**Target Journals:**

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

**Running Head:** *β* modifies leaf nitrogen content

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**Manuscript compilation details**

**Abstract:** XXX words

**Main text word count**: XXX words

Introduction: XXX words

Methods: XXX words

Results: XXX words (not including text in figures or tables)

Discussion: XXX words

**References**: XXX

**Tables and Figures**: XXX

**Supplemental Information**:

**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).

[PLCT explanation]

[theoretical expectations of nutrient-water use tradeoffs in response to soil nutrient availability; brief review of empirical support from patterns expected from theory]

[theoretical expectations of nutrient-water use tradeoffs in response to soil nutrient availability; brief review of empirical support for patterns expected from theory]

[theoretical expectations for the possible interaction between soil nutrient availability and water availability on nutrient-water use tradeoffs]

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 contains a diverse climatic gradient, indicated by 15-year mean annual precipitation totals ranging from XX to XX mm per year, mean annual temperatures ranging from XX to XX, and mean annual vapor pressure deficit ranging from XX to XX. We used eco-evolutionary optimality theory explained above to test the following hypotheses:

1. Soil nutrient availability will decrease the unit cost of acquiring and using nutrients relative to water. This should lead to an increase in water use efficiency and leaf nutrient content with increasing soil nutrient availability. However, we expect that species capable of forming associations with symbiotic nitrogen-fixing bacteria might be less sensitive to changes in soil nutrient availability and should therefore demonstrate weaker nutrient-water use tradeoffs expected from the theory.
2. Soil moisture will increase the unit cost of acquiring and using nutrients relative to water. This should result in a reduction in water use efficiency that corresponds with a reduction in leaf nutrient content with increasing soil moisture. However, we expect that species that undergo C4 photosynthesis to be less sensitive to changes in soil moisture availability and should demonstrate weaker nutrient-water use tradeoffs expected from the theory compared to species that undergo C3 photosynthesis. We also expect species that undergo C4 photosynthesis to have increased water-use efficiency compared to their C3 counterparts irrespective of soil moisture availability.
3. In drier sites (i.e., ones with lower soil moisture), soil nutrient availability will induce stronger reductions in the unit cost of acquiring and using nutrients relative to water. This will result in stronger increases in water use efficiency and leaf nutrient content at drier sites than wetter sites. We expect that these patterns will be context dependent on the ability of species to associate with symbiotic nitrogen-fixing bacteria or to perform C4 photosynthesis. Specifically, we expected the interaction between soil moisture and soil nutrient availability to be strongest in C3 species without capabilities to associate with symbiotic nitrogen-fixing bacteria.

**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.

*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, mean annual temperature, and mean annual vapor pressure deficit for each site between 1991 and 2020 (Table 1). We also iteratively calculated total precipitation, mean daily air temperature, and mean daily vapor pressure deficit for the prior 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 60, 90, and 365 days leading up to each site visit. This was done to determine relevant climatic timescales that best explained *β* and *χ*.

*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 (Keeney & Nelson, 1983; Kachurina *et al.*, 2000). 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 (Mehlich, 1984). 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 similarly measured in a 1:2 soil: deionized water slurry after a 30-minute incubation period with a conductivity probe (Rhoades, 1983). Soil NO3-N concentration was chosen as the primary indicator of site soil nutrient availability. The rationale for this decision was that plants require nitrogen in the largest quantity of all macronutrients and because soil NO3-N concentrations had strong positive correlations 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). 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).

Soil moisture was estimated using the ‘Simple Process-Led Algorithms for Simulating Habitats’ model ('SPLASH'; Davis *et al.*, 2017). This model, derived from the STASH model (Cramer & Prentice, 1988), spins up a bucket model that calculates daily soil moisture (*W*n; mm) as a function of the previous day’s soil moisture (*W*n-1; mm), daily precipitation (*P*n; mm), condensation (*C*n; mm), actual evapotranspiration (; mm), and runoff (RO; mm):

(Eqn. 4)

This equation is solved on a daily timestep using a 150mm bucket size (Cramer & Prentice, 1988) by first equilibrating the previous day’s soil moisture using successive model iterations with daily mean air temperature, precipitation, the number of daily sunlight hours, and latitude as model inputs to estimate daily solar radiation, condensation, evapotranspiration, and runoff using equations explained in Cramer & Prentice (1988) and Davis *et al.* (2017). Daily sunlight hours were estimated for each day at each site using the ‘getSunlightTimes’ function in the ‘suncalc’ R package, which estimated sunrise and sunset times of each property using date and site coordinates (Thieurmel & Elmarhraoui, 2019). We used daily soil moisture outputs from the SPLASH model for each site to iteratively calculate mean daily soil moisture for the prior 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 60, and 90 days leading up to each site visit. This was done to determine relevant climatic timescales that best explained *β* and *χ*.

*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 by hand 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 set to 6.3‰, and was derived from:

(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.

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 mean vapor pressure deficit of the seven days leading up to each site visit, *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 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 constructed a series of separate linear mixed-effects models to investigate environmental drivers of *β*, χ, and *N*area. To explore environmental drivers of *β*, we built a model that included soil moisture, soil NO3-N availability, and plant functional type as fixed effect coefficients. Species was 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. We used an information-theoretic model selection approach to determine whether 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean daily 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 (Table S1; Fig. S2).

To explore environmental drivers of χ, we constructed two separate linear mixed effects models. In the first model, we included mean daily air temperature, vapor pressure deficit, *β*, and plant functional group as fixed effect coefficients. We also included two-way interaction terms between plant functional group and daily air temperature, vapor pressure deficit, and *β*. Species was designated as a random intercept term. Because χ is an input into the calculation of *β*, and is therefore autocorrelated with *β*, we constructed a second linear mixed effects model that included daily air temperature, vapor pressure deficit, soil moisture, soil NO3-N availability, and plant functional group as fixed effect coefficients. We also included all possible interactions between soil moisture, soil NO3-N availability, and plant functional group, and two-way interactions between plant functional group and daily air temperature or vapor pressure deficit as additional fixed effect coefficients. In both models, we used an information-theoretic model selection approach to determine whether 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean daily air temperature and vapor pressure deficit conferred the best model fit for *χ* using the same approach explained for the model investigating primary drivers of *β*. In the second model, we used the same soil moisture timescale as determined relevant for *β*.

To explore environmental drivers of *N*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, we conducted a path analysis to examine direct and indirect pathways that drive variance in *N*area. The path analysis included plant functional group, soil nitrogen availability, *β*, and *χ* as direct predictors of *N*area. Vapor pressure deficit, Air temperature, *β*, and plant functional group were included as direct predictors of *χ*, while plant functional group, soil moisture, and soil nitrogen availability were included as direct predictors of *β*. We also included soil moisture as a direct predictor of soil nitrogen availability, and air temperature as a direct predictor of vapor pressure deficit. The structural equation model was build using the ‘sem’ function in the ‘lavaan’ R package (Rosseel, 2012).

In all linear mixed-effects 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)*

Model selection indicated that 3-day soil moisture was the timescale that conferred the best model fit for *β* (Table S1; Fig. S2). Variance in *β* across sites was driven by a strong two-way interaction between soil moisture and plant functional group (Table 2), which indicated a negative effect of increasing soil moisture on *β* in C4 non-legumes (Tukey: p=0.001) and no effect of soil moisture in C3 legumes (Tukey: p=0.641) and non-legumes (Tukey: p=0.415; Fig. 2A). We also found a strong negative effect of increasing soil nitrogen availability on *β* (Table 2; Fig. 2B), a pattern observed across all plant functional groups. Finally, C4 non-legumes generally had lower *β* values than both C3 legumes (Tukey: p=0.005) and C3 non-legumes (Tukey: p<0.001) when averaged across soil moisture and soil nitrogen availability values.

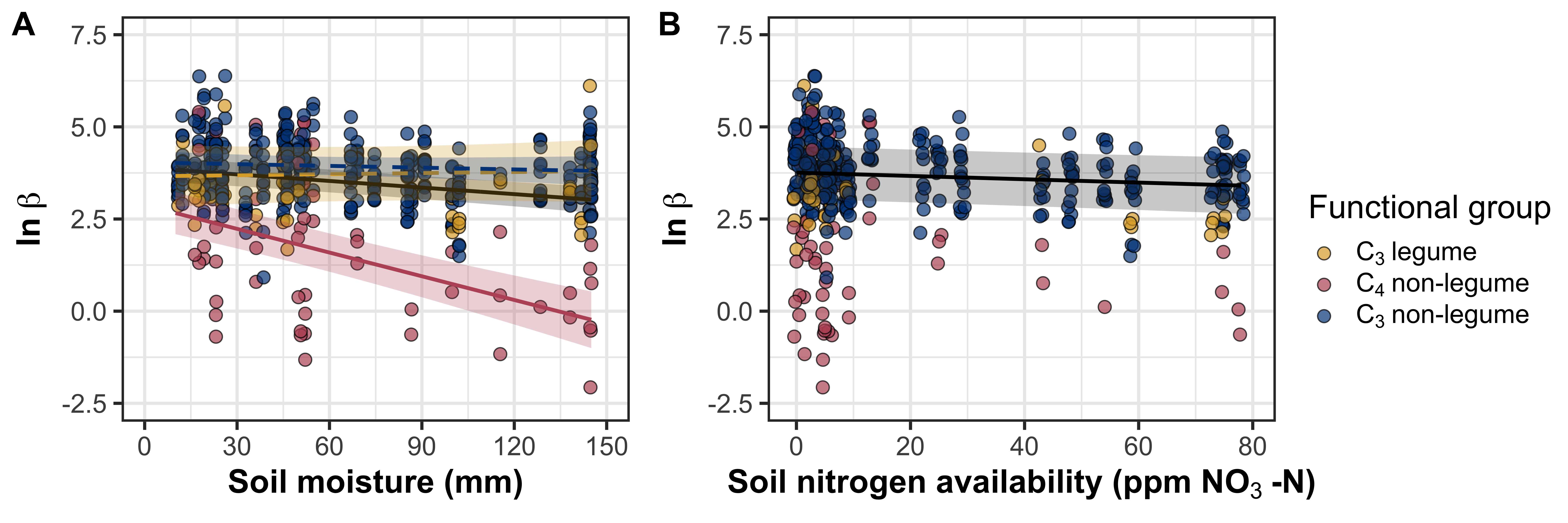
[SEM results]

**Table 2** Effects of soil moisture, soil nitrogen availability, and plant functional group on *β*\*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | df | Coefficient | χ2 | *P*-value |
| Intercept |  | 3.786 |  |  |
| Soil moisture (SM) | 1 | 0.001 | 12.846 | **<0.001** |
| Soil NO3-N (N) | 1 | -0.021 | 9.670 | **0.002** |
| PFT | 2 | - | 65.020 | **<0.001** |
| SM \* N | 1 | <0.001 | 0.062 | 0.803 |
| SM \* PFT | 2 | - | 37.695 | **<0.001** |
| N \* PFT | 2 | - | 4.070 | 0.131 |
| SM \* N \* PFT | 2 | - | 0.037 | 0.982 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold. Model coefficients are on natural log scale, as beta was natural log transformed prior to fitting

**Figure 2**



**Fig. 2** Effects of soil moisture (panel A) and soil nitrogen availability (panel B) on *β*. Yellow shaded points and trendlines indicate C3 legumes, red shaded points and trendlines indicate C4 non-legumes, and blue shaded points and trendlines indicate C3 non-legumes. Black trendlines indicate the bivariate relationship between soil moisture or soil nitrogen availability and *β*. Solid trendlines indicate relationships where *P*<0.05 and dashed trendlines indicate relationships where *P*>0.05. Plant functional group-specific trendines in panel B are not included as there was no two-way interaction between soil nitrogen availability and plant functional group.

*Chi*

Model selection indicated that 4-day vapor pressure deficit and 4-day air temperature were the timescales that conferred the best model fit for *χ* (Table S1; Fig. S2). When the unit cost ratio was included as a direct predictor of *χ*, *χ* was driven by a series of two-way interactions between plant functional group and 4-day vapor pressure deficit, 4-day air temperature, and the unit cost ratio *β* (Table 3). Specifically, a two-way interaction between functional group and *β* revealed that, while increasing *β* increased *χ* across all functional groups, *χ* in C4 non-legumes was less sensitive to changes in *β* than C3 nonlegumes and C3 legumes (Tukey: p<0.001 in both cases). An additional two-way interaction between plant functional group and vapor pressure deficit indicated that increasing vapor pressure deficit increased χ in C4 nonlegumes (Tukey: p=0.002), decreased χ in C3 nonlegumes (Tukey: p<0.001), and did not change χ in C3 legumes (Tukey: p=0.271). A final two-way interaction between functional group and 4-day air temperature indicated that increasing air temperature increased χ in C4 nonlegumes (Tukey: p=0.002) and C3 legumes (Tukey: p=0.013) but did not influence χ in C3 nonlegumes (Tukey: p=0.509).

Similar patterns were observed when *β* was substituted for soil moisture and soil nitrogen availability (Table 3). Specifically, a two-way interaction between plant functional group and 3-day soil moisture indicated a negative effect of increasing soil moisture on χ in C4 nonlegumes (Tukey: p<0.001), with no apparent effect of soil moisture on χ in C3 legumes (Tukey: p=0.689) and C3 nonlegumes (Tukey: p=0.731). A weak two-way interaction between functional group and soil nitrogen availability indicated a marginal positive effect of increasing soil nitrogen availability on C4 nonlegumes (Tukey: p=0.082), with again no apparent effect on χ in C3 legumes (Tukey: p=0.401) and C3 nonlegumes (Tukey: p=0.849). A two-way interaction between functional group and 4-day vapor pressure deficit also indicated the same patterns as observed from the previous model, where increasing 4-day vapor pressure deficit increased χ in C4 nonlegumes (Tukey: p=0.002), decreased χ in C3 nonlegumes (Tukey: p<0.001), and did not change χ in C3 legumes (Tukey: p=0.262). Finally, a two-way interaction between functional group and 4-day air temperature indicated that increasing air temperature increased χ in C4 nonlegumes (Tukey: p<0.001) and C3 legumes (Tukey: p=0.027) but did not influence χ in C3 nonlegumes (Tukey: p=0.427).

**Table 3** Analysis of variance results exploring effects of vapor pressure deficit, temperature, the unit cost ratio, drivers of the unit cost ratio, and plant functional group on χ\*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | χ with β | | | χ without β | | |
| Treatment | df | Coefficient | χ2 | *P*-value | Coefficient | χ2 | *P*-value |
| Intercept | - | 0.457 | - | - | 0.445 | - | - |
| VPD | 1 | -0.008 | 34.332 | **<0.001** | -0.013 | 10.890 | **<0.001** |
| Temperature (T) | 1 | 0.020 | 0.603 | 0.437 | - | 3.430 | *0.064* |
| Unit cost ratio (*β*) | 1 | 0.001 | 327.005 | **<0.001** | - | - | - |
| Soil moisture | 1 | - | - | - | <0.001 | 17.503 | **<0.001** |
| Soil N | 1 | - | - | - | -0.001 | 1.209 | 0.272 |
| PFT | 2 | - | 122.582 | **<0.001** | - | 87.142 | **<0.001** |
| SM \* N | 1 | - | - | - | <0.001 | 0.441 | 0.507 |
| VPD \* PFT | 2 | - | 20.848 | **<0.001** | - | 24.786 | **<0.001** |
| T \* PFT | 2 | - | 16.407 | **<0.001** | - | 42.059 | **<0.001** |
| *β* \* PFT | 2 | - | 157.257 | **<0.001** | - | - | - |
| SM \* PFT | 2 | - | - | - | - | 56.680 | **<0.001** |
| N \* PFT | 2 | - | - | - | - | 9.128 | **0.010** |
| SM \* N \* PFT | 2 | - | - | - | - | 0.464 | 0.793 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold and 0.05<*P*<0.1 are italicized. The set of model coefficients, χ2 values, and *P* values on the left indicate results from a linear mixed-effect model where the unit cost ratio is included as a predictor of χ. The rightmost set of model coefficients, χ2 values, and *P* values indicate a linear mixed-effect model where the unit cost ratio is substituted for soil nitrogen availability and soil moisture, mimicking the structure of the model used to detect predictors of the unit cost ratio in Table 2. χ was not transformed prior to model fitting, so model coefficients are on the response scale.

*Leaf nitrogen content*

Variance in leaf nitrogen per leaf area was driven by a two-way interaction between the unit cost ratio *β* and plant functional group (Table 4). This interaction indicated a negative effect of increasing *β* on leaf nitrogen per leaf area in C3 legumes (Tukey: p=0.002) and a marginal negative effect in C3 nonlegumes (Tukey: p=0.083). There was no effect of *β* on leaf nitrogen per leaf area in C4 nonlegumes (Tukey: p=0.244). We also observed a negative effect of increasing *χ* on leaf nitrogen per leaf area, a pattern observed regardless of plant functional group (Table 4). Finally, increasing soil nitrogen availability had a marginal positive effect on leaf nitrogen per leaf area, a pattern observed regardless of plant functional group (Table 4).

**Table 4** Analysis of variance results exploring effects of soil nitrogen availability, the unit cost ratio, χ, and plant functional group on *N*area\*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Treatment | df | Coefficient | χ2 | P-value |
| Intercept | - | 0.865 | - | - |
| Unit cost ratio (*β*) | 1 | -0.003 | 6.617 | **0.010** |
| *χ* | 1 | 0.503 | 5.451 | **0.020** |
| Soil N (N) | 1 | 0.003 | 3.247 | *0.072* |
| PFT | 2 | - | 50.839 | **<0.001** |
| *β* \* PFT | 2 | - | 7.816 | **0.020** |
| *χ* \* PFT | 2 | - | 2.511 | 0.285 |
| N \* PFT | 2 | - | 2.147 | 0.342 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold and 0.05<*P*<0.1 are italicized. Coefficients are expressed on natural-log scale, as *N*area was natural log transformed prior to model fitting

**Figure 4**

**Figure 5**

Diagram

Description automatically generated

**Figure 5** Structural equation model results exploring direct and indirect drivers of *β*, χ, and leaf nitrogen per unit leaf area. Blue solid arrows indicate bivariate relationships that are positively correlated and red solid arrows indicate bivariate relationships that are negatively correlated (P<0.05), while grey dashed lines indicate bivariate relationships where there is no correlation (P>0.05). Numbers indicate z-scores of each bivariate relationship and are noted in bold font when the correlation between the bivariate relationship occurs at P<0.05. Arrow thickness corresponds with the magnitude of the z-score.

**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.

**Cramer W, Prentice IC**. **1988**. Simulation of regional soil moisture deficits on a European scale. *Norsk Geografisk Tidsskrift - Norwegian Journal of Geography* **42**: 149–151.

**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.

**Davis TW, Prentice IC, Stocker BD, Thomas RT, Whitley RJ, Wang H, Evans BJ, Gallego-Sala A V, Sykes MT, Cramer W**. **2017**. Simple process-led algorithms for simulating habitats (SPLASH v.1.0): robust indices of radiation, evapotranspiration and plant-available moisture. *Geoscientific Model Development* **10**: 689–708.

**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.

**Huber ML, Perkins RA, Laesecke A, Friend DG, Sengers J V, Assael MJ, Metaxa IN, Vogel E, Mareš R, Miyagawa K**. **2009**. New international formulation for the viscosity of H2O. *Journal of Physical and Chemical Reference Data* **38**: 101–125.

**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*.

**Kachurina OM, Zhang H, Raun WR, Krenzer EG**. **2000**. Simultaneous determination of soil aluminum, ammonium- and nitrate- nitrogen using 1 M potassium chloride. *Communications in Soil Science and Plant Analysis* **31**: 893–903.

**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.

**Keeney DR, Nelson DW**. **1983**. Nitrogen—Inorganic Forms. In: Page AL, ed. Methods of Soil Analysis. Madison, WI, USA: ASA and SSSA, 643–698.

**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.

**Mehlich A**. **1984**. Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. *Communications in Soil Science and Plant Analysis* **15**: 1409–1416.

**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.

**Rhoades JD**. **1983**. Soluble Salts. In: Page AL, ed. Methods of Soil Analysis. Madison, WI, USA: ASA and SSSA, 167–179.

**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.

**Rosseel Y**. **2012**. lavaan : An R Package for Structural Equation Modeling. *Journal of Statistical Software* **48**.

**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.

**Thieurmel B, Elmarhraoui A**. **2019**. suncalc: Compute sun position, sunlight phases, moon position, and lunar phase.

**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.