**Increasing whole-plant nitrogen demand reduces leaf nitrogen responses to soil nitrogen addition in grasslands**

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**Abstract**

Terrestrial carbon and nitrogen cycles are closely coupled. As such, the land surface components of Earth System Models are now beginning to include explicit nitrogen cycles and this has been shown to alter carbon cycling dynamics and, thus, climate feedbacks. An important aspect of this coupling within these models is the assumed positive correlations between soil nitrogen, leaf nitrogen per leaf area (*N*area), and photosynthetic capacity, which results in greater simulated leaf assimilation capacity in areas with more soil nitrogen. While these relationships have some empirical support, other studies have shown that *N*area and photosynthetic capacity are primarily determined by climate and that soil nitrogen availability, instead, leads to increased tissue development and/or storage. Here, we reconcile these differences using theory and data from a globally distributed experimental nitrogen addition network (Nutrient Network). We show that, across the network, soil nitrogen addition increases both *N*area and aboveground plant biomass. However, we find that soil nitrogen addition is a poor predictor of *N*area, which can be well modeled from leaf mass per leaf area and climate alone. Additionally, we find that the positive *N*area response to soil nitrogen decreases as the stimulation of biomass by soil nitrogen increases. These results reconcile discrepancies between past studies and shows that *N*area is the product of both soil nitrogen availability as well as whole-plant nitrogen demand. That is, in cases where plants use added soil nitrogen to grow new tissues, *N*area is primarily the product of climate and leaf structure. Whereas, a positive relationship between soil and leaf nitrogen is observed when added nitrogen is not used for growth. These dynamics will be important to include in the next generation of ESMs.

**Introduction**

Globally, carbon and nitrogen cycles are closely coupled. This coupling has a strong influence on carbon fluxes between the atmosphere and the Earth’s surface (Thornton et al., 2007). For instance, land plants rely on nitrogen to build photosynthetic enzymes (Evan 1989). Thus, nitrogen is an important regulator of carbon fluxes into terrestrial ecosystems, as indicated by Earth System Models (ESMs) that simulate reduced plant carbon assimilation when nitrogen constraints are considered (Wieder et al. 2015, Thomas et al., 2015, Thornton et al., 2007). Given ongoing addition of nitrogen to terrestrial ecosystems (Vitousek et al. 1997, Galloway et al. 2004, 2008), it is critical to understand how this will manifest itself in terrestrial ecosystems to reliably predict the rate and magnitude of future climate change.

ESMs typically assume a positive relationship between soil nitrogen availability, leaf nitrogen on an area basis (*N*area), and photosynthetic capacity. The positive correlation between *N*area and photosynthetic capacity is commonly observed (Walker et al., 2014, Kattge et al., 2009, Evan 1989) and is thought to be the result of the fact that photosynthetic enzymes are typically nitrogen-rich (Evans and Seeman 1989, Evans and Clarke 2019). However, the positive correlation between soil nitrogen availability and *N*area is not as straightforward. This is because plant nitrogen allocation is dynamic (Onoda et al., 2017) and is likely the product of both soil nitrogen availability and tissue or organ-specific plant nitrogen demand (Paillassa et al., 2020), which itself is environmentally dependent (Perkowski et al., in prep).

A few recent studies have highlighted the significantly positive relationship between soil nitrogen availability and *N*area (Firn et al., 2019, Li et al., 2020, Liang et al., 2020). These studies generally reason that this positive correlation stems from the fact that leaf photosynthesis is limited by nitrogen available to build nitrogen-rich proteins such as Ribulose-1,5-bisphosphate (Rubisco) that are involved in carboxylation. This generally follows previous conclusions from leaf-level analyses (Walker et al., 2014, Kattge et al., 2009). However, analyses on Rubisco carboxylation suggest that leaves are not carboxylation-limited and are instead well set up to maximize the utilization of available light in a given environment (Smith et al., 2019, Smith et al., 2020). So, an increase in leaf nitrogen to build Rubisco under nitrogen addition would be a wasteful process in the sense that the extra Rubsico would not increase photosynthesis unless it was accompanied by a similar increase in light energy. Nonetheless, a plant may choose to extra available nitrogen to build Rubisco as a means to maintain similar rates of photosynthesis at a lower stomatal conductance, effectively reducing nutrient use efficiency to increase water use efficiency (Wright et al., 2003). Global studies have found empirical support for this response in some contexts (Prentice et al., 2014, Paillassa et al., 2020).

Other studies have highlighted the importance of nitrogen demand for predicting *N*area (Dong et al., 2017, Onoda et al., 2017). Variations in demand is typically tied to variations in aboveground climatic conditions (Smith et al., 2020). Both ecophysiological theory and data (Smith et al., 2019, Dong et al., 2017) suggest that plant demand for nitrogen to build photosynthetic proteins decreases with temperature (Smith et al., 2020, Smith et al., 2018, Rogers et al., 2017, Hinojo-Hinojo, 2018, Ali et al., 2015, Smith et al., 2019, Dong et al., 2017, Paillassa et al., 2020) and CO2 (Smith et al., 2020, Ainsworth and Long, 2005, Ainsworth and Rogers, 2007, Leakey et al., 2009) and increases with light availability (Smith et al., 2019, Dong et al., 2017, Niinemets et al., 2015, Paillassa et al., 2020) and leaf-atmosphere vapor pressure deficit (Smith et al., 2019, Dong et al., 2017, Wang et al., 2017, Paillassa et al., 2020). In fact, previous data on Rubisco carboxylation capacity (Smith et al., 2019, Paillassa et al., 2020) and leaf nitrogen (Dong et al., 2017, Firn et al., 2019, Paillassa et al., 2020) suggest that this climate-driven changes in demand may be as, or even more important than soil nitrogen availability.

Plant ecophysiological theory (Wright et al., 2003, Franklin et al., 2020, Paillassa et al., 2020) provides a framework for reconciling the impact of soil nitrogen availability and plant nitrogen demand on *N*area. This theory suggests that a change in *N*area (∆*N*area) is the result of relative changes in nitrogen availability (∆*N*supply) and nitrogen demand (∆*N*demand):

∆*N*area ~ ∆*N*supply/∆*N*demand                                                                                                                (1)

where *N*supply is the nitrogen available for uptake and *N*demand is whole plant demand to build new tissues. Thus, an increase in *N*supply would increase *N*area as a means to increase water use efficiency only when there is a smaller accompanying increase in *N*demand (Figure 1 grey dashed line). If *N*demand changes in concert with *N*supply, we would expect no change in *N*area because all of the N would be allocated to build new tissues (Figure 1 black solid line). Different environmental contexts (e.g., canopy openness) may dictate how *N*demand varies with *N*supply and the resulting impact on *N*area.

Here, we use leaf-level and biomass data from a globally distributed grassland nutrient addition experiment, Nutrient Network (NutNet; Lind 2016), alongside ecophysiological theory to better understand the response of *N*area to nitrogen addition. Our aims were to (1) quantify and separate the impact of soil nitrogen, leaf traits, and climate on *N*area and (2) separate the impacts of nitrogen demand and nitrogen availability on *N*area. We hypothesized that soil nitrogen availability, leaf traits, and climate would have significant separate impacts on *N*area, but that the effect of soil nitrogen availability would be relatively weak due to the alternative ways in which plants can allocate available nitrogen. Following from this, we expected that *N*area responses would be weaker than leaf area index (LAI) responses because the optimal strategy for increasing productivity in grasslands would be to increase LAI rather than increase water use efficiency. Finally, we hypothesized that the *N*area response to soil nitrogen availability would be greatest in species that did not show a large increase in biomass, as these species likely were not exhibiting a concomitant increase in nitrogen demand.

**Methods**

*Nutrient Network Description*

The Nutrient Network (NutNet; Lind 2016) is a network of >100 replicated nutrient addition experiments in grassland worldwide. Each site in the network has followed a similar nutrient addition protocol, factorially adding nitrogen (N), phosphorus (P), and potassium plus a mix of macro- and micronutrients (K+µ). At each site, the experiment is set up as a randomized split-plot design with 3 replicate blocks each containing 10, 5m x 5m plots. N, P, and K were added as urea, triple super phosphate, and potassium sulphate, respectively, at each site annually at a rate of 10 g m-2 yr-1. The macro- and micronutrient mix (i.e., Fe, S, Mg, Mn, Cu, Zn, B, Mo, and Ca) was added to all K plots once. The oldest sites in the network have been adding nutrients since 2008.

*Datasets*

To test our hypotheses we utilized two datasets from the NutNet: (1) a leaf trait dataset (Firn et al., 2019) and (2) the NutNet core dataset (Lind 2016). The leaf trait dataset used consisted of leaf elemental, isotopic, and morphological variables. Samples were collected from up to five randomly selected individuals per plot, typically 3-4 years after nutrient addition at each site (see Firn et al., 2019). For our analyses, we selected samples that contained each of nitrogen concentration (*N*mass; g g-1), leaf mass per area (*M*area; m2 g-1), and δ13C (‰). *N*mass was converted to *N*area (g m-2) using *M*area:

*N*area = *N*mass / *M*area                                                                                                                          (2)

We calculated the ratio of intercellular to extracellular CO2 concentration (χ; Pa Pa-1) from δ13C following Farquhar et al. (1989) as:

∆13C = δ13Cair - δ13C / 1 + δ13C                                                                                                       (3)

where ∆13C (‰) is the leaf discrimination relative to air (δ13Cair; ‰), assumed to be -8 ‰. For leaves of C3 species, ∆13C was converted to χ as:

χ = ∆13C - a / bC3 - a                                                                                                                       (4)

where a and b were assumed to be 4.4‰ and 27‰, respectively (Farquhar et al., 1989). For leaves of C4 species, ∆13C was converted to χ as:

χ = ∆13C - a / bC4 - a                                                                                                                       (5)

where

bC4 = c + dφ                                                                                                                                   (6)

where c and d were assumed to be -5.7‰ and 30‰, respectively (Farquhar et al., 1989). The bundle sheath leakiness term (φ) was assumed to be 0.4. For use in our analyses, we selected individuals with χ values between 0.2 and 0.95. This resulted in 2048 individuals from 195 species at 22 sites (Figure 2).

The second NutNet dataset used was the NutNet “core” dataset. This dataset consisted of data collected similarly at each NutNet site, typically on a yearly basis. From this data we selected plot level biomass and aboveground interception of photosynthetically active radiation (PAR). Aboveground biomass (AGB; g) was sampled by hand within 0.2 m2 (two 10 x 100 cm) strips in each plot. Aboveground interception of PAR was typically sampled with a single PAR sensor or a light meter with multiple PAR sensors. To do this, a PAR measurement was taken above the canopy (PARabove; µmol m-2 s-1) and two PAR measurements were taken below the canopy (PARbelow; µmol m-2 s-1). The PARbelow measurements were averaged to a single value. We used these measurements to estimate the leaf area index (LAI; m2 m-2) of each plot following standard protocols (METER Group, Inc., Pullman, WA, USA) as:

LAI = -ln(PARbelow / PARabove) / 0.86                                                                                             (7)

To aid in comparison of the leaf data and AGB and LAI data, we only used biomass and LAI data from the same sites in the same year as the leaf data for our analyses.

*Climate Data*

The latitude and longitude of each site were used to extract mean annual growing season temperature (*T*g; °C), atmo- spheric vapour pressure deficit (*D*g; Pa) and incoming PAR (*I*g; µmol m2 s1) for each site from monthly, 1901–2015, 0.5° resolution data provided by the Climatic Research Unit (CRU TS3.24.01) (Harris et al. 2014). Growing season was operationally defined as months with mean temperatures greater than 0 °C. The elevation (*z*; m) at each site at 0.5° resolution was obtained from the WFDEI meteorological forcing dataset (Weedon et al. 2014).

*Analyses*

To assess the drivers of *N*area and their relative importance, we followed an analysis protocol similar to that described by Dong et al. (2017). First, we fit a linear mixed effects model with *N*area as the dependent variable and soil treatment variables (soil N treatment, soil P treatment, soil K+µ treatment, and their respective interactions), climate (*T*g, *D*g, and *I*g), leaf traits (χ and *M*area), and species characteristics (photosynthetic pathway and whether the plant has the known capacity to biologically fix nitrogen) as fixed effects. Soil treatment and species characteristics were categorical fixed effects and climate and leaf traits were continuous fixed effects in the model. Species identity, species identity by site, and species identity by site by block were included as categorical random intercept terms. *N*area was natural log transformed to meet normality assumptions. Predictors *D*g, *I*g, and *M*area were also natural log transformed.

We also analyzed the drivers of *N*area from a more predictive perspective, again following the approach by Dong et al. (2017). To do this, we first calculated a prediction of the nitrogen used for photosynthesis (*N*photo) as:

*N*photo = *N*Rubisco + *N*bioenergetics + *N*PEP                                                                                                  (8)

for C3 plants and

*N*photo = *N*Rubisco + *N*bioenergetics + *N*PEP                                                                                                  (9)

for C4 plants. To do this, we first calculated predicted optimal rates of photosynthetic processes following Smith et al. (2019) as modified in Smith and Keenan (2020) for C3 plants and an analogous model for C4 plants by Scott and Smith (in prep). Specifically, these models used measured χ and climate variables to calculate predicted optimal maximum rates of Rubisco carboxylation (*V*cmax,25; µmol m-2 s-1),  photosynthetic electron transport (*J*max,25; µmol m-2 s-1), and phosphoenolpyruvate (PEP) carboxylation (*V*pmax,25; µmol m-2 s-1; C4 plants only), all standardized to 25°C. Then, we calculated the predicted amount of nitrogen in Rubisco (*N*Rubisco)based on the model and parameterizations of Harrison et al. (2009):

*N*Rubisco  = *V*cmax,25*M*r*M*n[*N*r] / *k*cat,r*n*r                                                                                                  (9)

where *M*r is the molecular mass of Rubisco, 0.55 g Rubisco (μmol Rubisco)−1; [*N*r] is the nitrogen concentration of Rubisco, 0.0144 mol nitrogen (g Rubisco)−1; *M*n is the molecular mass of nitrogen, 14 g nitrogen (mol nitrogen)−1; *k*cat is the catalytic turnover at 25°C, 3,500,000 μmol CO2 (mol Rubisco sites\*seconds)−1; and *n*r is the catalytic sites per mol Rubisco, 8 mol sites (mol Rubisco)−1. We used *J*max,25 to estimate nitrogen in bioenergetics (*N*bioenergetics) following the approach by Niinemets and Tenhunen (1997):

*N*bioenergetics = *J*max,25*N*cyt / *j*mc                                                                                                           (10)

where *N*cyt is the nitrogen investment in bioenergetics (0.124 g N/ (μmol cyt f)) and *j*mc is the activity of electron transport at 25°C (156 μmol e− (μmol cyt f\*s)−1; Niinemets & Tenhunen, 1997). *N*PEP was calculated in a similar manner to *N*Rubisco, but with PEP-specific constants:

*N*PEP  = *V*pmax,25*M*p*M*n[*N*p] / *k*cat,p*n*p                                                                                                  (11)

where *M*p is the molecular mass of PEP, 0.41 g PEP (μmol PEP)−1; [*N*p] is the nitrogen concentration of PEP, assumed to be similar to Rubisco (Sage et al., 1987), 0.0144 mol nitrogen (g PEP)−1; *k*cat is the catalytic turnover at 25°C, 5,440,000 μmol CO2 (mol Rubisco sites\*seconds)−1(Boyd et al., 2015); and *n*r is the catalytic sites per mol PEP, assumed to be 2 mol sites (mol PEP)−1. We also calculated the nitrogen in structural tissue (*N*structure) using *M*area following the empirical approach described in Dong et al. (2017):

*N*structure = 10-2.67*M*area0.99                                                                                                                 (12)

We then fit a second linear mixed effects model with *N*area as the dependent variable and soil treatment variables (soil N treatment, soil P treatment, soil K+µ treatment, and their respective interactions), predicted nitrogen components (*N*photo and *N*structure), and species characteristics (photosynthetic pathway and whether the plant has the known capacity to biologically fix nitrogen) as fixed effects. Soil treatment and species characteristics were categorical fixed effects and predicted nitrogen components were continuous fixed effects in the model. Species identity, species identity by site, and species identity by site by block were included as categorical random intercept terms. *N*area was natural log transformed to meet normality assumptions.

To examine the response of AGB and LAI to the soil treatments, we fit a third and fourth linear mixed effects models with AGB and LAI as the dependent variables, respectively. In both models, soil treatment variables (soil N treatment, soil P treatment, soil K+µ treatment, and their respective interactions) were included as independent categorical variables. Site and site by block were included as categorical random intercept terms. In both cases, dependent variables were natural log transformed to meet normality assumptions.

In a final analysis, we explored the effect of soil nitrogen supply in relation to community nitrogen demand on *N*area. To do this, we calculated treatment type average *N*area, χ, *M*area, and AGB values for all plots at all sites. Within a site and P by K+µ treatment, we calculated the percent change in in *N*area (∆*N*area; %), χ (∆χ; %), *M*area (∆*M*area; %), and AGB (∆AGB; %) from the added soil N plots to the ambient soil N plots. We used mean absolute deviation (MAD; Leys et al., 2013) to remove instances where any ∆ values were 3 times higher than the MAD. We then fit a linear mixed effects model with ∆*N*area as the dependent variable and ∆AGB, ∆χ, ∆*M*area, and their interactions were included as independent variables. Soil treatment variables (soil P treatment, soil K+µ treatment, and their respective interactions) were also included as independent variables. Species identity, species identity by site, and species identity by site by block were included as categorical random intercept terms.

Throughout, all models were fit using the “lmer” package (Bates et al., 2015) in R version 3.5.3 (R Core Team, 2019; this R version was used for all analyses). We used Wald’s χ2 tests to test the statistical significance of each fixed effect term in the models using the “car” package (Fox & Weisberg, 2011) in R. Post hoc analyses were done using the “emmeans” package (Lenth, 2018) in R. For the first two models, relative importance of each variable was calculated as the R2 partitioned by averaging over orders (Lindemann et al., 1980) using the “calc.relimp” function in the “relaimpo” package (Grömping, 2006) in R.

**Results**

* Do these based on each question
  + Does soil N impact leaf Narea?
  + How does the impact of soil N on leaf Narea compare to other drivers, such as climate and LMA?
  + How does the leaf Narea response to soil N compare to the LAI response to soil N?
  + Does the impact of soil N on leaf Narea vary with leaf N demand, as indexed through the LAI response?

**Discussion**