. Plants closest to the donor were most enriched, and ^{15}N-enrichment decreased over time (Fig. S4, Fig. S5). Although the rainout shelters had limited effect, there were major differences across the latitudinal gradient represented by the sites in temperature and soil moisture availability (Dawson et al., 2022; Reed et al., 2019); however, neither treatment nor site affected our results.

The major predictors of differences in allocation of ^{13}C and ^{15}N to roots and subsequent transfer to "receiver" species were the intrinsic difference in plant functional types and correlated traits, including measured leaf C:N. Previous studies in northern California under environmental conditions similar to those found in our southernmost experimental site showed rapid (days to weeks) transfer of ^{15}N applied to the leaves of ectomycorrhizal pines to surrounding annual AM plant receivers (He et al., 2006). Those results demonstrated "direct fungal connections are not necessary for N transfer among plants" and, similar to our results, the "leaves of the annual plants had greater ^{15}N derived from source and were more enriched (^{15}N at % excess and δ^{15}N values) than perennial receivers, irrespective of the mycorrhizal type." Similarly, as proposed by He et al. (2006), our data suggest that annual plants were a strong sink for N which could be explained by stoichiometric gradients that affect root exudation and recapture of N-containing materials from rhizodeposition (Høgh-Jensen & Schjoerring, 2001; Mayer et al., 2003). Although our study included two species with symbiotic N fixation ability that could have altered some of the baseline data, even if a plot was fully dominated by legumes that difference would represent a minor effect relative to the pulse label application. Our data corroborate rapid transfer among AM plants, with no detectable enrichment in root or soil ^{13}C near roots 21 days post-labelling, but do not allow us to determine general mechanisms that are responsible for the ^{15}N transfers. Other methods of transfer–such as by fungi of other functional guilds, bacteria, or water flow in the soil–were possible given that plants shared a common growing medium in each plot.

We inferred a high connectivity between plants within each given treatment and site given the highly similar fungal composition in the root systems of both perennial and annual plants (Fig. 4.4). Given the constraints of ASV-identified data, we did this analysis on a strain-level scale, and it is possible that separate spores of the same ASV separately infected plants within the same plot. However, we are reasonably confident in our use of fungal ASVs as a proxy for connectivity given the strong overlap in our community and because individuals of one ASV can anastomose in the soil (Mikkelsen et al., 2008). This overlap could explain the lack of support for the kinship hypothesis in our dataset and offers further support for stoichiometric gradients in general, and C:N gradients in particular, as a principal control of terms of trade in

CMNs (Kiers et al., 2011). Because we observed such high rates of shared ASVs (~97%) and we observed high levels of N-enrichment in receivers at our first post-label sampling point four days after application, we could not test whether receivers connected to donors or whether plants were connected to the network affected ^{15}N transfer. We found unexpectedly low soil isotopic enrichment (Appendix C: Fig. S4.8) which suggested that the labels did not remain in the fungal network. This low level was likely driven by the fact that we did not sample soils until 21 days after labeling and hyphal turnover for grass-associated AM fungi can be less than one month (See et al., 2022). Because N is a major limiting nutrient in this system, according to the current paradigm of CMNs, it would be quickly taken up and recycled or transferred rather than accumulating in the soil.

We found that plant-soil stoichiometric gradients and functional traits were the strongest predictors of resource sharing in grassland CMNs. We interpret this finding as evidence of biochemical and biophysical sinks, in which nutrients are allocated to plants with the greatest need for those nutrients, either through a 'passive' mycorrhizal network as suggested by the high number of shared fungal ASVs or direct uptake from soil or water flow. Expanding on previous studies, we propose that AM fungi facilitate spatiotemporal dynamics of carbon and nitrogen transfer through CMNs in ways that are neither plant- nor fungi-centric. That is, plants and fungi that are located closer together in space and with stronger demand for resources over time are more likely to receive larger amounts of those limiting resources.

CHAPTER 5:

CONCLUSION

Leaf traits are an important metric for understanding how plants interact with their environment and for predicting how plants can influence processes such as carbon and water cycles. However, leaf traits have a complex relationship with the environment and scaling from trait to process is not straightforward. In this thesis, I addressed three influences on leaf trait variation: leaf ontogeny, environmental stress, and cross-kingdom interactions. Chapter 2 compares leaf traits for current and previous year growth in two evergreen dwarf shrubs (*Empetrum nigrum* and *Vaccinium vitis-idaea*). I found that the two cohorts had contrasting traits that could affect how results are interpreted and scaled from individual to landscape levels. Chapter 3 tests if experimental drought stress affects the relationship between morphological and functional traits. I found no difference in Pacific Northwest grassland herbaceous plants. Chapter 4 explores the transfer of carbon and nitrogen through a potential common mycorrhizal network in natural systems. I found a stoichiometric sink effect, where annual plants with lower leaf nitrogen content received a higher portion of the nitrogen tracer label compared to perennial plants with higher leaf nitrogen content. Overall, this thesis provides insight into how leaf trait variation affects plants' carbon and water relations in a changing environment.

APPENDIX A:

CHAPTER 2 SUPPLEMENTARY INFORMATION

Supplementary Figures

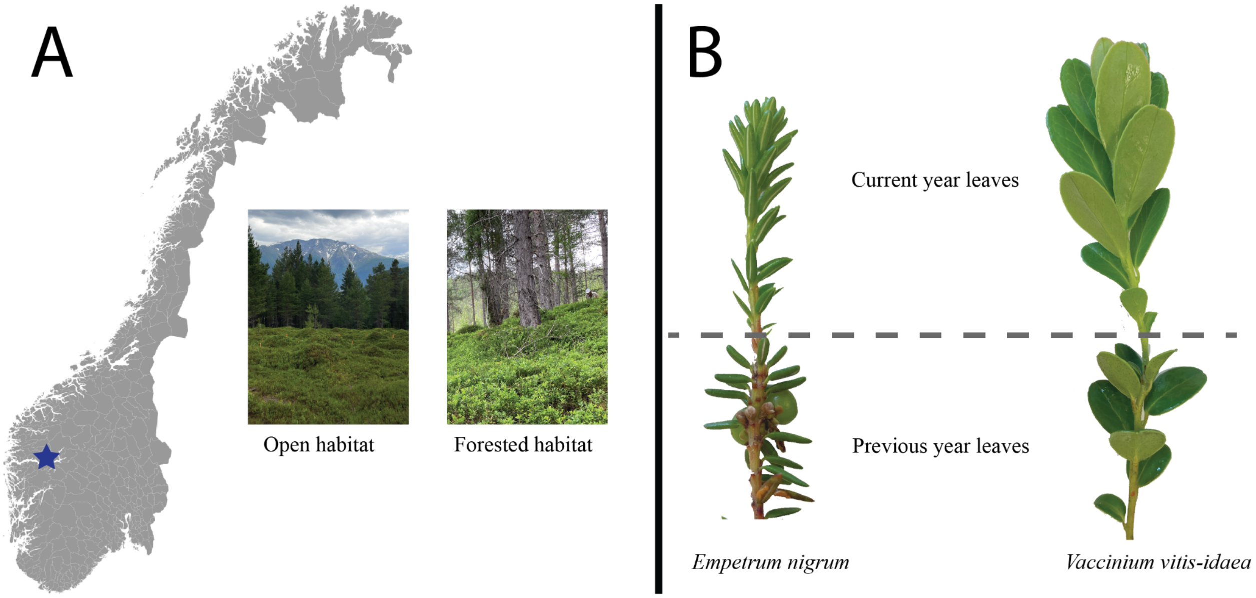
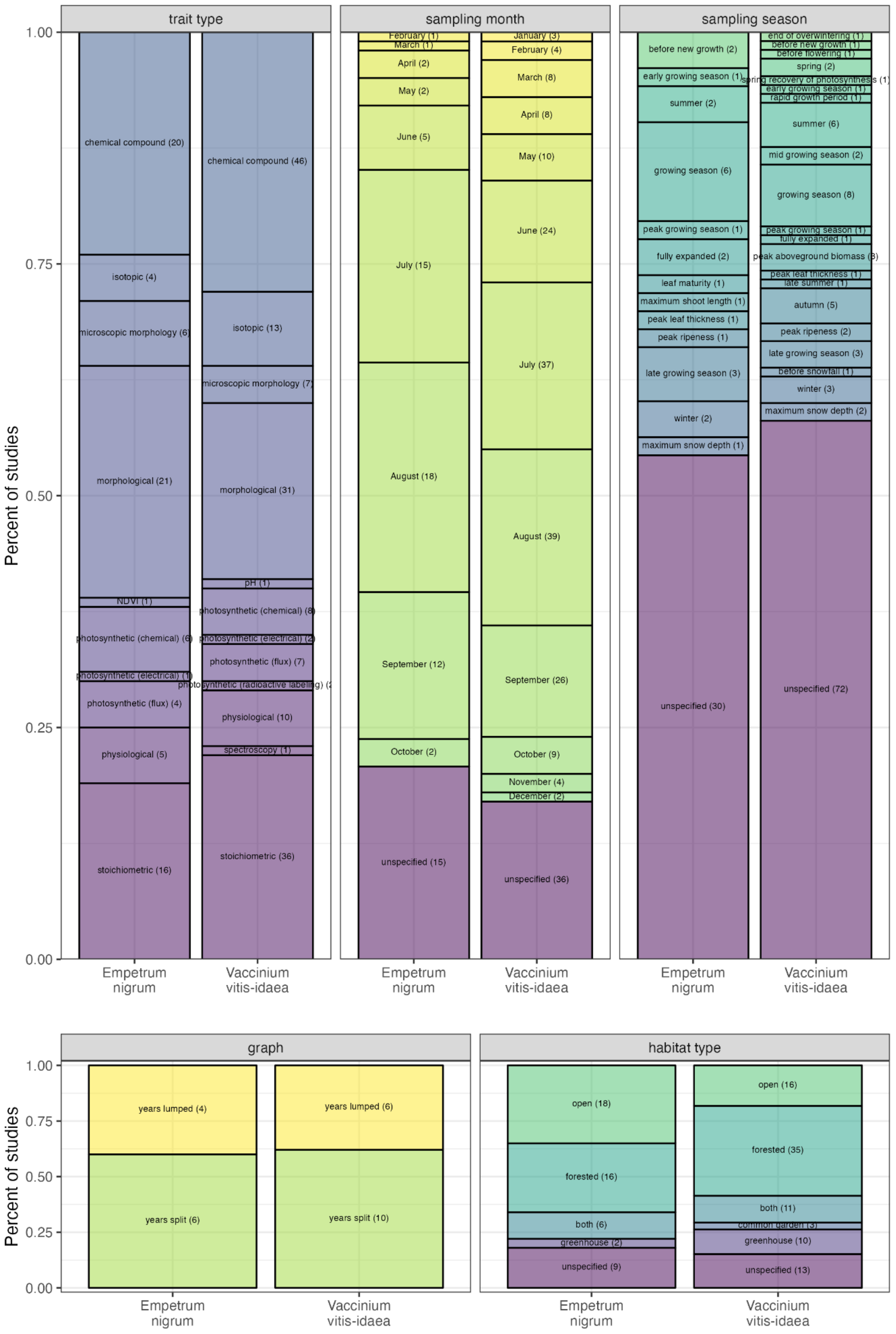


Figure S2.1: Experimental setup visuals including A) Location of the site in Sogndal and habitat photographs, and B) Current and previous year leaves of both species.

Figure S2.2 (next page): Additional variables from the systematic literature review. Values on bars indicate the number of studies for each category. Note that for some categories the numbers may total to more than the number of studies available if an individual study used more than one source or measured leaves more than once (e.g., one study measured leaves in every month of the year). Explanations of each variable are in Table S2.1.


trait type
sampling month
sampling season
graph
habitat type
Percent of studies
1.00
0.75
0.50
0.25
0.00
Empetrum nigrum
Vaccinium vitis-idaea
chemical compound (20)
isotopic (4)
microscopic morphology (6)
morphological (21)
NDVI (1)
photosynthetic (chemical) (6)
photosynthetic (electrical) (1)
photosynthetic (flux) (4)
physiological (5)
stoichiometric (16)
chemical compound (46)
isotopic (13)
microscopic morphology (7)
morphological (31)
pH (1)
photosynthetic (chemical) (8)
photosynthetic (electrical) (2)
photosynthetic (flux) (7)
photosynthetic (radioactive labeling) (2
physiological (10)
spectroscopy (1)
stoichiometric (36)
February (1)
March (1)
April (2)
May (2)
June (5)
July (15)
August (18)
September (12)
October (2)
unspecified (15)
January (3)
February (4)
March (8)
April (8)
May (10)
June (24)
July (37)
August (39)
September (26)
October (9)
November (4)
December (2)
unspecified (36)
before new growth (2)
early growing season (1)
summer (2)
growing season (6)
peak growing season (1)
fully expanded (2)
leaf maturity (1)
maximum shoot length (1)
peak leaf thickness (1)
peak ripeness (1)
late growing season (3)
winter (2)
maximum snow depth (1)
unspecified (30)
end of overwintering (1)
before new growth (1)
before flowering (1)
spring (2)
spring recovery of photosynthesis (1)
early growing season (1)
rapid growth period (1)
summer (6)
mid growing season (2)
growing season (8)
peak growing season (1)
fully expanded (1)
peak aboveground biomass (3)
peak leaf thickness (1)
late summer (1)
autumn (5)
peak ripeness (2)
late growing season (3)
before snowfall (1)
winter (3)
maximum snow depth (2)
unspecified (72)
years lumped (4)
years split (6)
years lumped (6)
years split (10)
open (18)
forested (16)
both (6)
greenhouse (2)
unspecified (9)
open (16)
forested (35)
both (11)
common garden (3)
greenhouse (10)
unspecified (13)

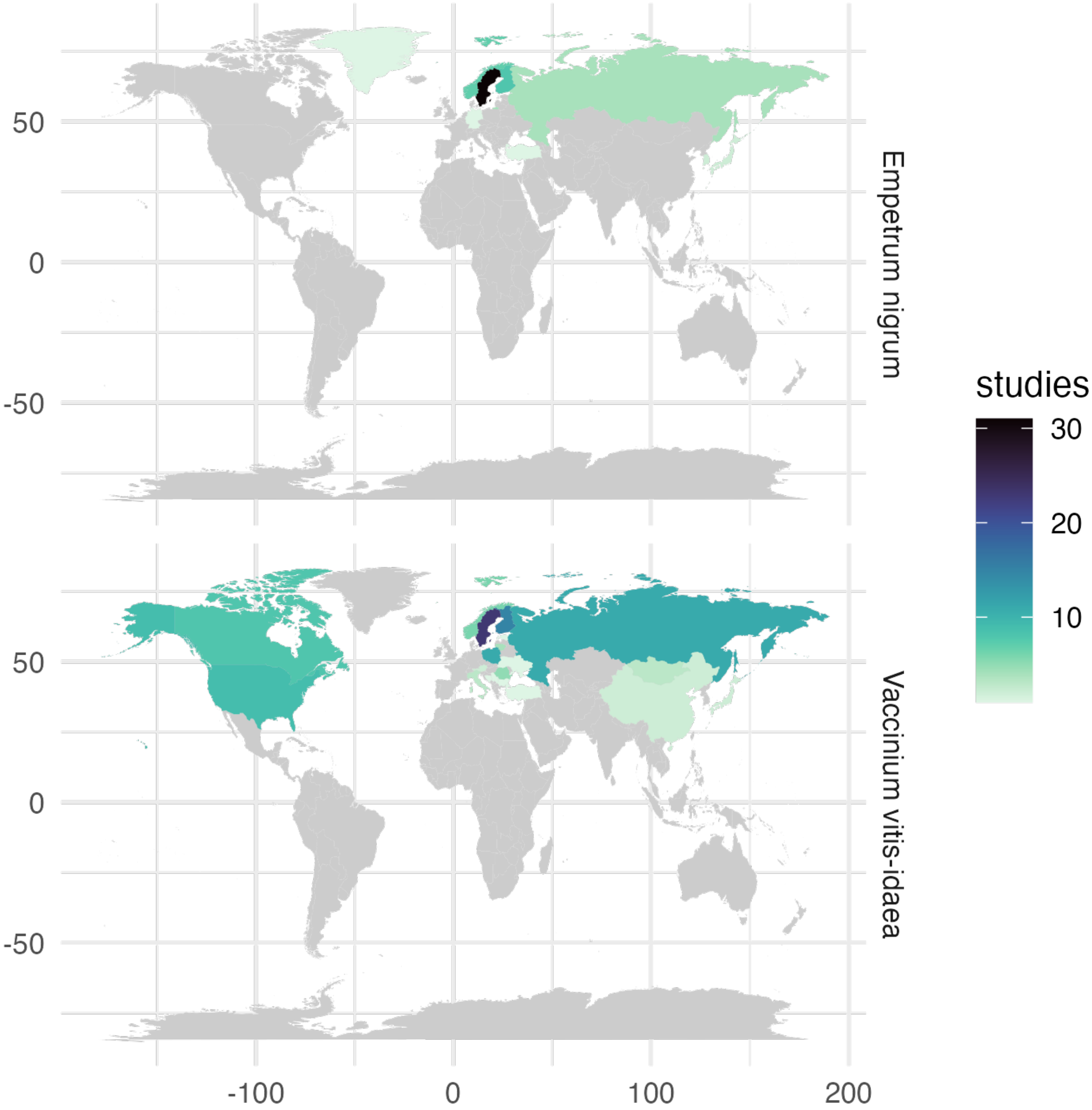


Figure S2.3: Location of studies in the systematic literature review. Location represents where plants were located, and leaves collected from to a country level. Not shown are seven studies of *V. vitis-idaea* that did not specify where they were performed.

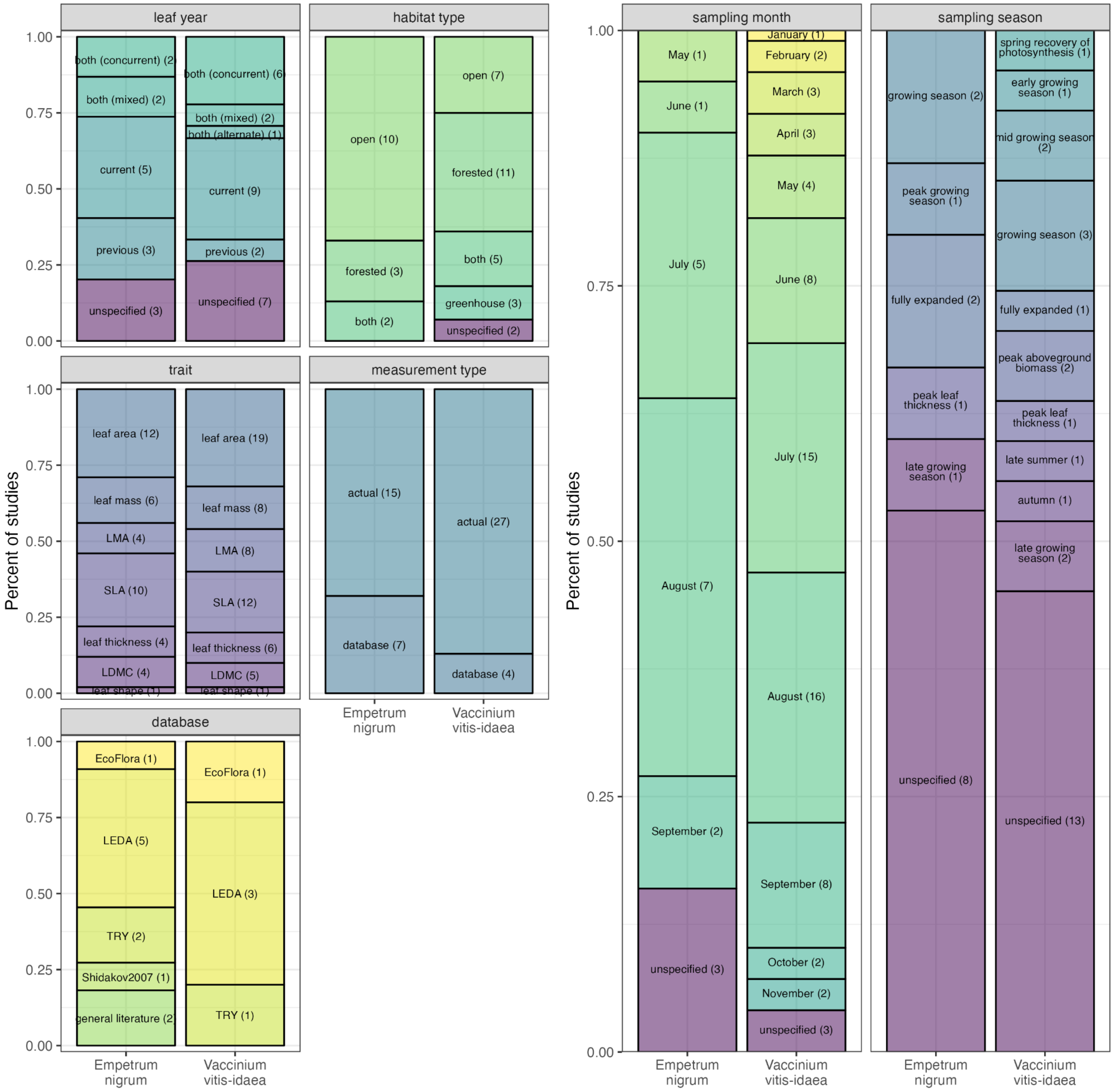


Figure S2.4: Comparison of how leaves are measured for morphological traits in the literature of *Empetrum nigrum* and *Vaccinium vitis-idaea*. Values on bars indicate the number of studies for each category. The data presented here are a subset of the data presented in Figs. 2.1, S2.1, and S2.2. Note that for some categories the numbers may total to more than the number of studies available if an individual study used more than one source or measured leaves more than once (e.g., one study measured leaves in every month between February and July). The panels for measurement type, trait, and database are the only panels with information about database-based papers. All others (as well as measurement type and trait) are based solely upon studies that measured traits.

Supplementary Tables

Table S2.1: Detailed code info for literature review tags and criteria

Variable	Value	Description	Multiple values?	Applies to…
database	[varies]	The name of the database authors used for their data	Y	Morphological trait studies that use database data
database source	multiple/single	Whether authors referenced more than one database	N	Morphological trait studies that use database data
extractable data	dataset	Whole dataset available through external link or SI	N (finest resolution reported)	Morphological trait studies
	means/median/points	Means, median, or individual points can be extracted from a table or figure in the manuscript for a trait of interest (LDMC, leaf area, leaf mass, LMA/SLA, dry mass)		
	no	No data can be extracted		
graph	years lumped	The study authors measured leaf cohorts separately, but all the graphs/tables show the data as a single category	N	Studies that measured cohorts concurrently
	years split	The study authors measured leaf cohorts separately and displayed the data separated by cohort in at least one graph/table		
habitat type	open	The study took place in the open (no tree cover described)	N	Morphological trait studies
	forested	Study took place in a forest (tree cover described)		
	both	Study included both open and forested locations		
	greenhouse	Study took place in a greenhouse		

Table S2.1: Detailed code info for literature review tags and criteria

Variable	Value	Description	Multiple values?	Applies to…
	unspecified	The study did not specify whether there was a tree cover		
justification	appearance	Leaf cohorts looked different	N	Studies that specified which leaf year/leaf cohort they used.
	both were present	Both cohorts were present, so the researchers separated them		
	data quality	Researchers were concerned that using more than one cohort would lower data quality		
	experimental constraints	The experiment relied on leaves of a certain cohort (usually young leaves developing)		
	leaves too young	Previous cohort was used because the current cohort was underdeveloped		
	missing other cohort	One cohort was preferred, but the other substituted when necessary		
	none	No reason was given or implied		
	only current leaves available	Previous year cohort wasn't present on the plants sampled		
	representation of the plant	Including both cohorts better represented what the plant was doing		
	standardization	Using just one cohort reduced the potential variation		
	testing physiology by age	The study focused on the difference between cohorts		
leaf year	both (alternate)	One cohort was preferred, but the other substituted when necessary	N	All included studies

Table S2.1: Detailed code info for literature review tags and criteria

Variable	Value	Description	Multiple values?	Applies to…
	both (concurrent)	Both cohorts were measured but separately		
	both (mixed)	Both cohorts were measured without differentiating		
	current	Leaves from the current year's growing season were measured		
	previous	Leaves from the previous years' growing season were measured		
	unspecified	Study did not say which cohort they measured		
location	[various countries]	The country where the study was located, as reported	Y	All included studies
	greenhouse	The study was conducted in a greenhouse. The country where the greenhouse was located is also reported.		
	unspecified	The authors did not report where they conducted the study		
measurement type	actual	The study measured leaves	N	Definitely included for morphological trait studies. Actual measurements are reported for other trait types as well, but databases were not necessarily included for non-trait studies.
	database	The study pulled values from a database		
sampling month	[various months]	All the months that leaves were collected and measured.	Y	All included studies
sampling season	spring/summer/ autumn/winter	As reported by the study authors for their location.	Y	All included studies
	before flowering	Before the study species flowered		

Table S2.1: Detailed code info for literature review tags and criteria

Variable	Value	Description	Multiple values?	Applies to…
	before new growth	Before the study species began growing the current year leaf cohort		
	before snowfall	Before snow fell		
	early growing season	As stated by authors. See growing season.		
	end of overwintering	When the overwintering period was ending		
	fully expanded	When the current year's leaf cohort was fully expanded		
	growing season	As stated by authors. When the plant was actively putting on biomass.		
	late growing season	As stated by authors. See growing season.		
	late summer	As stated by authors.		
	leaf maturity	When the authors determined leaves were fully mature.		
	maximum aboveground biomass	When aboveground biomass had fully accumulated for the season.		
	maximum shoot length	When the current year's shoot was fully extended.		
	maximum snow depth	When snow was at its deepest expected for winter.		
	mid growing season	As stated by authors. See growing season.		
	peak growing season	As stated by authors. See growing season.		

Table S2.1: Detailed code info for literature review tags and criteria

Variable	Value	Description	Multiple values?	Applies to…
	peak leaf thickness	When the authors determined leaves were fully bulked out		
	peak ripeness	When the most fruit was ripe		
	rapid growth period	While the plants were rapidly expanding		
	spring recovery of photosynthesis	When photosynthesis was back to full strength		
	unspecified	No season was specified or implied		
trait	LDMC	Leaf dry matter content (dry mass divided by wet mass).	Y	Morphological trait studies
	leaf area	The area of a discrete leaf/leaves.		
	leaf mass	The mass of a discrete leaf/leaves		
	leaf shape	The silhouette of a leaf		
	leaf thickness	The thickness of a leaf		
	LMA	Leaf mass per area.		
	SLA	Specific leaf area (leaf area divided by mass)		
trait type	chemical compound	Metric of how much of a chemical compound was in a leaf. Examples: phenolics, cellulose. Excludes chlorophyll (see photosynthetic (chemical)).	Y	All included studies
	isotopic	Isotopic content within a leaf. Could be radioactive or stable.		

Table S2.1: Detailed code info for literature review tags and criteria

Variable	Value	Description	Multiple values?	Applies to…
	microscopic morphology	Morphological measurements that require a microscope. Not included in morphological unless macro traits were also measured.		
	morphological	Measurements of a leaf's shape and dimensions		
	NDVI	Normalized Differentiation Vegetation Index. Only included if measured on the leaf scale.		
	pH	Leaf pH		
	photosynthetic (chemical)	A measure of photosynthetic efficiency using chemistry, including chlorophyll content.		
	photosynthetic (electrical)	A measure of photosynthetic efficiency using changes in electrical charges.		
	photosynthetic (flux)	A measure of the photosynthetic efficiency using an IRGA or other carbon flux metric.		
	photosynthetic (radioactive labeling)	A measure of photosynthetic efficiency using a radioactive label.		
	physiological	Any physiological metric not otherwise mentioned. Usually freeze tolerance.		
	spectroscopy	Spectral signature of a leaf.		
	stoichiometric	Measure of element concentration within a leaf (usually C, N, or P).		

Table S2.2: List of literature sources of morphological trait data.

Lead Author	Year	metric	trait	leaf_age	Vaccinium vitis-idaea	Empetrum nigrum
Johanson	(1995)	mean	dry_mass_g	current	1	1
			leaf_area	current	1	0
Gragalia	(1997)	mean	leaf_area	current	1	1
				previous	1	1
			SLA	current	1	1
				previous	1	1
Suzuki	(1997)	mean	SLA	current	5	1
Kudo	(1999)	mean	SLA	previous	3	4
Gerdol	(2000)	mean	SLA	previous	6	0
			SLA	current	5	0
Bruun	(2005)	mean	SLA	unspecified	1	1
Kolari	(2006)	mean	SLA	unspecified	1	0
Sundqvist	(2011)	mean	LDMC	unspecified	6	6
			SLA	unspecified	6	6
Fletcher	(2012)	mean	SLA	unspecified	0	6
Jagerbrand	(2012)	mean	leaf_area	previous	1	1
Kumordzi	(2014)	mean	LDMC	current	1	0
			SLA	current	1	0
Palmroth	(2014)	raw	SLA	previous	27	0
			SLA	current	27	0

Table S2.2: List of literature sources of morphological trait data.

Lead Author	Year	metric	trait	leaf_age	Vaccinium vitis-idaea	Empetrum nigrum
			SLA	unspecified	138	0
Solanki	(2019)	mean	leaf_area	current	3	0
				previous	3	0
			SLA	current	3	0
				previous	3	0
Iturrate-Garcia	(2020)	raw	leaf_area	unspecified	90	0
			SLA	unspecified	90	0
Ritz	(2020)	raw	SLA	current	10	12
Bjedov	(2021)	mean	leaf_area	both	4	0
Eckert	(2021)	raw	SLA	both	27	0
Wakui	(2021)	raw	dry_mass_g	current	645	0
			leaf_area	current	645	0
			SLA	current	645	0
Rajewicz	(2023)	raw	SLA	previous	30	0
Standen	(2024)	raw	SLA	unspecified	23	0

Table S2.3: Comparison of data available in three multi-national databases, the literature, and collected in this study.

	DURIN		**LEDA**		**TRY**		**TTT**		**Literature**		***Total***
trait	***E. nigrum***	***V. vitis-idaea***	***E. nigrum***	***V. vitis-idaea***	***E. nigrum***	***V. vitis-idaea***	***E. nigrum***	***V. vitis-idaea***	***E. nigrum***	***V. vitis-idaea***	
Leaf area	180	179	8	30	16	0	230	274	3	749	1668
SLA	178	179	8	10	54	187	457	334	32	1053	2492
LDMC	177	179	6	6	59	143	241	246	6	7	1070
Dry mass	178	179	3	15	4	21	0	0	1	646	1047
Total	713	716	25	61	133	351	928	854	42	2454	6277

APPENDIX B:

CHAPTER 3 SUPPLEMENTARY MATERIAL

Supplementary Figures

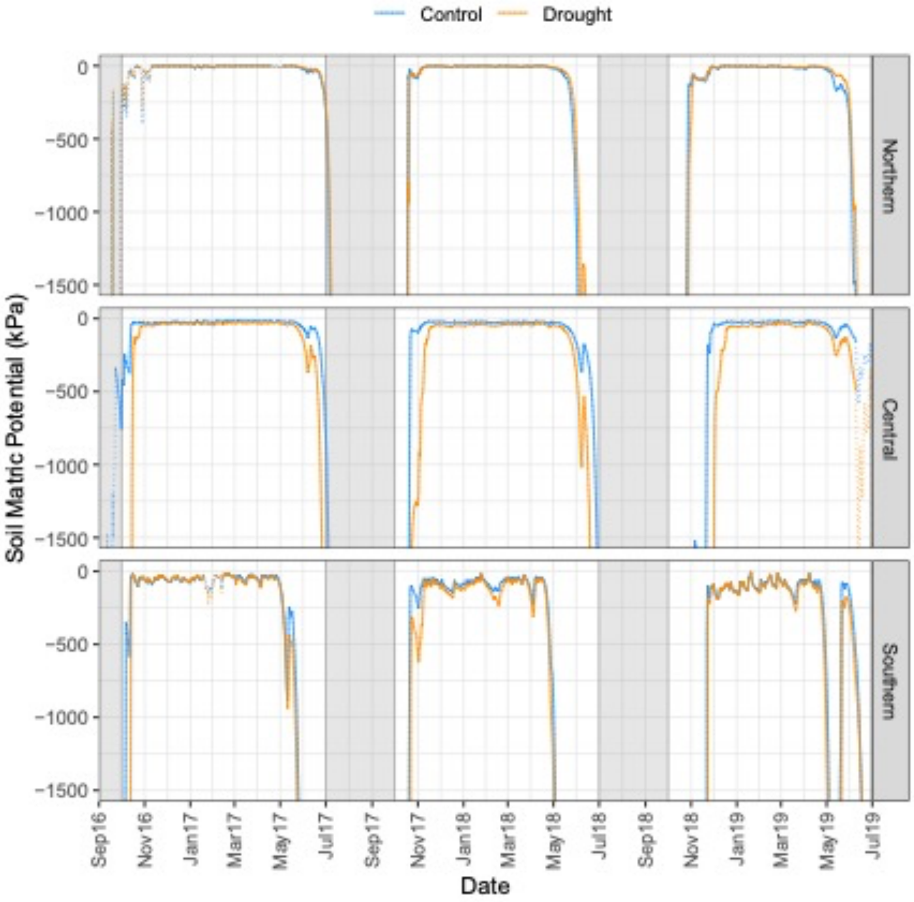


Fig. S2.1. Daily variation in effect of rain exclusion treatment on soil matric potential (SMP) at each site.

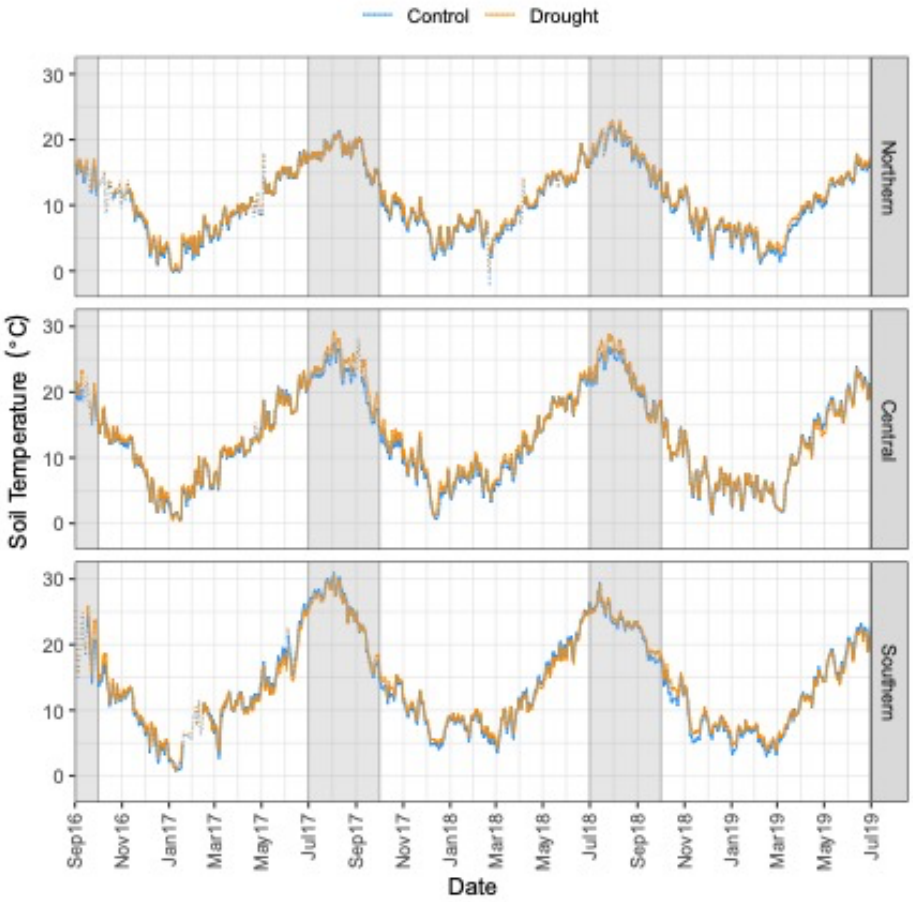


Fig. S2.2 Daily variation in effects of rain exclusion treatment on soil temperature at each site.

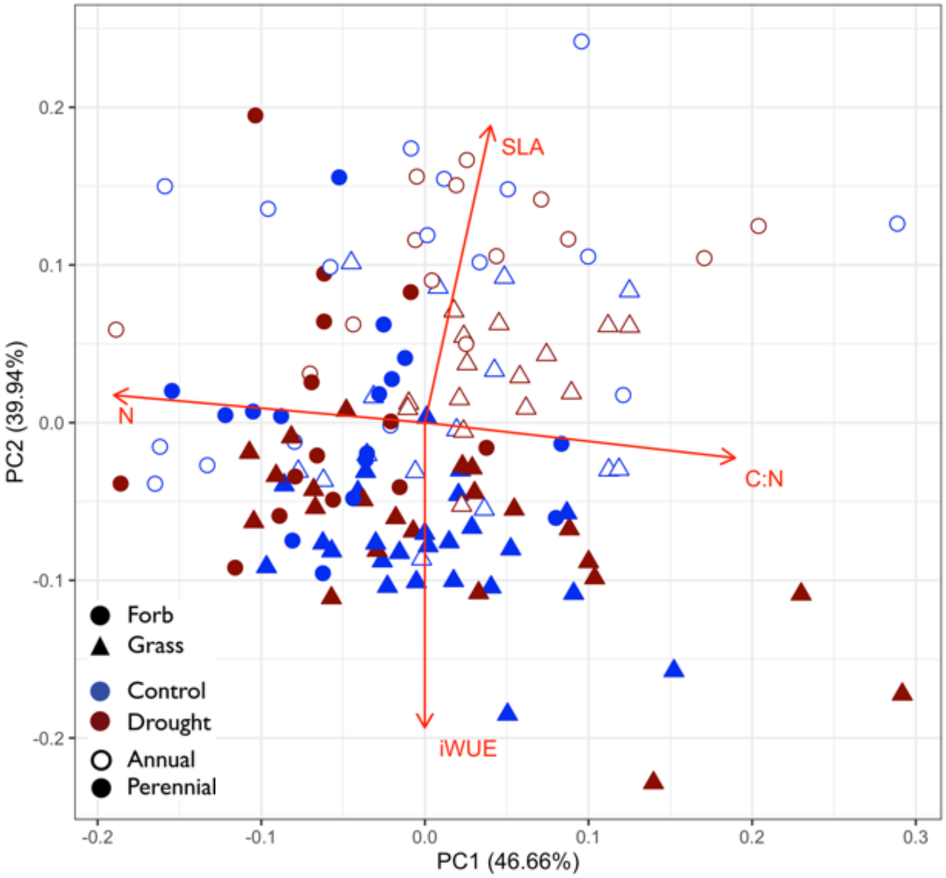


Fig. S2.3 Principal component analysis of specific leaf area (SLA) and three functional traits. SLA has a nearly orthogonal relationship with N content (N), intrinsic water-use efficiency (iWUE), and carbon to nitrogen ratio (C:N). Life history strategy (annual, perennial) is divided along PC2 while functional group (grass, forb) is divided along PC1. Model explained 86.6% of variation.

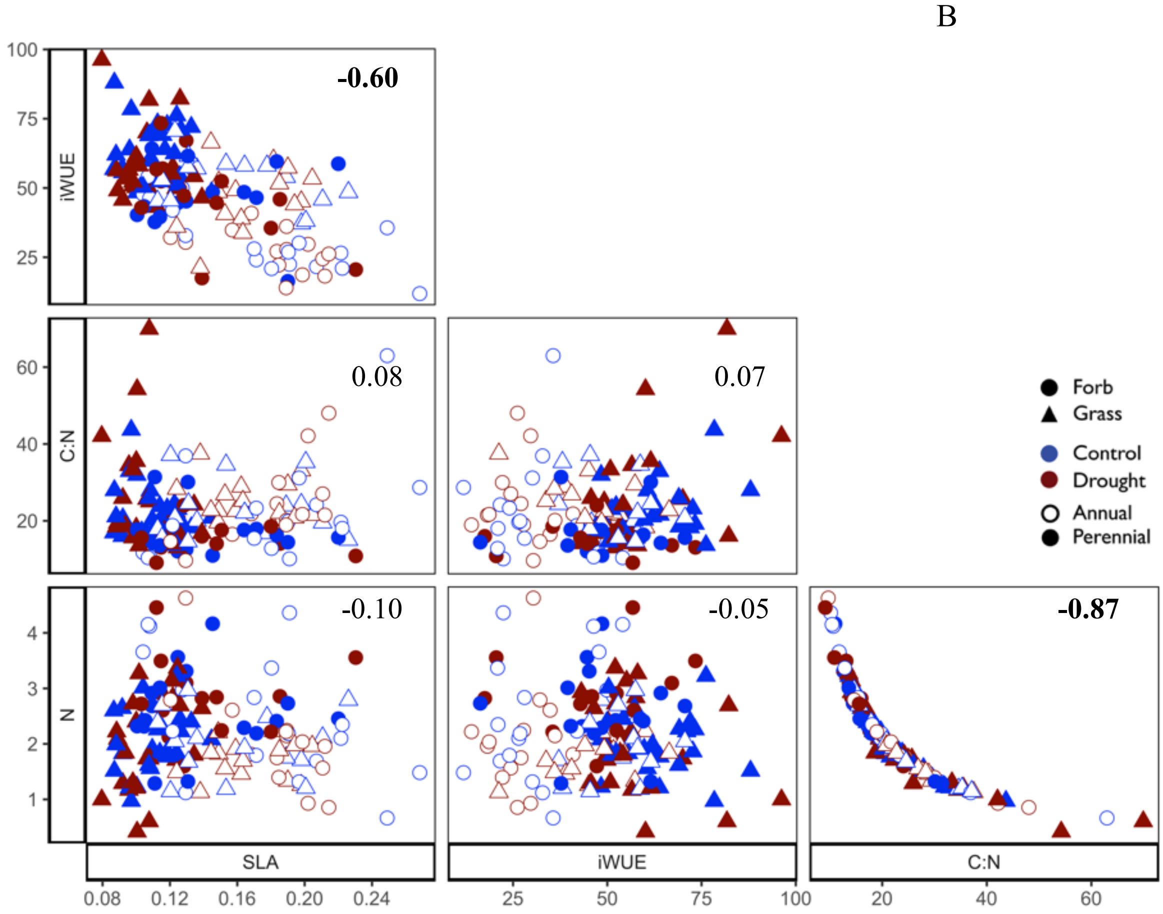


Fig. S2.4 Correlation between the four traits measured including specific leaf area (SLA), intrinsic water-use efficiency (iWUE), carbon to nitrogen ratio (C:N), and nitrogen (N). Each point represents a single species in each plot. Values shown are R^2, significance indicated in bold. Plot made with GGally package in R (Schloerke et al. 2020).

Supplementary Tables

Table S2.1. Complete list of species included in this study, replicates of each species, and plant strategies.

Notes: Nutrient-use strategy data derived from [1]Chaudhary et al. (2016), [2]Wang and Qui (2006), and [3]Dickie et al. (2013). * indicates “to genus,” that strategy was extrapolated from a sister species given a lack of data on this species. Nutrient-use strategies include arbuscular mycorrhizal (AM) and nitrogen fixation (N-fixer). Totals are given followed by the number of specific leaf area (SLA) replicates in parentheses.

Species name	**Family**	**Life strategy**	**Functional group**	**Total**	**Number sampled (SLA replicates)**			**Nutrient-use strategy**
					Northern	*Central*	*Southern*	
Agrostis capillaris L.	Poaceae	Perennial	Grass	9 (16)	0	9 (16)	0	AM[1]
Alopecurus pratensis L.	Poaceae	Perennial	Grass	19 (50)	19 (50)	0	0	AM[2]
Bromus diandrus Roth	Poaceae	Annual	Grass	9 (26)	0	9 (26)	0	AM[1]*
Bromus hordeaceus L.	Poaceae	Annual	Grass	21 (50)	5 (15)	4 (12)	12 (23)	AM[1]*
Daucus carota L.	Apiaceae	Annual	Forb	2 (6)	0	0	2 (6)	AM[1]
Elymus repens (L.) Gould	Poaceae	Perennial	Grass	10 (31)	0	0	10 (31)	AM[1]*
Geranium dissectum L.	Malvaceae	Annual	Forb	13 (36)	10 (28)	3 (8)	0	AM[2]
Lotus corniculatus L.	Fabaceae	Perennial	Forb	3 (9)	3 (9)	0	0	AM, N-fixer[1]
Schedonorus arundinaceus (Schreb.) Dumort.	Poaceae	Perennial	Grass	10 (23)	0	0	10 (23)	AM[1]
Sidalcea malviflora (DC.) A. Gray ex Benth.	Malvaceae	Perennial	Forb	27 (81)	10 (30)	8 (23)	9 (28)	AM[3]
Trifolium subterraneum L.	Fabaceae	Annual	Forb	11 (34)	0	9 (31)	2 (3)	AM, N-fixer[1]
Vicia sativa L.	Fabaceae	Annual	Forb	5 (15)	5 (15)	0	0	AM, N-fixer[1]*
Totals				**139 (368) 12 species**	**52 (138) 6 species**	**42 (116) 6 species**	**45 (114) 6 species**	

Table S2.2 Distribution of functional groups and life history strategies across sites.

	Annual		Perennial	
Site	**Grass**	**Forb**	**Grass**	**Forb**
Northern	1	2	1	2
Central	2	2	1	1
Southern	1	2	2	1

Note: Counts represent individual species from each group sampled at each site.

Table S2.3 AICc values used in intrinsic water-use efficiency (iWUE) model selection.

	Model	**K**	**AICc**	**ΔAICc**	**Model Likelihood**	**AICc weights**	**Log likelihood**	**Cumulative AIC weights**
Model 4	SLA+MPD+C:N+N+ life history strategy	11	1086.406	0.000	1.000	0.948	-531.164	0.948
Model 2	SLA+MPD	8	1092.723	6.317	0.042	0.040	-537.808	0.988
Model 3	SLA+C:N+N+MPD	10	1096.007	9.601	0.008	0.008	-537.144	0.996
Model 5	MPD+life history strategy	8	1097.425	11.019	0.004	0.004	-540.159	1.000
Model 7	SLA+C:N+N+life history strategy	10	1109.732	23.326	0.000	0.000	-544.007	1.000
Model 1	SLA	7	1111.642	25.236	0.000	0.000	-548.394	1.000
Model 6	SLA+C:N+N	9	1112.294	25.888	0.000	0.000	-546.449	1.000

Note: All models include plot as random effect and rain exclusion treatment and site as a fixed effect. Models were selected using biologically likely hypotheses. Variables include specific leaf area (SLA), mean phylogenetic distance (MPD), C:N, N content, and life history strategy (annual, perennial).

Table S2.4 Statistical difference of rain exclusion treatment on soil matric potential.

Year	**Predictor**	***F***	***P***
2017	Site	179.79	**< 0.001**
	Rain excl. trt	3.98	*0.057*
	Site x Rain excl. trt	2.70	*0.087*
2018	Site	146.40	**< 0.001**
	Rain excl. trt	5.79	**0.024**
	Site x Rain excl. trt	2.16	0.137
2019	Site	91.59	**< 0.001**
	Rain excl. trt	4.12	*0.054*
	Site x Rain excl. trt	4.42	**0.023**

Notes: Significance tested with an ANOVA. Growing season is defined as 1 Oct to 30 Jun and each season is labeled by the spring months of the growing year (e.g. 2018 is 1 Oct 2017 to 30 Jun 2018).

Table S2.5 Statistical difference of rain exclusion treatment on soil temperature.

Year	**Predictor**	***F***	***P***
2017	Site	216.97	**< 0.001**
	Rain excl. trt	4.58	**0.043**
	Site x Rain excl. trt	0.91	0.417
2018	Site	230.89	**< 0.001**
	Rain excl. trt	15.94	**0.001**
	Site x Rain excl. trt	1.52	0.239
2019	Site	168.30	**< 0.001**
	Rain excl. trt	5.45	**0.028**
	Site x Rain excl. trt	3.32	*0.053*

Notes: Significance tested with an ANOVA. Growing season is defined as 1 Oct to 30 Jun and each season is labeled by the spring months of the growing year (e.g. 2018 is 1 Oct 2017 to 30 Jun 2018).

Table S2.6 Summary statistics of effects of rain exclusion treatment on soil matric potential (SMP).

Year	Site	Treatment	N	T-ratio	*P*	mean SMP	SD
2017	Southern	Control	5	0.702	0.490	-90.803	47.501
		Drought	5			-114.127	68.523
	Central	Control	5	2.975	**0.007**	-27.233	1.863
		Drought	5			-54.831	4.762
	Northern	Control	5	-0.220	0.828	-4.057	1.953
		Drought	5			-3.554	0.409
2018	Southern	Control	5	1.163	0.256	-237.932	111.111
		Drought	5			-356.978	205.041
	Central	Control	5	2.958	**0.007**	-44.947	6.258
		Drought	5			-97.448	14.419
	Northern	Control	5	0.045	0.964	-11.435	5.276
		Drought	5			-10.864	2.540
2019	Southern	Control	5	0.662	0.514	-312.293	189.753
		Drought	5			-393.405	245.943
	Central	Control	5	3.481	**0.002**	-66.543	13.957
		Drought	5			-174.131	31.206
	Northern	Control	5	-0.629	0.535	-24.686	8.689
		Drought	5			-20.215	6.247

Notes: Growing season is defined as 1 Oct to 30 Jun and each season is labeled by the spring months of the growing year (e.g. 2018 is 1 Oct 2017 to 30 Jun 2018). T-ratios and P-values are for a Tukey post hoc test on the ANOVA results presented in Table S4.

Table S2.7. Summary statistics of effects of rain exclusion treatment on soil temperature.

Year	Site	Treatment	N	T-ratio	*P*	Mean soil temp	Sd
2017	Southern	Control	5	-0.37	0.714	10.974	0.271
		Drought	5			11.033	0.248
	Central	Control	5	-1.08	0.293	10.787	0.251
		Drought	5			10.958	0.373
	Northern	Control	5	-2.26	**0.033**	8.736	0.113
		Drought	5			9.094	0.168
2018	Southern	Control	5	-0.97	0.344	11.615	0.263
		Drought	5			11.779	0.219
	Central	Control	5	-3.39	**0.002**	10.683	0.099
		Drought	5			11.261	0.505
	Northern	Control	5	-2.55	**0.017**	8.968	0.104
		Drought	5			9.402	0.206
2019	Southern	Control	5	-1.56	0.131	11.018	0.233
		Drought	5			11.306	0.260
	Central	Control	5	0.57	0.573	10.583	0.239
		Drought	5			10.478	0.448
	Northern	Control	5	-3.05	**0.005**	8.570	0.279
		Drought	5			9.132	0.226

Notes: Growing season is defined as 1 Oct to 30 Jun and each season is labeled by the spring months of the growing year (e.g. 2018 is 1 Oct 2017 to 30 Jun 2018). T-ratios and P-values are for a Tukey post hoc test on the ANOVA results presented in Table S5.

Table S2.8. Statistical significance of each factor influencing intrinsic water-use efficiency (iWUE) model shown in Fig. 4.

Predictor	χ^2	*P*
Fixed effects		
Specific leaf area	13.869	**<0.001**
Mean phylogenetic distance	28.212	**<0.001**
C:N	2.726	*0.099*
N	1.435	0.231
Life history strategy (annual/perennial)	12.491	**<0.001**
Treatment	4.938	**0.026**
Site	2.745	0.254
Random effect		
Plot		1

Note: Significance tested with a Type II ANOVA.

APPENDIX C:

CHAPTER 4 SUPPLEMENTARY MATERIAL

Supplementary Figures

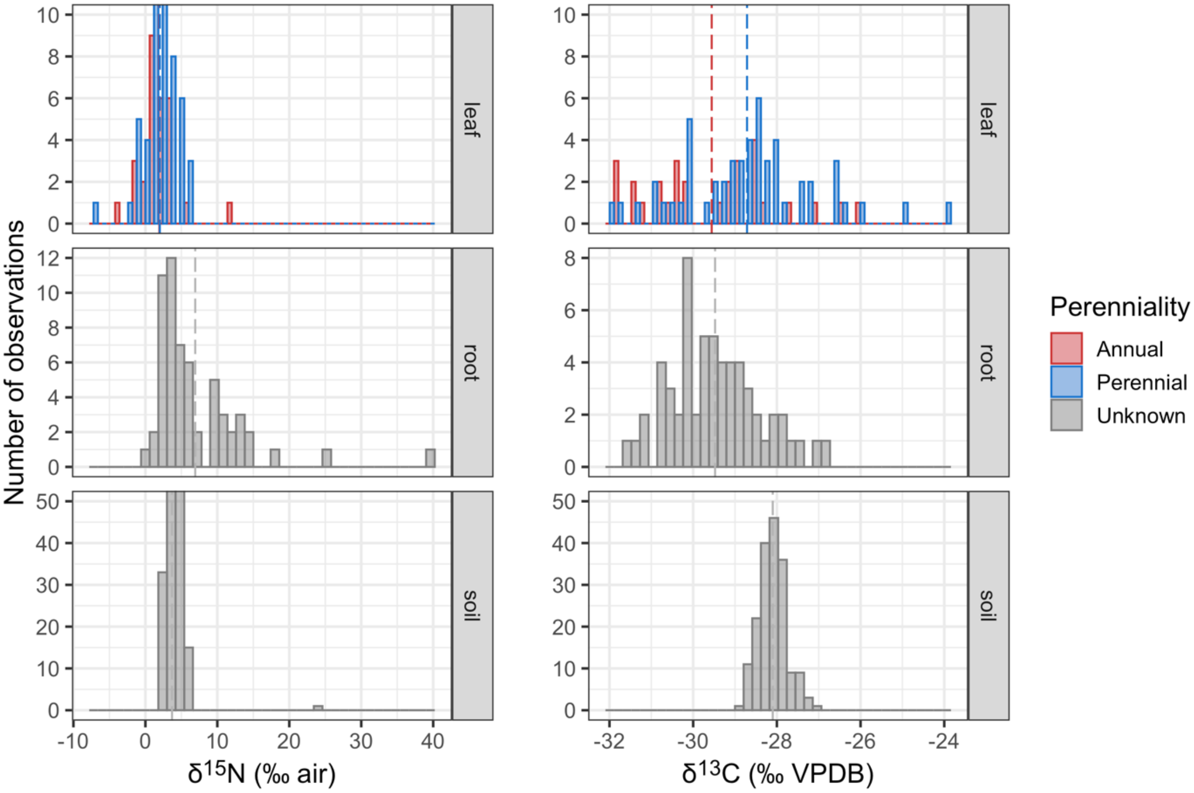


Figure S4.1. Natural abundance of stable isotopes in leaves, roots, and soil before labelling. Dashed lines indicate mean value. Exact values used to calculate derivation from label (Eq. 1 – Eq. 4) are available in Table S3.

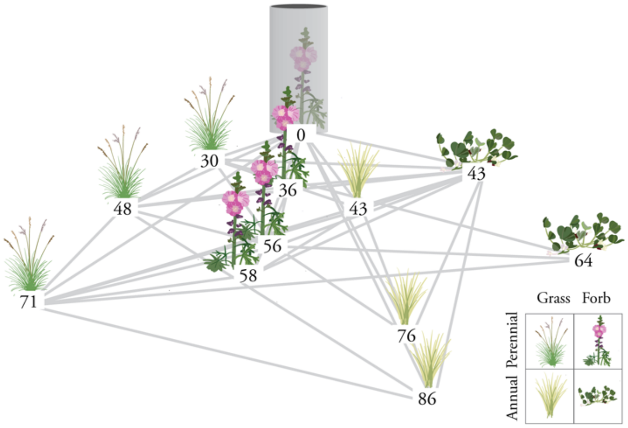


Figure S4.2. Example of how networks were constructed for each plot. The grey cylinder indicates the donor plant for the plot. Numbers beneath the receivers are the distance (in centimeters) from the donor. Degrees were calculated as how many plants each individual plant was connected to by shared fungal ASVs; for example, the perennial grass at 71 cm has 7 degrees. We visualized individual plot networks in CytoScape.

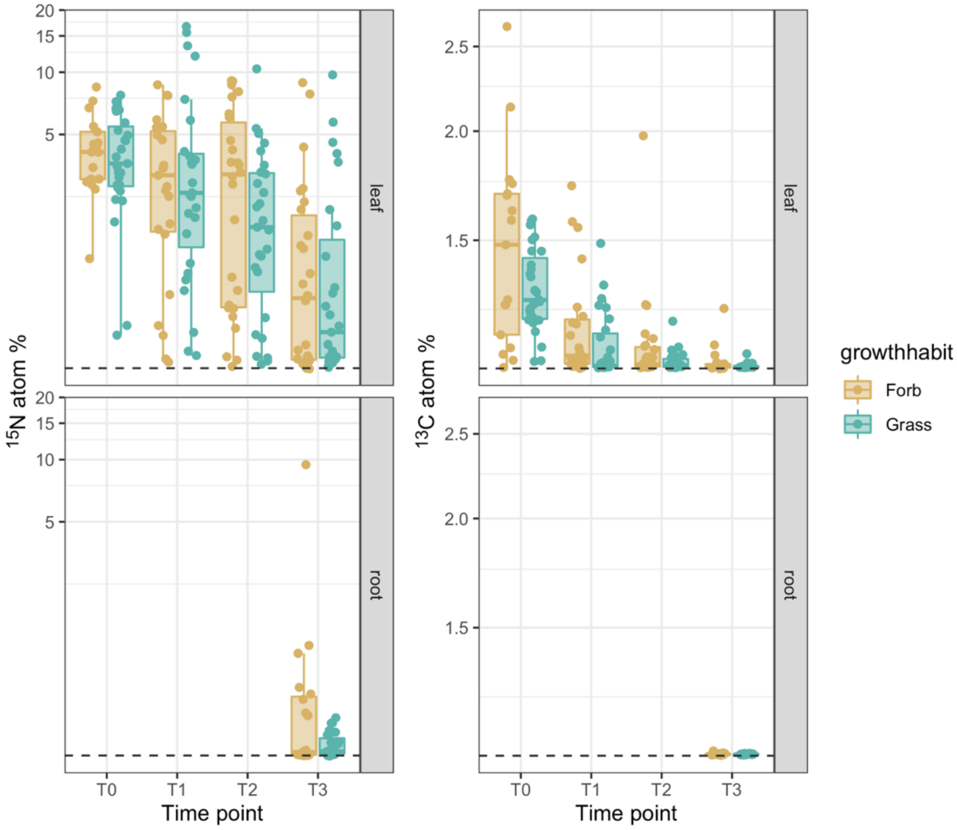


Figure S4.3. Enrichment decreases over time in labelled plants. Time points roughly correspond with T0 = time of labelling, T1 = 4 days later, T2 = 10 days later, T3 = 21 days later. Y-axis is in log10 scale.

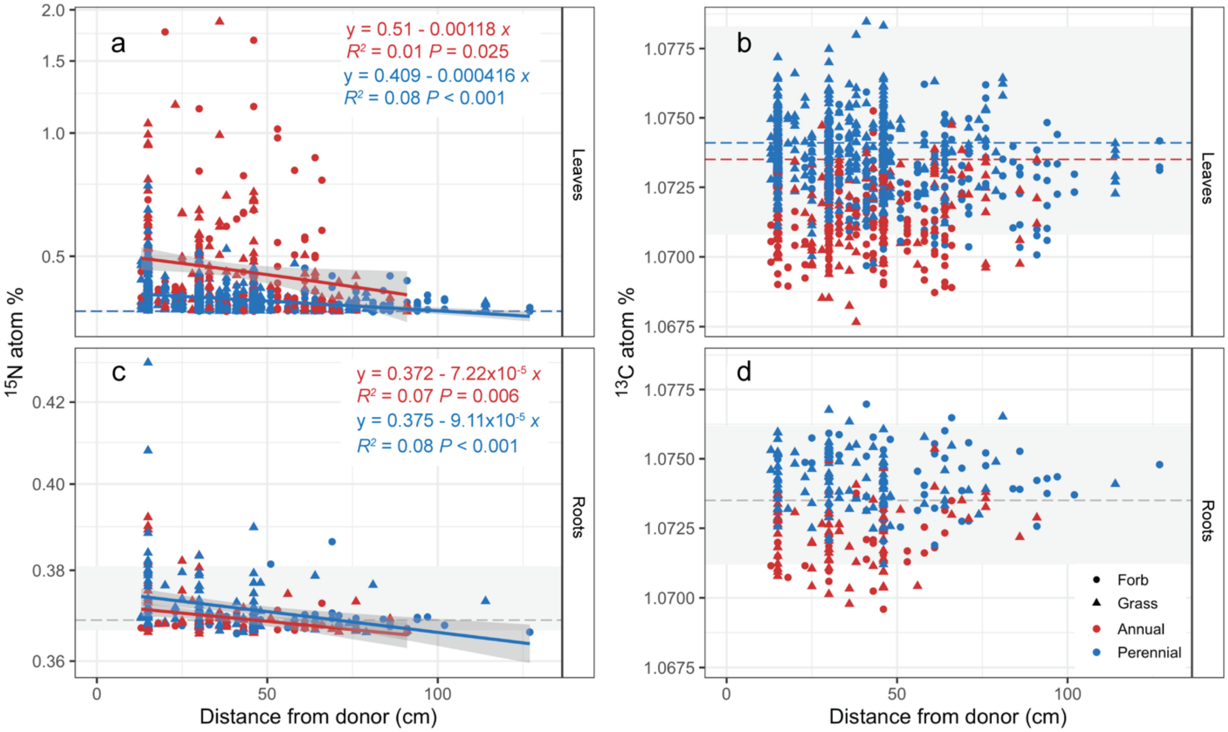


Figure S4.4. Decreased receiver enrichment with distance from donor in leaves and roots. Dashed lines indicate natural abundance means; grey boxes indicate range of natural abundance variation shown in Fig. 2. There is no systematic enrichment of ^{13}C; however, there is high ^{15}N enrichment in leaves. Y-axes are $\log_{10}$ scale. Note that the y-axis scales are different between ^{15}N leaves and ^{15}N roots. See Fig. 2 for boxplots of enrichment data by grass/forb and annual/perennial.

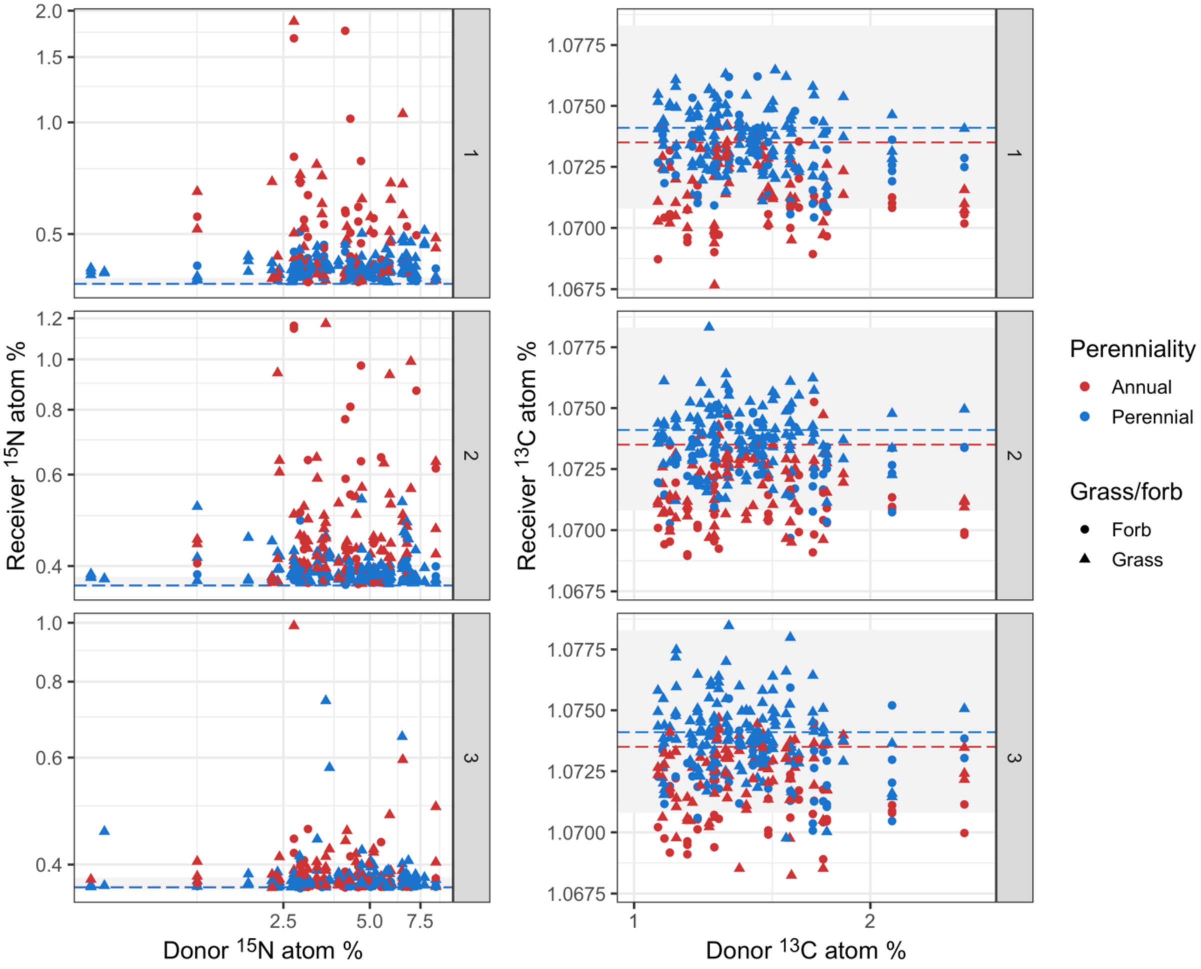


Figure S4.5. Receiver enrichment did not correlate with initial donor enrichment. Dashed lines indicate natural abundance means; grey boxes indicate range of natural abundance variation shown in Figure S1. (Grey boxes are too small to be noticeable for ^{15}N.) ^{15}N axes are log10 scaled. Note there is a sizeable difference between X- and Y-axes of both elements. Time point facets are time point 1 (~4 days post label), time point 2 (~10 days post label), and time point 3 (~21 days post label). Donors are all sampled at time point 0 (immediately after labelling).

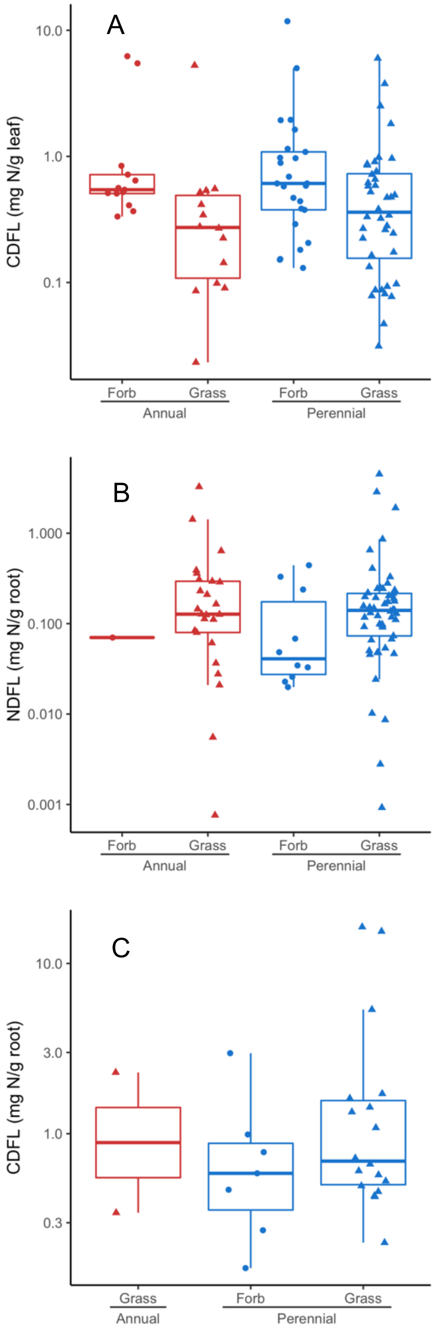


Figure S4.6: Enrichment derived from label for A) CDFL in leaves, B) NDFL in roots, and C) CDFL in roots. Points represent enriched individual receiver plants with associated DNA data at one time point of sampling. In A all three post-enrichment time points are shown; in B and C only time point 3 is shown. Y-axis is log10 scale. See Fig. S** for values over distance and Fig. 4 for NDFL in leaves.

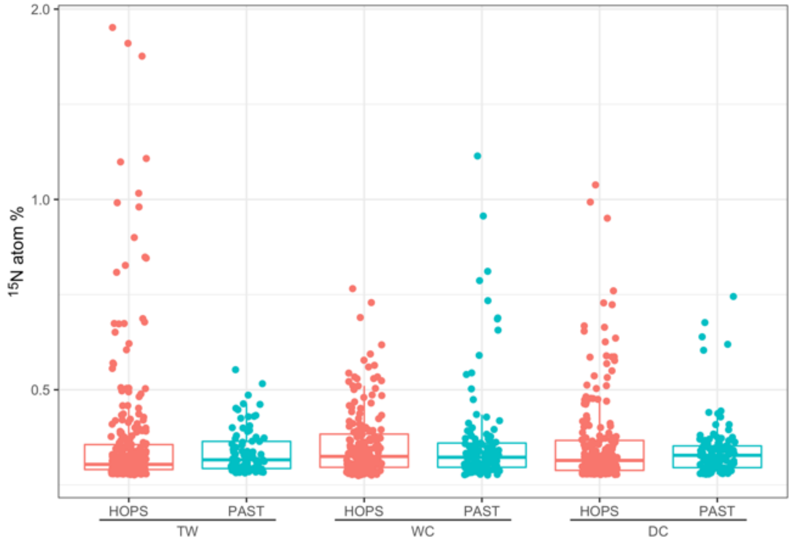


Figure S4.7. No trend in ^{15}N enrichment across site and diversity treatment. Y-axis is in log10 scale. HOPS indicates restored prairie sites, PAST indicates pasture sites. Sites are ordered from north (TW) to central (WC) to south (DC).

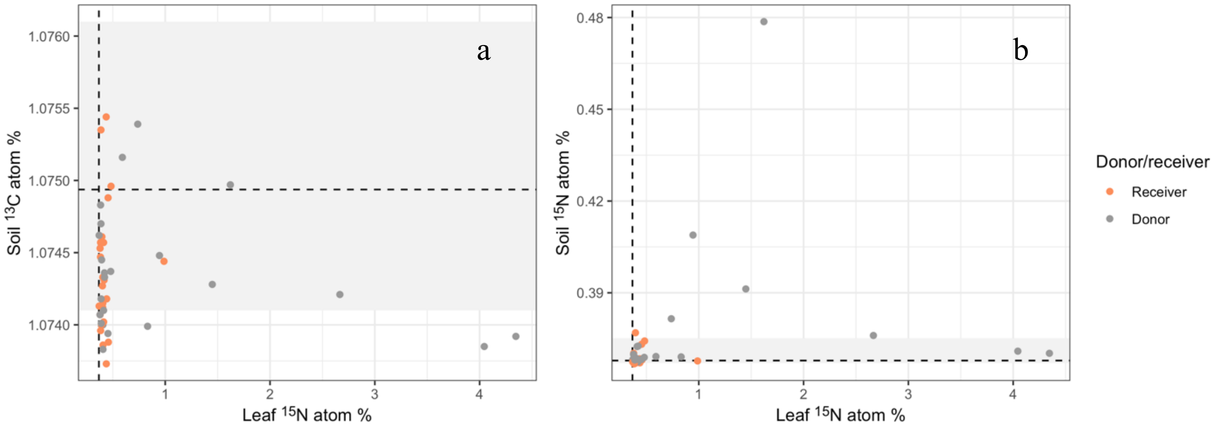


Figure S4.8. Limited soil enrichment 21 days after labelling. Dashed lines indicate average baseline enrichment. Grey boxes indicate range of baseline enrichment. Samples are subset to individuals most highly enriched with foliar ^{15}N at each site by restored prairie/introduced pasture as well as their associated donors.

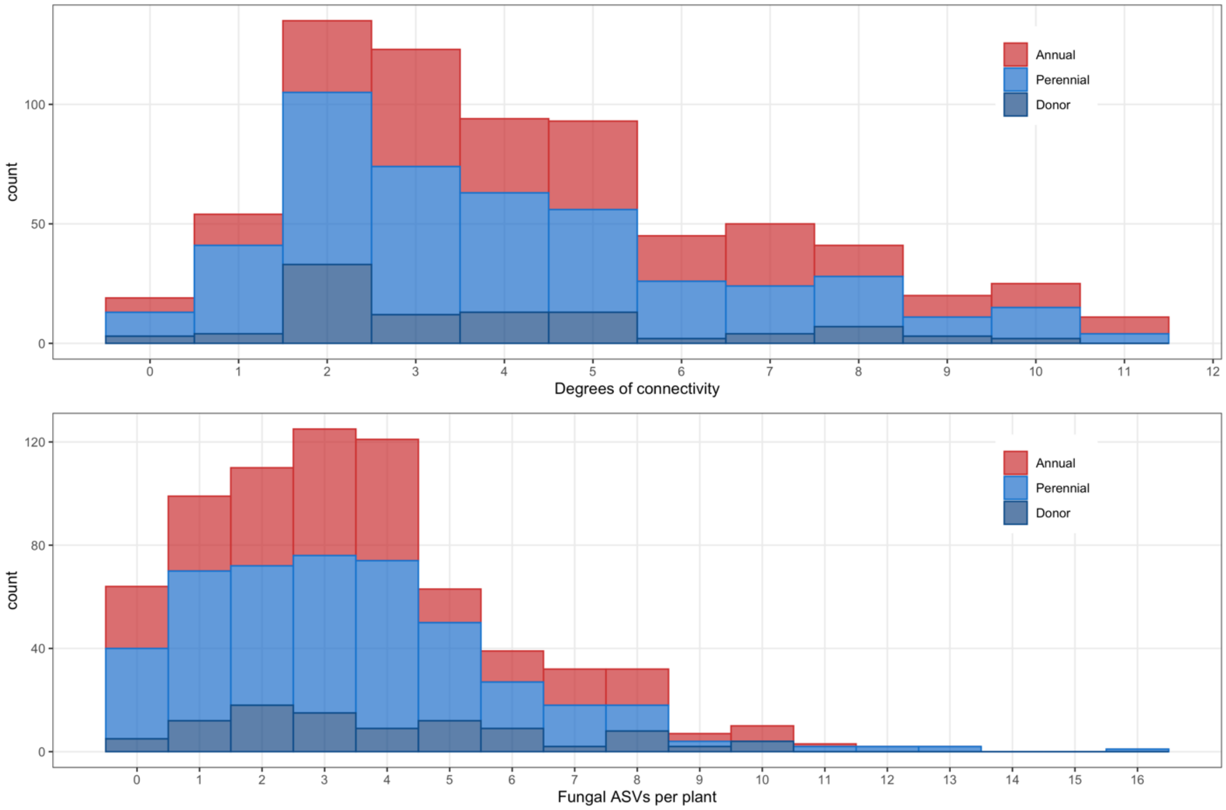


Figure S4.9. Histogram of connectivity metrics showing left skew. Degrees of connectivity indicate how many plants an individual shared at least one fungal ASV with in the same plot. Data are from time point 1 (approximately four days after labeling). Donors are the plants that we applied the isotopic label to.

Supplementary Tables

Table S1. List of species at each site with number of leaf samples per species. Donor species are indicated in bold.

			Site			
Species	**Annual/ perennial**	**Grass/forb**	**Northern**	**Central**	**Southern**	**Fungal symbiont**
***Agrostis capillaris* L.**	Perennial	Grass	121	--	--	AM[1]
***Alopecurus pratensis* L.**	Perennial	Grass	--	210	--	AM[1]
Aphanes occidentalis L.	Annual	Forb	3	--	--	AM*[2]
Bromus diandrus Roth	Annual	Grass	49	--	--	AM*[1]
Bromus hordeaceus L.	Annual	Grass	35	42	96	AM*[1]
Elymus repens (L.) Gould	Perennial	Grass	--	--	63	AM*[1]
Eriophyllum lanatum (Pursh) Forbes	Perennial	Forb	8	--	--	AM[2]
Festuca idahoensis Elmer ssp. *roemeri*	Perennial	Grass	18	--	--	AM*[1]

Table S1. List of species at each site with number of leaf samples per species. Donor species are indicated in bold.

			Site			
Species	**Annual/ perennial**	**Grass/forb**	**Northern**	**Central**	**Southern**	**Fungal symbiont**
(Pavlick) S. Aiken						
Geranium dissectum L.	Annual	Forb	15	81	--	AM[2]
Holcus lanatus L.	Perennial	Grass	--	6	--	AM[1]
Koeleria macrantha (Ledeb.) Schult.	Perennial	Grass	18	--	--	AM[2]
Lotus corniculatus L.	Perennial	Forb	--	9	--	AM[1]
***Schedonorus arundinaceus* (Schreb.) Dumort.**	Perennial	Grass	--	--	130	AM[1]
***Sidalcea malviflora* ssp. *virgata* (DC.) A. Gray ex Benth.**	Perennial	Forb	66	129	119	AM[3]
Trifolium subterraneum L.	Annual	Forb	48	--	--	AM[1]
Veronica arvensis L.	Annual	Forb	3	--	--	AM[1]
Vicia sativa L.	Annual	Forb	--	14	--	AM*[1]
Vulpia spp.	Annual	Grass	54	8	21	AM[1]
		Total samples	438	499	429	

Notes: Fungal symbiont data derived from [1]Chaudhary et al. (2016), [2]Soudzilovskaia et al. (2020), and [3]Dickie et al. (2013). * indicates "to genus," that strategy was extrapolated from a sister species given a lack of data on this species. Nutrient-use strategies include arbuscular mycorrhizal (AM) and nitrogen fixation (N-fixer).

Table S2. Collection dates for each time point at each site. Days since labeling given in parentheses after date.

Site	**Label application (time point 0)**	**Time point 1**	**Time point 2**	**Time point 3**
Ideal days since labeling	*0*	*4*	*10*	*21*
Northern	June 10	June 14 (4)	June 20 (10)	July 2 (22)
Middle	May 29	June 3 (5)	June 11 (13)	June 18 (20)
Southern	May 9	May 13 (4)	May 22 (9)	May 30 (21)

Table S3. Baseline values used to calculate amount derived from label (DFL).

		Roots						**Leaves**			
Site	*Drought treatment*	*N atm%*	*±SD*	*C atm%*	*±SD*	*Annual/perennial*	*Grass/forb*	*N atm%*	*±SD*	*C atm%*	*±SD*
North	Control	0.370	0.001	1.073	0.001	Annual	Forb	0.367	0.001	1.07	0.001
							Grass	0.367	0.000	1.071	0.003
						Perennial	Forb	0.367	0.000	1.072	0.001
							Grass	0.368	0.000	1.075	0.001
	Rain exclusion treatment	0.370	0.001	1.073	0.001	Annual	Forb	0.367	0.001	1.072	0.001
							Grass	0.367	0.000	1.073	0.000
						Perennial	Forb	0.367	0.000	1.072	0.002
							Grass	0.368	0.000	1.075	0.001
Central	Control	0.368	0.001	1.073	0.001	Annual	Forb	0.367	0.001	1.071	0.001
							Grass	0.366	0.001	1.073	0.001
						Perennial	Forb	0.368	0.003	1.071	0.001
							Grass	0.367	0.003	1.074	0.002
	Rain exclusion treatment	0.368	0.001	1.073	0.001	Annual	Forb	0.367	0.001	1.071	0.001
							Grass	0.367	0.001	1.072	0.001
						Perennial	Forb	0.366	0.000	1.071	0.001
							Grass	0.366	0.000	1.075	0.002
South	Control	0.368	0.000	1.074	0.001	Annual	Forb	0.366	0.000	1.071	0.000
							Grass	0.368	0.001	1.074	0.001
						Perennial	Forb	0.367	0.000	1.074	0.001
							Grass	0.367	0.000	1.074	0.000
	Rain exclusion treatment	0.368	0.001	1.074	0.001	Annual	Forb	0.366	0.000	1.071	0.000
							Grass	0.367	0.001	1.074	0.002
						Perennial	Forb	0.367	0.001	1.074	0.001
							Grass	0.367	0.000	1.074	0.001

Table S4. Mixed-effects ANOVA results effects on leaf carbon derived from label (%CDFL).

	F-statistic	DF	P-value
Annual/perennial	22.74	1	**<0.001**
Grass/forb	12.43	1	**0.001**
Same species as donor	11.64	1	**0.001**
Degree of connectivity	1.74	1	0.192
iWUE	0.17	1	0.678
C:N	2.28	1	0.136
Site	3.16	2	0.064
Drought treatment	1.27	1	0.274
Restoration treatment	2.79	1	0.107
Distance from donor	0.00	1	0.958
Time from labelling	1.37	1	0.246
Annual/perennial: Grass/forb interaction	NA	0	NA

Notes: Random effect is plot nested within site. Only receiver leaves enriched with ^{13}C with associated DNA data were included in the analysis (n = 92). Note that since 13C enrichment was limited, not all treatments were replicated when filtered to only enriched leaves and the results here should be treated with caution. Leaf %NDFL results (which are more robust as most leaves were enriched with 15N) are available in Table 1.

Table S5. List of fungal ASVs by annuals and perennials.

Fungal taxon	No. of perennial plants assoc. with	No. of annual plants assoc. with
Acaulospora sp877	13	0
Claroideoglomus sp744	20	7
Claroideoglomus sp745	18	5
Claroideoglomus sp746	18	7
Claroideoglomus sp749	14	4
Claroideoglomus sp751	25	10
Claroideoglomus sp757	11	1
Claroideoglomus sp758	11	1
Claroideoglomus sp781	18	13
Claroideoglomus sp782	59	36
Claroideoglomus sp783	38	20
Claroideoglomus sp784	47	21
Claroideoglomus sp811	19	9
Claroideoglomus sp812	12	4
Claroideoglomus sp813	15	10
Claroideoglomus sp815	21	11
Claroideoglomus sp816	19	7
Claroideoglomus sp817	30	8
Glomus sp1024	11	1
Glomus sp1025	12	4
Glomus sp1029	23	4
Glomus sp1031	20	7
Glomus sp104	12	3
Glomus sp113	23	10
Glomus sp114	6	16
Glomus sp115	21	15
Glomus sp1349	13	8
Glomus sp1351	18	8

Table S5. List of fungal ASVs by annuals and perennials.

Fungal taxon	No. of perennial plants assoc. with	No. of annual plants assoc. with
Glomus sp1352	15	5
Glomus sp148	22	11
Glomus sp149	12	6
Glomus sp150	27	14
Glomus sp168	21	12
Glomus sp169	13	6
Glomus sp170	15	6
Glomus sp177	11	1
Glomus sp191	14	10
Glomus sp470	15	6
Glomus sp472	14	4
Glomus sp488	13	2
Glomus sp539	13	3
Glomus sp541	19	3
Glomus sp565	15	7
Glomus sp566	12	1
Glomus sp612	13	3
Glomus sp614	11	6
Glomus sp648	12	2
Glomus sp651	25	10
Glomus sp661	11	4
Glomus sp662	14	3
Glomus sp663	12	8
Glomus sp667	13	1
Glomus sp668	14	3
Glomus sp669	14	5
Glomus sp670	20	2
Glomus sp692	14	1
Glomus sp700	11	2

Table S5. List of fungal ASVs by annuals and perennials.

Fungal taxon	**No. of perennial plants assoc. with**	**No. of annual plants assoc. with**
Glomus sp701	12	12
Glomus sp703	19	13
Glomus sp83	11	0
Glomus sp85	12	3
Glomus sp904	18	3
Glomus sp905	11	2
Glomus sp906	17	6
Glomus sp917	29	12
Glomus sp918	85	48
Glomus sp919	45	32
Glomus sp92	11	0
Glomus sp920	53	33
Glomus sp921	17	3
Glomus sp938	14	8
Glomus sp986	12	1
Paraglomus sp301	19	4
Paraglomus sp302	26	5
Paraglomus sp303	15	4
Paraglomus sp304	25	5
Scutellospora sp1080	12	6
Unknown sp21	12	4

Notes: Table only includes fungal species associated with at least 10 plants.

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Appendix B

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