**Target Journals:**

**Title**:Leaf nitrogen content is driven by the unit cost of nitrogen and water use in Texan grasslands

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**Abstract**

Terrestrial biosphere models commonly predict leaf photosynthesis based on positive relationships between soil nitrogen, leaf nitrogen, and photosynthetic capacity. While positive empirical relationships between soil nitrogen and leaf nitrogen are common, recent work leveraging photosynthetic least-cost theory indicates that interactions between aboveground climate and soil nitrogen may be a more reliable predictor of leaf nitrogen allocation than soil nitrogen alone. Specifically, the theory predicts that increasing aridity will increase leaf nitrogen allocation, which will allow individuals to maintain photosynthesis at lower water use. It also predicts that soil nitrogen availability should increase the positive effect of aridity on leaf nitrogen allocation and water-use efficiency. However, few direct tests of this theory exist, and the timescale that plants respond to is unknown. To test the theory, we measured leaf nitrogen and water-use efficiency at 25 sites scattered across a precipitation and soil nitrogen availability gradient in Texan grasslands. We found that soil nitrogen availability increased the positive effect of aridity on leaf nitrogen per leaf mass, but only in C3 graminoid and herbaceous species. We also observed that soil nitrogen availability increased the positive effect of aridity on water use efficiency, a pattern also only observed in C3 graminoid and herbaceous species. These results support patterns expected from photosynthetic least-cost theory and support a mechanism explaining increased leaf nitrogen allocation in arid or dryland systems.

**Introduction**

Terrestrial biosphere models, which comprise the land surface component of Earth system models, are sensitive to the formulation of photosynthetic processes (). This is because photosynthesis is the largest carbon flux between the atmosphere and terrestrial biosphere, and is constrained by ecosystem carbon and nutrient biogeochemical cycles (Hungate *et al.*, 2003; LeBauer & Treseder, 2008; IPCC, 2013; Fay *et al.*, 2015). Many terrestrial biosphere models formulate photosynthesis by parameterizing photosynthetic capacity within plant functional types through positive relationships between soil nitrogen availability, leaf nitrogen allocation, and photosynthetic capacity (Rogers, 2014; Rogers *et al.*, 2017). While empirical support for these relationships is abundant (Brix, 1971; Evans & Seemann, 1989; Evans, 1989; Walker *et al.*, 2014; Firn *et al.*, 2019), plant acclimation responses to changing environments (e.g., increasing CO2, light availability, temperature, precipitation variability, etc.) can alter leaf nitrogen allocation and photosynthetic capacity independent of soil nutrient availability (Poorter *et al.*, 2019, 2022; Luo *et al.*, 2021). The inability of terrestrial biosphere models to capture such acclimation responses to environmental change casts uncertainty in the ability of these models to accurately simulate accurate and robust photosynthetic responses to global change (Smith & Dukes, 2013; Harrison *et al.*, 2021).

[why leaf nitrogen allocation? What does it tell us and what do we need to understand about its plasticity in response to environmental change across time and space?]

[existing frameworks for predicting leaf nitrogen allocation across time and space]

Contemporary analyses using photosynthetic least-cost theory suggest that leaf nutrient allocation, photosynthetic capacity, and relationships between leaf nutrient allocation and photosynthetic capacity may be better predicted through factors that influence leaf nutrient demand to build and maintain photosynthetic machinery (Dong *et al.*, 2017, 2020, 2022; Smith *et al.*, 2019; Paillassa *et al.*, 2020). Indeed, studies show that leaf nitrogen and photosynthetic capacity can be reliably predicted through mean growing season irradiance, growing season temperature, growing season vapor pressure deficit, or edaphic characteristics such as soil pH (Dong *et al.*, 2017, 2020; Paillassa *et al.*, 2020). However, relationships between leaf nitrogen and photosynthetic capacity may also be determined through costs of leaf construction, commonly evidenced through leaf mass per area, or other species identity traits, such as whether a species associated with nitrogen-fixing bacteria (Dong *et al.*, 2017)

Photosynthetic least-cost theory provides a useful framework for understanding when and where factors that influence leaf nutrient demand to build and maintain photosynthesis modify leaf nitrogen allocation and photosynthetic capacity. The theory predicts that plants acclimate to their environment by maximizing photosynthetic carbon gain at the lowest summed cost of nitrogen and water usage (Wright *et al.*, 2003; Prentice *et al.*, 2014). Nitrogen and water use can be substituted for each other to maintain the lowest summed cost of nitrogen and water use, such that less efficient use of a relatively more abundant resource should be traded for more efficient use of a relatively less abundant resource. For example, plants growing in arid or semiarid systems should acclimate to their growing conditions by increasing leaf nitrogen allocation and decreasing stomatal conductance, allowing a given photosynthetic rate to be maintained with less efficient nitrogen use and more efficient water use. This strategy is particularly useful in arid or semiarid systems because it allows for plants to save water without needing to sacrifice productivity (Paillassa *et al.*, 2020).

While there is a clear need to understand when and where factors influence leaf nutrient demand to build and maintain photosynthetic machinery, it is also imperative that we understand the relevant timescales plants use to acclimate to their environment. Smith & Dukes (2017) used model selection techniques to show that photosynthetic biochemical process rates were best predicted by the mean temperature of the seven days leading up to a measurement. This was done across temperate and tropical forest sites spanning a large latitudinal gradient, and their results are limited to said ecosystem types. Understanding whether these timescales differ across other ecosystem types is also important, especially in grassland systems because they occupy approximately XX% of land in the coterminous United States and XX% of land globally.

In the summer 2020 and 2021, we measured leaf traits of XX individuals spanning XX species scattered across 24 grassland sites in Texas, USA. Texas is home to a diverse climatic gradient, with mean annual precipitation ranging from XX to XX, mean annual temperature ranging from XX to XX, and mean annual vapor pressure deficit ranging from XX to XX (Table 1; Fig. 1). Texas is also home to diverse soils and nutrient availability thresholds. Following the approach explained in Smith & Dukes (2018), we used model selection to determine whether mean 15-year climatic factors, iterations of short-term climatic factors (one day leading up to thirty days pre-measurement), edaphic characteristics such as soil nutrient availability, cation exchange capacity, or soil pH, or leaf and species identity traits such as leaf mass per area or nitrogen-fixing potential were . Given the best model fits, we hypothesized that increasing aridity would increase leaf nitrogen allocation, which would allow individuals to maintain photosynthesis at lower water usage. We also hypothesized that soil nutrient availability would increase the positive effect of aridity on leaf nitrogen allocation and water use efficiency.

**Methods**

*Site descriptions and sampling methodology*

This environmental gradient experiment was conducted through the Texas Ecological Laboratory program, which grants researchers access to privately owned properties in the state of Texas. We collected leaf and soil samples from 24 sites scattered across central and eastern Texas in summer 2020 and summer 2021 (Fig. 1). Twelve sites were visited between June and July 2020 and 14 sites (11 unique from 2020) were visited between May and June 2021 (Table 1). We explicitly chose sites that maximized climatic and edaphic variability between sites and contained dominant open savanna or grassland components (Table 1). Any site with personally communicated or anecdotal evidence of grazing or disturbance (e.g., mowing, feral hog activity, etc.) was excluded from our analysis.

**Table 1** Site locality information, sampling year(s), 2006-2020 mean annual precipitation (MAP; mm), mean annual temperature (MAT; °C), and mean annual vapor pressure deficit (MAV). Rows are arranged by longitude to visualize precipitation variability across sites

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Property** | **Latitude** | **Longitude** | **Sampling year** | **MAP** | **MAT** | **MAV** |
| Edwards\_2019\_17 | 29.95 | -100.36 | 2020 | 563.5 | 19.0 | 1.21 |
| Uvalde\_2020\_02 | 29.59 | -100.09 | 2020, 2021 | 648.5 | 19.5 | 1.23 |
| Menard\_2020\_01 | 30.91 | -99.57 | 2020 | 641.9 | 18.3 | 1.29 |
| Kerr\_2020\_03 | 30.06 | -99.34 | 2021 | 672.4 | 18.3 | 1.19 |
| Bandera\_2020\_03 | 29.85 | -99.30 | 2021 | 789.4 | 18.8 | 1.22 |
| Sansaba\_2020\_01 | 31.29 | -98.62 | 2020 | 733.0 | 18.8 | 1.26 |
| Comal\_2020\_21 | 29.79 | -98.43 | 2020 | 878.5 | 19.9 | 1.21 |
| Blanco\_2019\_16 | 29.99 | -98.43 | 2020 | 833.0 | 19.2 | 1.15 |
| Bexar\_2019\_13 | 29.24 | -98.43 | 2020 | 759.3 | 21.5 | 1.33 |
| Burnet\_2020\_14 | 30.84 | -98.34 | 2021 | 763.3 | 19.5 | 1.24 |
| Comal\_2020\_19 | 30.01 | -98.32 | 2021 | 845.0 | 19.3 | 1.15 |
| Hays\_2021\_54 | 29.96 | -98.17 | 2021 | 861.3 | 20.0 | 1.17 |
| Burnet\_2020\_12 | 30.82 | -98.06 | 2021 | 815.1 | 19.4 | 1.17 |
| Williamson\_2019\_09 | 30.71 | -97.86 | 2020 | 867.7 | 19.7 | 1.18 |
| Williamson\_2019\_10 | 30.54 | -97.77 | 2020 | 819.5 | 19.9 | 1.20 |
| Bell\_2021\_08 | 31.06 | -97.55 | 2021 | 937.3 | 19.6 | 1.20 |
| Fayette\_2021\_12 | 29.86 | -97.21 | 2021 | 985.7 | 20.4 | 1.12 |
| Fayette\_2019\_04 | 30.09 | -96.78 | 2020 | 1017.4 | 20.6 | 1.10 |
| Fayette\_2020\_09 | 29.86 | -96.71 | 2021 | 1002.7 | 20.8 | 1.10 |
| Washington\_2020\_08 | 30.28 | -96.41 | 2021 | 1077.4 | 20.4 | 1.08 |
| Austin\_2020\_03 | 29.78 | -96.24 | 2021 | 1108.7 | 20.6 | 1.01 |
| Brazos\_2020\_16 | 30.93 | -96.23 | 2021 | 1078.0 | 20.1 | 1.09 |
| Brazos\_2020\_18 | 30.52 | -96.21 | 2020, 2021 | 1099.4 | 20.4 | 1.12 |
| Harris\_2020\_03 | 29.88 | -95.31 | 2020, 2021 | 1492.0 | 21.6 | 1.00 |

**Figure 1**

**Chart

Description automatically generated**

**Figure 1** Maps that detail site locations along 2006-2020 mean annual precipitation (panel A) and mean annual temperature (panel B) gradients in Texas, USA. Precipitation and temperature data were plotted at a 4-km grid resolution and are masked to include only grid cells that occur in the Texas state boundary in the United States. In both panels, open circles refer to sites visited in 2020, open triangles to sites visited in 2021, and closed circles to sites visited in 2020 and 2021. The scale bar in panel A applies to both panels.

*Sampling methodology and leaf trait measurements*

At each site, we collected leaf material from three individuals each of the five most abundant species at random locations in the property. All collected leaves were fully expanded, with no visible herbivory or damage, and free from shading by nearby shrubs or trees. Five soil samples were collected from 0-15cm soils at random locations in the property. Soil samples were then mixed together to create one composite soil sample per site.

Images of each leaf were taken immediately following each site visit using a flat-bed scanner. Fresh leaf area was determined from each image using the 'LeafArea' R package (Katabuchi, 2015), which automates leaf area calculations using ImageJ software (Schneider *et al.*, 2012). Each leaf was then dried at 65C for at least 48 hours, weighed for dry biomass, and manually ground in a mortar and pestle until homogenized. Subsamples of dried and homogenized leaf tissue were used to measure leaf nitrogen content (*N*mass; gN g-1) through elemental combustion analysis (Costech-4010, Costech Instruments, Valencia, CA). We calculated leaf mass per area (*M*area; g m-2) as the ratio of fresh leaf area to dry leaf biomass, and leaf nitrogen per leaf area (*N*area; gN m-2) as the product of *N*mass and *M*area.

Subsamples of dried and homogenized leaf tissue were also sent to the University of California-Davis Stable Isotope Facility to determine leaf δ13C. Leaf δ13C values were determined using an elemental analyzer (PDZ Europa ANCA-GSL; Sercon Ltd., Chestshire, UK) interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20 Isotope Ratio Mass Spectrometer, Sercon Ltd., Chestshire, UK). We used leaf δ13C values to estimate the ratio of intercellular (*C*i) to extracellular (*C*a) CO2 (χ; unitless) following the approach of Farquhar *et al.* (1989) described in Cernusak *et al.* (2013). We derived χ as:

(Eqn. 1)

Δ13C represents the relative difference between leaf δ13C (‰) and air δ13C (‰), and is calculated from the following equation:

(Eqn. 2)

where δ13Cair is assumed to be -8‰ (Keeling *et al.*, 1979; Farquhar *et al.*, 1989), *a* represents the fractionation between 12C and 13C due to diffusion in air, assumed to be 4.4‰, and *b* represents the fractionation caused by Rubisco carboxylation, assumed to be 27‰ (Farquhar *et al.*, 1989). For C4 species, *b* in Eqn. 1 was calculated as:

(Eqn. 3)

Where c was set to -5.7‰ and d was set to 30‰ (Farquhar *et al.*, 1989). φ, which is the bundle sheath leakiness term, was set to 0.4. All χ values less than 0.2 and greater than 1.0 were removed.

*Site edaphic characteristics*

Composite soil samples were sent to the Texas A&M Soil, Water and Forage Laboratory to quantify macronutrient concentrations, pH, and electrical conductivity. Soil nitrate-nitrogen (NO3-N; ppm) was extracted in 1 M KCl and measured spectrophotometrically at 520 nm through the end product of a NO3-N to NO2-N cadmium reduction reaction. Soil phosphorus and potassium were extracted in a solution containing 0.2 M acetic acid, 0.25 M ammonium nitrate, 0.015 M ammonium fluoride, 0.013 M nitric acid, and 0.001 M EDTA (cite Mehlich III). Soil pH was measured in a 1:2 soil: deionized water slurry after a 30-minute incubation period with a hydrogen selective electrode. Electrical conductivity was similar measured in a 1:2 soil: deionized water slurry after a 30-minute incubation period with a conductivity probe. Soil NO3-N concentration was chosen as the primary indicator of site soil fertility because NO3-N. This decision was made on the basis that plants require nitrogen in the largest quantity out of all macronutrients, and because soil NO3-N concentrations were positively correlated with soil phosphorus concentrations (Pearson’s r=0.44, p=0.006; Fig. S1) and electrical conductivity (Pearson’s r=0.74, p<0.001; Fig. S1). However, there was no correlation between soil NO3-N concentration and soil pH (Pearson’s r=-0.02, p=0.908; Fig. S1) or soil potassium concentration (Pearson’s r=0.04, p=0.819; Fig. S1).

*Site climate data*

We used the Parameter-elevation Regressions on Independent Slopes Model (PRISM; Daly *et al.*, 2008) climate product to access gridded daily temperature and precipitation data for the coterminous United States at a 4-km grid resolution between January 1, 1991 and July 31, 2021 (PRISM Climate Group, Oregon State University, <https://prism.oregonstate.edu>, data created 4 Feb 2014, accessed 24 Mar 2022). Daily mean air temperature, mean vapor pressure deficit, and total precipitation data were extracted from the grid cell that contained the latitude and longitude of each property using the ‘extract’ function in the ‘terra’ R package (Hijmans, 2022). PRISM data were used in lieu of local weather station data because several rural sites did not have a local weather station present within a 20-km radius of the property. Daily site climate data were used to estimate mean annual precipitation and mean annual temperature for each property between 1991 and 2020 (Table 1).

*Unit cost of resource use*

We derived the unit cost of resource use (*β*) using leaf χ and site climatic data with equations described in Prentice et al. (2014) and simplified in Lavergne et al. (2020):

(Eqn. 4)

η\* is the viscosity of water relative to 25ºC, calculated using elevation and mean annual temperature of each site following equations in Huber et al. (2009). D represents vapor pressure deficit (Pa), set to the 2006-2020 mean annual vapor pressure deficit of each site, *C*a represents atmospheric CO2 concentration, set to 420 μmol mol-1 CO2. K (μmol mol-1) is the Michaelis-Menten coefficient for Rubisco affinity to CO2 and O2, calculated as:

(Eqn. 5)

where Kc (μmol mol-1) and *K*o (μmol mol-1) are the Michaelis-Menten coefficients for Rubisco affinity to CO2 and O2, respectively, and Oi is the intercellular O2 concentrations, assumed to be 210 μmol mol-1. Γ\* (μmol mol-1) is the CO2 compensation point in the absence of dark respiration. We determined *K*c, *K*o, and *Γ*\* using leaf temperature equations described in Medlyn *et al.* (2002) and derived in Bernacchi *et al.* (2001):

(Eqn. 6a)

and

(Eqn. 6b)

and

(Eqn. 6c)

In all three equations, *T*k is the leaf temperature (in Kelvin), which was approximated using the mean daily air temperature of the seven days leading up to each site visit. R is the universal gas constant (8.314 J mol-1 K-1).

*Plant functional type assignments*

Plant functional types were assigned and used as the primary descriptor of species identity. Specifically, we assigned plant functional types based on photosynthetic pathway (C3, C4) and ability to form associations with symbiotic nitrogen-fixing bacteria. The ability to form associations with symbiotic nitrogen-fixing bacteria was assigned based on whether species were in the *Fabaceae* family. This created three distinct plant functional groups within our dataset: C3 nitrogen-fixers (n=53), C3 non nitrogen-fixers (n=353), and C4 non nitrogen-fixers (n=114).

*Data analysis*

We used an information-theoretic model selection approach to determine whether 365-, 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day soil moisture conferred the best model fit for β. To do this, we constructed 16 linear mixed-effects models for each response variable where each soil moisture timestep was included as a single continuous fixed effect. Species was also included as a random intercept term. We used corrected Akaike Information Criterion (AICc) to select the precipitation timescale that conferred the best model fit, indicated by the model with the lowest AICc score. The soil moisture timescale that conferred the best model fit for *β* was fed into subsequent models to predict drivers of *β*, χ, *N*area, *N*mass, and *M*area. We also used an information-theoretic model selection approach to determine whether 365-, 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean vapor pressure deficit and air temperature conferred the best model fit for χ. This was done following the same approach as described for selecting the relevant soil moisture timescale for *β.*

We constructed linear mixed-effects models to investigate environmental drivers of *β*, χ, *N*area, *N*mass, *M*area, and *N*area:χ. To explore environmental drivers of *β*, we built a model that included the relevant soil moisture timescale, soil NO3-N availability, and plant functional type as fixed effect coefficients with species designated as a random intercept term. Interaction coefficients between all possible combinations of the three fixed effect coefficients were also included. *β* was natural log transformed to satisfy residual normality assumptions.

To explore environmental drivers of χ, we included the relevant mean daily air temperature and vapor pressure deficit timescale, soil moisture, soil NO3-N availability, and plant functional type as fixed effect coefficients, with species designated as a random intercept term. Interaction coefficients between soil moisture, soil NO3-N availability, and plant functional type were also included. We did not include *β* as a predictor of χ because χ is an input into the calculation of *β*, and is therefore highly autocorrelated with *β*.

To explore environmental drivers of *N*area, *N*mass, and *M*area, we constructed a series of linear mixed-effects models that included the relevant soil moisture timescale selected for *β*, soil nitrogen availability, natural log transformed *β*, χ, and plant functional group as fixed effect coefficients, with species again designated as a random intercept term. Interaction coefficients between soil moisture, soil NO3-N availability, and plant functional type were also included.

Finally, to explore environmental drivers of *N*area:χ, we included the relevant mean daily air temperature and vapor pressure deficit timescale for χ, the relevant soil moisture timescale for *β*, soil NO3-N availability, and plant functional type as fixed effect coefficients. Interaction terms between *β*, soil NO3-N availability, and plant functional type were also included. As with χ, we did not include *β* directly as a predictor of *N*area:χ because *β* is autocorrelated with χ.

In all models, including those to select relevant aridity timescales, we used the 'lmer' function in the 'lme4' R package (Bates *et al.*, 2015) to fit each model and the 'Anova' function in the 'car' R package (Fox & Weisberg, 2019) to calculate Type II Wald's χ2 and determine the significance level (α=0.05) of each fixed effect coefficient. We also used the 'emmeans' R package (Lenth, 2019) to conduct post-hoc comparisons using Tukey's tests, where degrees of freedom were approximated using the Kenward-Roger approach (Kenward & Roger, 1997). All analyses and plots were conducted in R version 4.1.1 (R Core Team, 2021).

**Results**

*Unit cost ratio (β)*

60-day soil moisture was the most explanatory aridity timescale for predicting *β* (Table S1). While there was an individual negative effect of increasing 30-day relative soil moisture on *β*, there was also a two-way interaction between 30-day relative soil moisture and photosynthetic pathway (Table 1). This interaction indicated that increasing 30-day relative soil moisture decreased *β* in C4 species (Tukey: p<0.001), but not C3 species (Tukey: p=0.714). We also observed a negative effect of increasing soil nitrogen availability on *β*; however, there was a marginal two-way interaction between soil nitrogen availability and photosynthetic pathway and a second marginal two-way interaction between soil nitrogen availability and the capacity of species to form associations with symbiotic nitrogen-fixing bacteria. These two-way interactions indicated that C4 species (soil N-ln *β* slope ± SE: -0.019±0.007) and species capable of forming associations with symbiotic nitrogen-fixing bacteria (-0.018±0.006) had marginally stronger negative *β* responses to increasing soil nitrogen availability than C3 species (-0.008±0.003; Tukey: p=0.096) and species not capable of forming associations with symbiotic nitrogen-fixing bacteria (-0.009±0.003; Tukey: p=0.083), respectively. Interestingly, there was no individual effect of the ability to form associations with symbiotic nitrogen-fixing bacteria on *β*.

**Table 1** Analysis of variance results explaining the effect of aridity, soil nitrogen availability, and plant functional type on *β*\*

|  |  |  |  |
| --- | --- | --- | --- |
|  | df | χ2 | p |
| **30-day relative soil moisture (SM30)** | 1 | 4.379 | **0.036** |
| **Soil nitrate-nitrite concentration (Nsoil)** | 1 | 9.976 | **0.002** |
| **Nitrogen fixation capability (BNF)** | 1 | 0.296 | 0.587 |
| **Photosynthetic pathway (Photo)** | 1 | 49.708 | **<0.001** |
| **SM30 \* BNF** | 1 | 0.083 | 0.774 |
| **SM30 \* Photo** | 1 | 31.529 | **<0.001** |
| **Nsoil \* BNF** | 1 | 3.034 | *0.082* |
| **Nsoil \* Photo** | 1 | 2.807 | *0.094* |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold and those between 0.05 and 0.10 are italicized. *β* was natural-log transformed prior to fitting model

*Chi*

Single-day daily mean vapor pressure deficit and 13-day mean daily temperature were the most explanatory vapor pressure deficit and temperature timescales for predicting χ (Table S2). We found that 13-day mean daily temperature had no effect on χ, although there was a strong negative effect of increasing single-day vapor pressure deficit (Table 1).

As with *β*, χ was driven by a two-way interaction between 30-day relative soil moisture and photosynthetic pathway (Table 2). This interaction indicated that the general marginal negative effect of increasing 30-day relative soil moisture on χ (Table 2) was driven by a negative effect of soil moisture on χ in C4 species (Tukey: p<0.001) and no change in C3 species (Tukey: p=0.116). There was also a two-way interaction between soil nitrogen availability and photosynthetic capacity that indicated a negative effect of increasing soil nitrogen availability χ in C4 species (Tukey: p=0.013), but not C3 species (Tukey: p=0.240). Finally, we observed a third two-way interaction between soil nitrogen availability and the capability of species to form associations with symbiotic nitrogen-fixing bacteria, which indicated a negative effect of increasing soil nitrogen availability on χ in species with the capability to form such associations (Tukey: p=0.009) and but no effect in species without the capability to form these associations (Tukey: p=0.113). Despite these interactions, there was no overall effect of soil nitrogen availability on χ (Table 2).

**Table 2** Analysis of variance results explaining the effect of aridity, soil nitrogen availability, and plant functional type on χ\*

|  |  |  |  |
| --- | --- | --- | --- |
|  | df | χ2 | p |
| **1-day vapor pressure deficit (VPD1)** | 1 | 12.809 | **<0.001** |
| **13-day average temperature (Tavg7)** | 1 | 0.150 | 0.698 |
| **30-day relative soil moisture (SM30)** | 1 | 3.273 | *0.070* |
| **Soil nitrate-nitrite concentration (Nsoil)** | 1 | 0.093 | 0.761 |
| **Nitrogen fixation capability (BNF)** | 1 | 0.079 | 0.779 |
| **Photosynthetic pathway (Photo)** | 1 | 62.615 | **<0.001** |
| **SM30 \* BNF** | 1 | 2.650 | 0.104 |
| **SM30 \* Photo** | 1 | 71.681 | **<0.001** |
| **Nsoil \* BNF** | 1 | 3.936 | **0.047** |
| **Nsoil \* Photo** | 1 | 4.774 | **0.029** |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold and those between 0.05 and 0.10 are italicized.

*Leaf nitrogen content*

15-year mean annual aridity was the most explanatory aridity timescale for predicting *N*area (Supplemental Information). Patterns of *N*area were primarily driven by a three-way interaction between 15-year aridity, β, and soil nitrogen fertilization (Table 2). This interaction indicated that increasing soil nitrogen availability increased the magnitude of the negative slope explaining the relationship between AI15yr and *N*area, although the extent of this magnitude was itself dependent on *β.*

This interaction indicated that increasing soil nitrogen availability generally increased the magnitude of the negative slope explaining the relationship between AI15yr and *N*area

increasing AI15yr generally

*N*area was driven by a two-way interaction between long-term aridity and plant functional type. This interaction indicated that *N*area generally increased with long-term aridity in C3 forbs (Tukey: p=), marginally increased with long-term aridity in C4 graminoids (Tukey: p=0.060), and decreased with long-term aridity in legumes (Tukey: p=0.011). Despite this, there was no overall effect of long-term aridity on *N*area when plant functional types were pooled (Table 1). There was also a two-way interaction between short-term aridity and plant functional type on *N*area, which revealed no effect of short-term aridity in any plant functional groups aside from an increase in *N*area with increasing aridity in legumes (Tukey: p=0.001). There was no overall effect of short-term aridity on *N*area when plant functional types were pooled (Table 1). Finally, there was a two-way interaction between plant functional type and soil nitrogen availability, which revealed a null effect of soil nitrogen availability in all plant functional types except for an increase in *N*area in legumes (Tukey: p=0.008). Nonetheless, soil nitrogen availability had an individual positive effect on *N*area when pooled across plant functional types (Table 1).

**Table 3** Analysis of variance results explaining the effect of aridity, soil nitrogen availability, and plant functional type on χ\*

|  |  |  |  |
| --- | --- | --- | --- |
|  | df | χ2 | p |
| **15-day vapor pressure deficit** | 1 | 0.038 | 0.846 |
| **21-day average temperature** | 1 | 5.172 | 0.023 |
| **30-day relative soil moisture (SM30)** | 1 | 0.429 | 0.513 |
| **Soil nitrate-nitrite concentration (Nsoil)** | 1 | 25.780 | <0.001 |
| **Nitrogen fixation capability (BNF)** | 1 | 7.671 | 0.006 |
| **Photosynthetic pathway (Photo)** | 1 | 31.625 | <0.001 |
| **SM30 \* BNF** | 1 | 0.646 | 0.422 |
| **SM30 \* Photo** | 1 | 1.797 | 0.180 |
| **Nsoil \* BNF** | 1 | 4.172 | 0.041 |
| **Nsoil \* Photo** | 1 | 0.304 | 0.581 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold and those between 0.05 and 0.10 are italicized.

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold. All response variables are natural log transformed. Key: AI15yr=mean annual aridity between 2006 and 2020, ln*β*=natural log transformed unit cost ratio;PFT=plant functional type

**Figure 1**

Chart, scatter chart

Description automatically generated

**Figure 1**. Effects of short-term aridity (90 days prior to measurement; panel A), long-term aridity (15-year mean annual aridity; panel B), and soil NO3-N availability (panel C) on log-transformed leaf nitrogen per leaf area. Yellow points and trendlines indicate measurements and model results for C3 forbs, red points and trendlines indicate measurements and model results for C3 graminoids, blue points and trendlines indicate measurements and model results for C4 graminoids, and grey points and trendlines indicate points and measurements and model results for legumes. The black trendline indicates the relationship between short-term aridity (panel A), long-term aridity (panel B), and soil NO3-N availability (panel C) when averaged across plant functional types. Solid trendlines indicate slopes that are statistically different from zero (p<0.05), while dashed trendlines indicate slopes that are not statistically different from zero (p>0.05). Plant functional type trendlines are only included when there is a two-way interaction with short-term aridity, long-term aridity, or soil nitrogen availability.

**Discussion**

**References**

**Bates D, Mächler M, Bolker B, Walker S**. **2015**. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**: 1–48.

**Bernacchi CJ, Singsaas EL, Pimentel C, Portis AR, Long SP**. **2001**. Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environment* **24**: 253–259.

**Brix H**. **1971**. Effects of nitrogen fertilization on photosynthesis and respiration in Douglas-fir. *Forest Science* **17**: 407–414.

**Cernusak LA, Ubierna N, Winter K, Holtum JAM, Marshall JD, Farquhar GD**. **2013**. Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist* **200**: 950–965.

**Daly C, Halbleib M, Smith JI, Gibson WP, Doggett MK, Taylor GH, Curtis J, Pasteris PP**. **2008**. Physiographically sensitive mapping of climatological temperature and precipitation across the conterminous United States. *International Journal of Climatology* **28**: 2031–2064.

**Dong N, Prentice IC, Evans BJ, Caddy-Retalic S, Lowe AJ, Wright IJ**. **2017**. Leaf nitrogen from first principles: field evidence for adaptive variation with climate. *Biogeosciences* **14**: 481–495.

**Dong N, Prentice IC, Wright IJ, Evans BJ, Togashi HF, Caddy-Retalic S, McInerney FA, Sparrow B, Leitch E, Lowe AJ**. **2020**. Components of leaf‐trait variation along environmental gradients. *New Phytologist* **228**: 82–94.

**Dong N, Wright IJ, Chen JM, Luo X, Wang H, Keenan TF, Smith NG, Prentice IC**. **2022**. Rising CO2 and warming reduce global canopy demand for nitrogen. *New Phytologist*.

**Evans JR**. **1989**. Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia* **78**: 9–19.

**Evans JR, Seemann JR**. **1989**. The allocation of protein nitrogen in the photosynthetic apparatus: costs, consequences, and control. *Photosynthesis* **8**: 183–205.

**Farquhar GD, Ehleringer JR, Hubick KT**. **1989**. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503–537.

**Fay PA, Prober SM, Harpole WS, Knops JMH, Bakker JD, Borer ET, Lind EM, MacDougall AS, Seabloom EW, Wragg PD, *et al.*** **2015**. Grassland productivity limited by multiple nutrients. *Nature Plants* **1**: 15080.

**Firn J, McGree JM, Harvey E, Flores-Moreno H, Schütz M, Buckley YM, Borer ET, Seabloom EW, La Pierre KJ, MacDougall AS, *et al.*** **2019**. Leaf nutrients, not specific leaf area, are consistent indicators of elevated nutrient inputs. *Nature Ecology & Evolution* **3**: 400–406.

**Fox J, Weisberg S**. **2019**. *An R companion to applied regression*. Thousand Oaks, California: Sage.

**Harrison SP, Cramer W, Franklin O, Prentice IC, Wang H, Brännström Å, de Boer H, Dieckmann U, Joshi J, Keenan TF, *et al.*** **2021**. Eco-evolutionary optimality as a means to improve vegetation and land-surface models. *New Phytologist* **231**: 2125–2141.

**Hijmans RJ**. **2022**. terra: Spatial Data Analysis.

**Hungate BA, Dukes JS, Shaw MR, Luo Y, Field CB**. **2003**. Nitrogen and climate change. *Science* **302**: 1512–1513.

**IPCC**. **2013**. *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*.

**Katabuchi M**. **2015**. LeafArea: An R package for rapid digital analysis of leaf area. *Ecological Research* **30**: 1073–1077.

**Keeling CD, Mook WG, Tans PP**. **1979**. Recent trends in the 13C/12C ratio of atmospheric carbon dioxide. *Nature* **277**: 121–123.

**Kenward MG, Roger JH**. **1997**. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**: 983.

**LeBauer DS, Treseder K**. **2008**. Nitrogen limitation of net primary productivity. *Ecology* **89**: 371–379.

**Lenth R**. **2019**. emmeans: estimated marginal means, aka least-squares means.

**Luo X, Keenan TF, Chen JM, Croft H, Prentice IC, Smith NG, Walker AP, Wang H, Wang R, Xu C, *et al.*** **2021**. Global variation in the fraction of leaf nitrogen allocated to photosynthesis. *Nature Communications* **12**: 4866.

**Medlyn BE, Dreyer E, Ellsworth DS, Forstreuter M, Harley PC, Kirschbaum MUF, Le Roux X, Montpied P, Strassemeyer J, Walcroft A, *et al.*** **2002**. Temperature response of parameters of a biochemically based model of photosynthesis. II. A review of experimental data. *Plant, Cell & Environment* **25**: 1167–1179.

**Paillassa J, Wright IJ, Prentice IC, Pepin S, Smith NG, Ethier G, Westerband AC, Lamarque LJ, Wang H, Cornwell WK, *et al.*** **2020**. When and where soil is important to modify the carbon and water economy of leaves. *New Phytologist* **228**: 121–135.

**Poorter H, Knopf O, Wright IJ, Temme AA, Hogewoning SW, Graf A, Cernusak LA, Pons TL**. **2022**. A meta-analysis of responses of C3 plants to atmospheric CO2: dose–response curves for 85 traits ranging from the molecular to the whole-plant level. *New Phytologist* **233**: 1560–1596.

**Poorter H, Niinemets Ü, Ntagkas N, Siebenkäs A, Mäenpää M, Matsubara S, Pons TL**. **2019**. A meta-analysis of plant responses to light intensity for 70 traits ranging from molecules to whole plant performance. *New Phytologist* **223**: 1073–1105.

**Prentice IC, Dong N, Gleason SM, Maire V, Wright IJ**. **2014**. Balancing the costs of carbon gain and water transport: testing a new theoretical framework for plant functional ecology. *Ecology Letters* **17**: 82–91.

**R Core Team**. **2021**. R: A language and environment for statistical computing.

**Rogers A**. **2014**. The use and misuse of Vc,max in Earth System Models. *Photosynthesis Research* **119**: 15–29.

**Rogers A, Medlyn BE, Dukes JS, Bonan G, von Caemmerer S, Dietze MC, Kattge J, Leakey ADB, Mercado LM, Niinemets Ü, *et al.*** **2017**. A roadmap for improving the representation of photosynthesis in Earth system models. *New Phytologist* **213**: 22–42.

**Schneider CA, Rasband WS, Eliceiri KW**. **2012**. NIH Image to ImageJ: 25 years of image analysis. *Nature methods* **9**: 671–675.

**Smith NG, Dukes JS**. **2013**. Plant respiration and photosynthesis in global-scale models: Incorporating acclimation to temperature and CO2. *Global Change Biology* **19**: 45–63.

**Smith NG, Keenan TF, Prentice IC, Wang H, Wright IJ, Niinemets Ü, Crous KY, Domingues TF, Guerrieri R, Ishida F oko, *et al.*** **2019**. Global photosynthetic capacity is optimized to the environment (S Niu, Ed.). *Ecology Letters* **22**: 506–517.

**Walker AP, Beckerman AP, Gu L, Kattge J, Cernusak LA, Domingues TF, Scales JC, Wohlfahrt G, Wullschleger SD, Woodward FI**. **2014**. The relationship of leaf photosynthetic traits - Vcmax and Jmax - to leaf nitrogen, leaf phosphorus, and specific leaf area: a meta-analysis and modeling study. *Ecology and Evolution* **4**: 3218–3235.

**Wright IJ, Reich PB, Westoby M**. **2003**. Least-cost input mixtures of water and nitrogen for photosynthesis. *The American Naturalist* **161**: 98–111.