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Physiological and structural tradeoffs underlying the leaf economics spectrum

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Summary

- The leaf economics spectrum (LES) represents a suite of intercorrelated leaf traits concerning construction costs per unit leaf area, nutrient concentrations, and rates of carbon fixation and tissue turnover. Although broad trade-offs among leaf structural and physiological traits have been demonstrated, we still do not have a comprehensive view on the fundamental constraints underlying the LES trade-offs.

- Here, we investigated physiological and structural mechanisms underpinning the LES by analysing a novel data compilation incorporating rarely-considered traits like dry-mass fraction in cell walls, nitrogen allocation, mesophyll CO_2 diffusion and associated anatomical traits for hundreds of species covering major growth forms.

- The analysis demonstrates that cell wall constituents are major components of leaf dry mass (18–70%), especially in leaves with high leaf mass per area (LMA) and long lifespan. A greater fraction of leaf mass in cell walls is typically associated with a lower fraction of leaf nitrogen (N) invested in photosynthetic proteins; and lower within-leaf CO_2 diffusion rates, due to thicker mesophyll cell walls.

- The costs associated with greater investments in cell walls underpin the LES: long leaf lifespans are achieved via higher LMA and in turn by higher cell wall mass fraction, but this inevitably reduces efficiency of photosynthesis.

Key words: anatomy, biodiversity, cell walls, leaf economics spectrum, mesophyll conductance, nitrogen allocation, photosynthesis.

Introduction

Leaf dry mass per area (LMA) varies globally several hundred-fold among the world's species (Poorter et al., 2009; Diaz et al., 2016), and up to c. 10-fold among co-occurring species (Wright et al., 2004). Low LMA, associated with leaves being thin or constructed of low density tissue (Witkowski & Lamont 1991; Niinemets, 2001), equates to a larger light-intercepting leaf area deployed for a given investment in leaf mass, which enables plants to grow faster as juveniles under ample supply of light and nutrients (Poorter, 1989). LMA is correlated with physiological traits such that on average species with lower LMA tend to have higher leaf nitrogen (N) concentration per unit mass (N_{mass}) and higher photosynthetic rate per unit N (photosynthetic N use efficiency, PNUE) (Poorter & Evans, 1998; Reich et al., 1998; Hikosaka, 2004; Wright et al., 2005), which also contribute to faster plant growth rates for a given condition (Poorter et al., 1990). At the same time, lower LMA leaves tend to have lower physical strength and shorter leaf lifespans (Wright & Westoby, 2002; Wright et al., 2004; Onoda et al., 2011). These leaf trait relationships largely underpin the worldwide 'leaf economic spectrum' (LES) (Reich et al., 1997; Wright et al., 2004). The LES captures about three-quarters of the interspecific variation in key traits concerning carbon fixation and nutrient use (Wright et al., 2004); at one end of the spectrum, species are characterized by low investment per unit area of light capturing surface (i.e. low LMA), high N and phosphorus (P) concentrations (N_{mass} and P_{mass}, respectively), high respiration rates and light-saturated photosynthetic rates per unit mass (A_{mass}), and short leaf lifespan – meaning that their faster photosynthetic returns are not sustained for long periods. Species at the other end of the spectrum have the opposite set of traits, implying that they are likely to have a longer income stream from investments in leaf tissue, compensating for their

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higher construction costs per unit leaf area (Chabot & Hicks, 1982; Mooney & Gulmon, 1982; Merino et al., 1984; Reich et al., 1991; 1997; Wright et al., 2004; Kikuzawa et al., 2013; Reich, 2014).

Despite broad recognition of the LES from local to global scales, we still do not have a comprehensive understanding on the fundamental constraints underlying the LES (Shipley et al., 2006; Blonder et al., 2011, 2013, 2015; Sack et al., 2013; Villar et al., 2013; Grubb, 2016; Mason et al., 2016). In this study we focus on how LMA is associated with physiological processes underpinning the LES by exploring variation in its underlying anatomical-physiological traits (e.g. mesophyll anatomy, mass allocation to cell walls, and N partitioning between metabolic and structural components), and their relationship to photosynthetic rates. As our focus is on physiological processes, here we start to compare traits on the same basis of expression, that is, area-based traits such as LMA, light-saturated photosynthetic rates per unit leaf area (hereafter A_{area}) and leaf N content per unit leaf area (hereafter N_{area}) (Table 1 shows the list of abbreviations). As a starting point we note that LMA and A_{area} are almost unrelated at the global scale while LMA and N_{area} are positively correlated (Wright et al., 2004). We argue that these tendencies can be understood as the outcome of two opposite effects that largely cancel out. First, all else being equal, leaves with higher-LMA might be expected to have higher A_{area}, if the greater thickness is due to thicker mesophyll layers (Niinemets, 1999). Second, leaves with higher LMA might be expected to have lower A_{area}, if the higher LMA connotes underlying anatomical variation such as greater cell wall mass per unit volume that reduces photosynthetic rates due to lower CO_2 diffusion and/or lower fraction of N in photosynthetic proteins (detailed in later section).

Shipley et al., (2006) explored trait relationships and trade-offs likely to underpin the LES using graphical methods and structural equation models (SEMs), suggesting that 'verbal' causal models (e.g. Wright et al., 2004) were not supported by the available data.

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Rather, their analysis indicated that key trait relationships were likely to operate via one or more unmeasured ('latent') variables, which they suggested might be the ratio of cell wall to cytosolic volume (a higher ratio leading to longer leaf lifespan and also a lower A_{mass}). While the importance of cell walls in the variation of photosynthetic rates has been suggested elsewhere (Rackham, 1966; Lambers & Poorter, 1992; Hikosaka et al., 1998; Onoda et al., 2004; Terashima et al., 2006), the hypothesis has not been tested widely due to a general lack of data. Scattered evidence suggests that cell walls (consisting of primary and secondary cell walls) constitute a considerable proportion of leaf dry mass (e.g. 30–50%, Merino et al., 1984; 20–60%, Read et al., 2003; 37–69%, Kitajima et al., 2012). The cell wall fraction (cell wall mass per unit leaf mass, CW_{mass}) tends to be higher in leaves with higher LMA (Read et al., 2003; Poorter et al., 2009). More cell walls increase physical strength (e.g. force-to-fracture) and therefore confer greater resistance to biotic and abiotic stresses such as herbivory, wind, drought and freezing (Onoda et al., 2011). At the same time, increasing the fraction of leaf mass invested in cell walls could decrease the efficiency of photosynthesis in two chief ways: (1) via a N allocation trade-off (Onoda et al., 2004), and/or (2) via CO_2 diffusion limitation (Evans et al., 2009; Terashima et al., 2011; Flexas et al., 2012). Later we describe these hypotheses in detail, with reference to the conceptual framework shown in Fig. 1.

Hypothesis 1: N allocation trade-off

In leaves, a large fraction of N is allocated to chloroplasts; as much as 70–80% in some vegetable and crop species (Evans & Terashima, 1987; Makino & Osmond, 1991). Rubisco, a key enzyme of photosynthesis, accounts for 30–40% of chloroplast N. However, these numbers cannot be automatically applied to wild plants. N allocation to Rubisco varies substantially among species and tends to be lower in woody than in herbaceous plant types (Evans, 1989; Hikosaka & Shigeno, 2009). This suggests that N allocation to

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nonphotosynthetic components may be important for understanding the leaf N economy (Field & Mooney, 1986; Lambers & Poorter, 1992). Cell walls account for a large fraction of leaf mass, and primary cell walls contain 2–10% proteins (c. 0.3–1.5%[N] in primary cell walls and little is known for secondary cell wall concentrations) (Lamport, 1965; Carpita & McCann, 2000; Held et al., 2015). Cell wall proteins function in defense, growth, development, signaling, intercellular communication and environmental sensing (Showalter, 1993; Kieliszewski et al., 2010). If cell walls contain substantial N, one might expect a trade-off between N allocation to cell walls and to photosynthetic proteins. Onoda et al. (2004) examined this possibility, indeed finding a trade-off between N allocation to cell walls and Rubisco among individuals of the deciduous herb Polygonum cuspidatum that differed in germination timing and therefore leaf lifespan. Further support has come from both intraspecific (Feng et al., 2009; Guan & Wen, 2011) and interspecific studies (Takashima et al., 2004). However, the generality of this trade-off is not yet clear since some studies did not find any such trend (Harrison et al., 2009; Hikosaka & Shigeno, 2009; Funk et al., 2013).

Hypothesis 2: CO_2 diffusion limitation

A higher mass of cell wall per leaf area (hereafter CW_{area}) could reflect thicker mesophyll cell walls, which would restrict CO_2 diffusion to chloroplasts; a larger total surface area of mesophyll cells per unit leaf area, which in theory would increase CO_2 diffusion to chloroplasts; or more structural tissues (e.g. vascular bundles, epidermis, and trichomes). The thickness of mesophyll cell walls ranges from 0.1 to 0.5 µm in angiosperms, generally with lower values reported for herbaceous plants and higher values for woody species, especially evergreen trees and shrubs (Evans et al., 2009; Terashima et al., 2011; Tosens et al., 2012). Some rather high values are also known for ferns and fern allies (up to 0.8 µm; Tosens et al., 2015). Thicker mesophyll cell walls increase resistance to CO_2 diffusion,

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thereby reducing mesophyll conductance and the CO_2 concentration at carboxylation sites (Rackham, 1966; Nobel et al., 1975; Terashima et al., 2006; Evans et al., 2009; Niinemets et al., 2009; Tosens et al., 2012). On the other hand, thicker leaves with more cell layers can have a greater total mesophyll surface area per unit leaf area (S_m) and therefore accommodate a greater chloroplast surface area facing the intercellular airspaces (S_c), thereby enhancing mesophyll conductance (Nobel et al., 1975). Thus, the nature of the relationship between LMA and mesophyll conductance may be subject to the balance between these opposing effects. Recent studies suggest that an inherently high LMA is generally associated with lower mesophyll conductance and lower chloroplast CO_2 concentration (Niinemets et al., 2009; Tosens et al., 2012, 2015), yet it has not been investigated how mesophyll conductance more broadly relates to the LES.

These two hypotheses are largely independent and not mutually exclusive, and both could contribute towards shaping the LES. To our knowledge, there is no study that has examined both the leaf N allocation trade-off and the CO_2 diffusion limitation in relation to the LES on the same set of species, therefore a strict test to quantify their relative importance on the LES is currently not feasible. That said, it is still possible to investigate these issues and to assess their relative importance via gathering data concerning each factor and analyzing them in relation to LMA (see the Materials and Methods section). Indeed, that is the approach taken in this study. Our overarching aim is to summarize current knowledge and data from the literature as well as unpublished works, in relation to these hypotheses. Both hypotheses are strongly related to cell walls, therefore first we summarize to what extent CW_{area} and CW_{mass} vary across species and how they are related to LMA. Second, we test how cell wall fractions are associated with leaf N allocation, in particular to cell wall and Rubisco. Third, we investigate how LMA influences CO_2 diffusion via changes in mesophyll anatomy including wall thickness. Fourth, we compare to what extent leaf N

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allocation and mesophyll CO_2 diffusion differ with respect to LMA. Based on these analyses, we then discuss the implications for understanding leaf trait variation among species along the leaf economics spectrum.

Materials and Methods

Analytical framework

In this section we formulate equations that describe how traits required for long leaf lifespan (i.e. high LMA, and underlying anatomical and physiological traits) are associated with lower efficiency of photosynthesis via (1) a N allocation trade-off (Eqn 6 for Hypothesis 1) and (2) CO_2 diffusion limitation (Eqn 8 for Hypothesis 2) (Fig. 1). First, we focus on cell walls, which are a critical component determining structural integrity and therefore leaf lifespan, but also play an important role in photosynthesis. Cell wall content per unit leaf area (CW_{area}) can be expressed as the product of LMA (mass/area) and cell wall mass per unit leaf mass (CW_{mass});

$$CW_{area} = CW_{mass} \, LMA$$

Eqn 1

CW_{area} varies in direct proportion to LMA when CW_{mass} is constant. When CW_{mass} is higher in high LMA leaves (e.g. Read et al., 2003), CW_{area} may disproportionally increase with LMA.

The light-saturated rate of photosynthesis per unit area (A_{area}) can be expressed according to Fick's law:

$$A_{area} = g_m \, (C_i - C_c) = g_s \, (C_a - C_i) = g_{tot} \, (C_a - C_c)$$

Eqn 2

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where C_i, C_c and C_a are CO_2 concentrations in the intercellular airspace, chloroplasts and ambient air respectively, and g_m, g_s and g_{tot} are mesophyll-, stomatal- and total (mesophyll + stomatal) conductances to CO_2. In this study, we largely focus on g_m because g_m is more tightly associated with leaf anatomy (Tosens et al., 2012), and g_m and g_{tot} are tightly correlated ($R^2 = 0.69$ in our dataset, see also Flexas et al., 2008; Warren, 2008).

Since we are interested in how an increase in LMA reduces the efficiency of photosynthesis (i.e. PNUE = A_{area}/ N_{area}), PNUE can be expressed with Eqn 2 as follows;

$$PNUE = \frac{g_m}{N_{area}}(C_i\text{-}C_c) \qquad\qquad \text{Eqn 3}$$

Eqn 3 can be further decomposed by considering N allocation to photosynthetic proteins and the CO_2 exchange surface, that is, the mesophyll cell surface where the chloroplasts are attached expressed per unit leaf area, S_c (Terashima et al., 2002);

$$PNUE = \frac{g_m}{S_c}\frac{S_c}{N_p}\frac{N_p}{N_{area}}(C_i\text{-}C_c) \qquad\qquad \text{Eqn 4}$$

where N_p is the amount of N associated with photosynthesis per unit area. This equation shows that PNUE can be expressed as the product of mesophyll conductance per chloroplast surface area (g_m/S_c), the ratio of chloroplast surface area to photosynthetic proteins ($S_c : N_p$), the fraction of N allocated to photosynthetic proteins (N_p/N_{area}), and CO_2 drawdown from intercellular spaces to chloroplasts (C_i - C_c). In this study, we pay particular attention to g_m /S_c and N_p/N_{area} because we hypothesized these two terms are responsible for low PNUE in high-LMA leaves, as explained later.

N allocation trade-off (Hypothesis 1)

Cell walls have a certain concentration of N (n_{CW}), thus cell wall N content per unit leaf area (N_{CW}) can be expressed as follows;

$$N_{CW} = n_{CW}CW_{area} \qquad\qquad \text{Eqn 5}$$

High CW_{area} leaves (with high LMA) are expected to have higher N_{CW}. Furthermore, when the cell wall fraction (CW_{mass}) is high, the fraction of total leaf N in cell walls (N_{CW}/N) is also expected to be high unless the concentration of N in cell walls (n_{CW}) itself decreases with CW_{mass} and overrides this effect. Since a substantial portion of leaf N up to 20% is thought to reside in cell walls (Lamport, 1965; Onoda et al., 2004; Takashima et al., 2004), cell walls can be considered as one of the major N fractions. Leaf N content can be considered as the sum of cell wall-N (N_{CW}), photosynthetic-N (N_p), and other cytosolic N (N_o). Therefore, if there is only limited variation in N allocation to other cytosolic N (N_o/N), the fraction of N allocated to cell walls (N_{CW}/N) must trade-off (be inversely proportional to) with the fraction of N allocated to photosynthetic proteins (N_p/N):

$$\frac{N_p}{N} = 1 - \frac{N_{CW}}{N} - \frac{N_o}{N} \qquad\qquad \text{Eqn 6}$$

In this study we assume that Rubisco-N represents about a half of all photosynthetic-N (Evans & Seemann, 1989). Rubisco-N content per unit leaf area (N_{Rub}) is calculated from the amount of Rubisco, assuming 16% of Rubisco mass consists of N (i.e. $N_{Rub} = 0.16$ Rubisco).

CO$_2$ diffusion limitation (Hypothesis 2)

CW_{area} can be considered as the sum of mesophyll, vascular and epidermal cell walls, considered per unit leaf area (putting aside the fact that some species may also have a small proportion of cell wall mass in other tissues such as trichomes or idioblasts). Little is known

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about the relative allocation of cell wall mass between these tissue-types (Poorter et al., 2009), although it does seem that the thickness of epidermal cell walls varies far more than that of mesophyll cell walls (Onoda et al., 2015). In this study, we assume a certain fraction of CW_{area} is associated with mesophyll cell walls ($CW_{m,area}$).

$CW_{m, area}$ can be expressed as the product of total mesophyll surface area per unit leaf area (S'_m), average thickness of mesophyll cell wall (T_{CW}), and the specific gravity of cell walls (ρ_{CW}).

$$CW_{m, area} = S'_m \, T_{CW} \, \rho_{CW} \qquad \text{Eqn 7}$$

A larger total mesophyll cell surface can accommodate more chloroplasts and increase mesophyll diffusion conductance if enough N is available to produce the chloroplasts. (Note that we use S_m for total mesophyll surface area exposed to the intercellular space per unit leaf area, which may be more closely associated with gas-exchange rates than S'_m while S'_m may be more closely associated with cell wall mass than S_m. S_m is smaller than S'_m by c. 30% (Milla-Moreno et al., 2016).) On the other hand, thicker mesophyll cell walls and greater ρ_{CW} may decrease mesophyll conductance per unit chloroplast surface area, g_m/S_c (Evans et al., 2009; Terashima et al., 2011; Tosens et al., 2012), which is one of the components determining PNUE as shown in Eqn 4. In this study we focus on S_m and T_{CW} but not ρ_{CW} because very few data are available for ρ_{CW}. We hypothesize that greater T_{CW} in leaves with greater LMA contributes to lower PNUE by reducing g_m/S_c, as another possible physiological mechanism underpinning the LES. This second hypothesis may be expressed as follows:

$$\frac{g_m}{S_c} = \frac{a}{T_{CW}} \qquad \text{Eqn 8}$$

where a represents a combination of other terms including diffusivity, porosity and membrane permeability (Tosens et al., 2012). Note that the total mesophyll diffusion conductance consists of the gas-phase conductance and the liquid phase conductance, including wall conductance (Niinemets & Reichstein, 2003; Evans et al., 2009; Tosens et al., 2012). In most plants the gas phase resistance (inverse of conductance) is negligible and most anatomical limitations (including cell wall) to CO_2 diffusion are in the liquid phase (Warren et al., 2008; Terashima et al., 2011), therefore we do not explicitly consider the gas-phase conductance in our analysis.

Data

This study used three existing datasets as well as newly compiled data. The Glopnet dataset (Wright et al., 2004) and its related dataset a 'Global Amax' database (Maire et al., 2015) were used to examine relationships among LMA, A_{area}, N_{area}, stomatal conductance and leaf longevity; and a 'Leaf Biomechanics' database (Onoda et al., 2011) was used to investigate relationships between LMA and leaf physical strength. Since the trend of the LES makes best sense for well-lit leaves, we only used data for well-lit leaves from the Leaf Biomechanics database.

The newly compiled data consists of three subdatasets: cell wall dataset, including CW_{area}, CW_{mass} and LMA (Supporting Information Table S2); leaf N allocation dataset, including A_{area}, CW_{area}, $N_{CW,area}$, LMA, N_{area}, and Rubisco (Table S3); and (mesophyll conductance dataset, including A_{area}, g_m, g_s, LMA and leaf anatomical traits (Table S4). A single unified dataset was not produced because each dataset was more or less independent, due to a lack of studies that covered these multiple datasets. These newly compiled datasets are available in Tables S2–S4. Data were extracted from literature as well as published and unpublished data provided by the authors.

LMA is defined as the oven dried mass of a leaf divided by its predried projected

area. In some studies the petiole is included when measuring LMA, in others it is excluded, and in others still the authors do not make clear what protocol was followed (see Supporting Information). In general, LMA including petiole can be higher by c. 10%, but there is considerable variation across species. In this study, we used the published/measured values without conversion, and we note that this difference can be a source of error in traits–LMA relationships. Total leaf N content was typically measured with a CN analyzer.

Cell walls are made up of complex macromolecules consisting of pectin, hemicellulose, cellulose, lignin and structural proteins. Cell walls can be extracted by various methods such as alcohol extraction, detergent extraction and organic solvent extraction from ground samples at cold or heated temperatures (Fry, 1988). Neutral detergent fiber (NDF) is the most popular indicator of cell wall content in plant ecological studies. NDF is typically extracted from ground dried material with neutral detergent at a boiling temperature for 1 h (Van Soest, 1994). NDF represents most of the cell wall components but pectin is inevitably removed by this method. Pectin accounts for 20–35% of primary cell walls in forb species and 2–10% in graminoid species but very little of secondary cell walls (Vogel, 2008), therefore the hot extraction method may underestimate the cell walls fraction by up to 10% of leaf mass if primary cell walls occupy 30% of leaf mass. On the other hand, some starch, cuticle compounds (e.g. cutin) and some minerals (e.g. silica) may also be contained in NDF. The fraction of leaf mass in cutin is usually small (0.5–4%, Goñi & Hedge, 1990) and some species such as some graminoids accumulate silica up to 9% (Cooke & Leishman, 2011). In short, NDF represents a conservative estimate of cell wall mass except for some graminoid species. Since it is not straightforward to convert cell wall mass data among different protocols due to variation in chemical composition among species, we used raw values without conversion in our analysis.

Cell wall N is normally measured from extracted cell walls with a CN analyzer (Onoda et al., 2004) or with amino acid analysis after digestion (Takashima et al., 2004). To

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measure cell wall N mass, it is important to extract cell walls without contamination of cytosolic proteins. In particular, cytosolic proteins can become insoluble with heat or drying or by binding with polyphenolics, and contaminate the cell wall N fraction. Therefore oven-dried samples should not be used for quantification of cell wall N for studies of leaf economy. Fresh frozen samples may be the best for the cell wall extraction but freeze-dried samples could be used (Harrison et al., 2009). Some cell wall proteins are strongly or covalently attached to the polysaccharides while others are weakly bound to cell walls, therefore cell wall N recovery rates depend on the strength of the solvents used (Lamport, 1965; Fry, 1988). If a weak extraction solvent is used, weakly-bound cell wall proteins can be extracted, but contamination of cell wall N fraction by cytosolic proteins will remain a concern. On the other hand, when a strong solvent is used, the amount of cell wall N is underestimated due to loss of weakly-bound cell wall proteins. Most data in this study were measured with strong solvents, therefore the value reported in this study may be a conservative estimate of cell wall N.

The amount of Rubisco can be measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) **[Author, please confirm inserted text 'sodium dodecyl sulfate polyacrylamide gel electrophoresis' is correct]**, capillary electrophoresis or immunoblotting method (Makino, 1986; Warren, 2004). Some studies have also estimated the amount of Rubisco from the maximum carboxylation capacity of Rubisco by inverting the Farquhar et al. (1980) photosynthesis model (Niinemets & Tenhunen, 1997).

Mesophyll conductance was variously measured via a combined chlorophyll fluorescence/gas exchange method (Harley et al., 1992), carbon isotope discrimination (Evans et al., 1986), and/or curve fitting to A_{area}–C_i relationship (Ethier & Livingston, 2004). Mesophyll surface area exposed to the intercellular space per unit leaf area (S_m) and chloroplast surface area exposed to the intercellular space per unit leaf area (S_c) were

generally measured on micrographs according to the methodology recommended by Evans et al. (1994) and Syvertsen et al. (1995). Mesophyll cell wall thickness (T_{CW}) was measured from electron micrographs (e.g. Tosens et al., 2012).

Most of these trait data were measured on outer-canopy leaves of field-grown plants, but we also include data from several growth experiments conducted in glasshouses or controlled environments where strong light (>500 μmol PPFD m^{-2} s^{-1}) was used for plant growth. For each species at each site or experiment, the mean value for each trait was used.

Analysis

Most data were log-transformed to improve the normality before the analysis. Pearson's correlation coefficient was used to quantify the degree of association between traits. When correlations were significant (P < 0.01), the log–log 'scaling' relationships were quantified with standardized major axis (SMA) slopes (Warton et al., 2006), fitted with the 'smatr' package in the R statistical software (version 3.2.2, R Foundation for Statistical Computing, Vienna, Austria). Species were categorized into four functional groups: evergreen woody species, deciduous woody species, graminoid species (e.g. Poaceae and Cyperaceae), and non-graminoid angiosperm herbaceous species (hereafter 'herbs'). Some but not all datasets included limited data for ferns; because of this poor representation these were excluded before analysis.

To assess the relative importance of the (1) N allocation trade-off and (2) CO$_2$ diffusion limitation in relation to the cross-species variation in PNUE, Eqn 4 can be expressed as follows after log-transformation:

$$log(PNUE) = log(\frac{g_m}{S_c}) + log(\frac{S_c}{N_p}) + log(\frac{N_p}{N_{area}}) + log(C_i - C_c) \qquad \text{Eqn 9}$$

This equation assumes that relative changes in N$_p$/N$_{area}$ and in g$_m$/S$_c$ have additive effects on

logPNUE. Since no study has measured all these components simultaneously on the same set of species, it is currently not possible to examine relative change in N_p/N_{area} and in g_m/S_c against logPNUE. Instead, the relative importance of these components can be explored by comparing the scaling slopes of g_m/S_c and N_{Rub}/N_{area} (assuming Rubisco content and photosynthetic protein contents are linearly correlated) across LMA. This analysis is possible when these trait data are available across a wide range of LMA. All analyses were run using the R statistical package.

Results

The overall relationships among A_{area}, N_{area} and LMA were largely similar between our newly compiled dataset and those fitted to the 'Global Amax' dataset (Maire et al., 2015; Fig. 1a–c). In that data compilation A_{area} was almost unrelated to LMA ($R^2 = 0.004$; Fig. 2a) and our dataset showed a weak negative correlation ($R^2 = 0.12$). N_{area} and LMA were positively correlated in both datasets ($R^2 = 0.43$ and 0.14; details given in caption to Fig. 2b). As a logical consequence, photosynthetic nitrogen use efficiency PNUE (A_{area}/N_{area}) was on average lower in high-LMA species in both datasets ($R^2 = 0.26$ and 0.49 for Maire et al., 2015 and our dataset respectively; Fig. 2c). On the other hand, high-LMA leaves had longer leaf lifespan (Wright et al., 2004; $R^2 = 0.42$; Fig. 2d). Note that our dataset included not only wild plants but also experimentally-grown plants that often had high A_{area} and N_{area} for a given LMA (presumably due to abundant nutrient supply), yet the negative correlation between PNUE and LMA was similar irrespective of plant growth conditions.

Leaf cell walls

As expected (Eqn 1; Fig. 1), there was a strong correlation between cell wall mass per unit leaf area (CW_{area}) and LMA across 416 species ($R^2 = 0.79$; Fig. 3a). The log–log scaling slope (SMA) of this relationship was 1.37 with 95% CI from 1.30 to 1.42 (ordinary least

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square regression slope 1.21 with 95% CI from 1.15 to 1.27), indicating that CW_{area} disproportionally increased with LMA: on average, a 10-fold increase in LMA was accompanied by a c. 23-fold increase in CW_{area}. In general, herbs, graminoids and deciduous woody species had low LMA and low CW_{area}; woody evergreen species had both high LMA and CW_{area}. High-LMA leaves had higher physical strength, that is, higher 'force to punch' as measured with a penetrometer ($R^2 = 0.30$; Fig. 3b; Onoda et al., 2011). CW_{mass} ranged from 19 to 66% (90% quantile range, hereafter 90%QR) with an average of 40%. Herbaceous and deciduous woody species typically had lower CW_{mass} than evergreen woody species (mean ± SD; 29 ± 10%, 28 ± 8% and 42 ± 12%, respectively). Graminoids had notably high CW_{mass} (60 ± 9%), reflecting the fact that many graminoid leaf blades are self-supporting (they lack stems) (Grubb et al., 2008). CW_{mass} and LMA were modestly correlated ($R^2 = 0.10$; Fig. 3c) and when graminoids were excluded the correlation was stronger ($R^2 = 0.17$).

Leaf N allocation

N concentration in cell walls (n_{CW}) ranged from 0.20 to 1.75% of cell wall mass (90%QR) and was unrelated to LMA (Fig. 4a). Cell wall N content per unit leaf area (N_{CW}) increased tightly with LMA ($R^2 = 0.55$; Fig. 4b), due to the higher CW_{area} in high LMA leaves. Leaf N allocation to cell walls (N_{CW}/N) ranged from 2.8 to 25% (90%QR) with an average of 11.2% (Fig. 4c). There was a positive correlation between LMA and the fraction of N allocated to cell walls ($R^2 = 0.37$; Fig. 4c), but also apparent in that scatterplot is the rather wide variation in N allocation at high LMA, whereas low-LMA species only showed low fractional N allocation to cell walls.

Rubisco content per unit leaf area was not significantly correlated with LMA (Fig. 4d). N allocation to Rubisco (N_{Rub}/N) varied from 9.2 to 26.6% (90%QR) with an average of 17.2%, and was significantly lower in high LMA leaves ($R^2 = 0.27$; Fig. 4e). On average,

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herbaceous plants allocated a slightly larger fraction of leaf N to Rubisco ($23.2 \pm 5.5\%$) than did woody deciduous or woody evergreen species ($21.1 \pm 4.0\%$ and $14.6 \pm 4.5\%$, respectively). There was a significant negative correlation between N allocation to cell walls and Rubisco with the slope of -0.69 ($R^2 = 0.10$; Fig. 4f), in support of the first hypothesis: N allocation to cell walls trades-off with N allocation to Rubisco. Three evergreen conifers (these are all the conifers in this dataset) were notable outliers in this relationship; they had both relatively high N allocation to cell walls as well as Rubisco. When these conifers were excluded from the analysis, the negative correlation was stronger ($R^2 = 0.29$) and the slope was steeper (slope = -0.83, 95%CI = 0.61-1.02), closer to our hypothesized value of -1 (Eqn 6).

Mesophyll conductance

Traits associated with CO_2 diffusion are shown in relation to LMA (Fig. 5a–f). Stomatal and mesophyll conductance for CO_2 diffusion (g_s and g_m) had similar ranges across species. Both were significantly negatively associated with LMA but the correlations were weak ($R^2 = 0.07$ and 0.11; Fig. 5a,b).

As expected from Eqn 7 LMA, which was positively associated with CW_{area}, was significantly positively correlated with both mesophyll cell wall thickness, T_{CW} ($R^2 = 0.41$; Fig. 5c) and mesophyll surface area per unit area, S_m ($R^2 = 0.28$; Fig. 5d). Across 15-fold variation in LMA, T_{CW} varied five-fold and S_m varied four-fold. Yet, the greater S_m in high LMA leaves did not directly translate into greater chloroplast surface area per unit leaf area (S_c); S_c was not correlated with LMA (Fig. 5e). The proportion of S_m covered by chloroplasts ($= S_c/S_m$) varied widely but was significantly lower in high LMA species ($R^2 = 0.11$; Fig. 5f). The overall correlation between S_c and N_{area} was not significant (Fig. 5g). Mesophyll cell wall thickness was tightly and negatively correlated with mesophyll conductance per unit S_c (g_m/S_c, $R^2 = 0.50$; Fig. 5h), supporting the second hypothesis, that mesophyll cell wall

thickness trades-off with CO_2 diffusion conductance.

Relative contributions to LES

To assess the relative importance of (1) the N allocation trade-off and (2) CO_2 diffusion limitation in relation to LES, fractional N allocation to Rubisco, g_m/S_c and C_i–C_c were each plotted against LMA and their scaling slopes were summarised (Fig. 6). The scaling slope of N allocation to Rubisco–LMA relationship was -0.59 and that of g_m/S_c–LMA relationship was -1.20 (Fig. 6d), indicating that CO_2 diffusion conductance per unit S_c declined more rapidly than N allocation to Rubisco with increases in LMA. C_i–C_c on the other hand was higher in high-LMA leaves, with a scaling slope of +0.57. Note that these relationships were all statistically significant, but there was scatter along each slope (R^2 = 0.28 and 0.20 for N_{Rub}/N–LMA relationship and g_m/S_c–LMA relationship, respectively).

Discussion

While the importance of cell walls in limiting the rate of leaf photosynthesis has been suggested by several studies, here we show across many species that a substantial portion of leaf mass is allocated to cell walls (19–67%, 90%QR) and relate this information to photosynthetic variables. The two proposed hypotheses for the fundamental trade-off in relation to the LES – first a N allocation trade-off between cell walls and photosynthetic proteins, and second a trade-off between mesophyll cell wall thickness and CO_2 permeability – were both supported by the available data. Later we first discuss these hypotheses in detail, and then we discuss ecological implications for leaf functional trait variation among species.

Leaf N allocation

Higher LMA leaves had higher cell wall N per unit area (N_{CW}) and also higher cell wall N per unit total leaf N (N_{CW}/N). It is known that cell walls have several kinds of structural

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proteins that are tightly cross-linked in cell walls (Cassab, 1998; Carpita & McCann, 2000), therefore a greater fraction of leaf mass in cell walls may be directly translated into a greater allocation of N to cell walls. Yet, N concentration in cell walls was quite variable across species (0.2–1.75%, 90%QR), and its functional importance is still poorly known. A part of the variation in N concentration in cell walls might be due to the differences in extraction methods because the recovery of cell wall proteins depends on extraction methods (see the Materials and Methods section), yet large variations in N concentration in cell walls were observed within studies where a single extraction method was used (Harrison et al., 2009; Hikosaka & Shigeno, 2009). Structural proteins are more abundant in primary cell wall rather than secondary cell walls (Carpita & McCann, 2000), therefore one might expect high-LMA species, which often have secondary wall thickening in epidermal and vascular tissues, to have lower N concentration in cell walls than low-LMA leaves. The current dataset did not show any such pattern, although one previous study reported such a relationship (Harrison et al., 2009). There is some evidence that graminoid species have lower concentrations of structural proteins in their cell walls than forb species (Cassab, 1998; Vogel, 2008), and this may be partly responsible for their relatively lower N allocation to cell walls. In addition, high silica accumulation in graminoids (0.1–10% of leaf dry mass, typically <2%; Epstein, 1999; Cooke & Leishman, 2011) could also reduce cell wall N concentrations in graminoids.

There was a significant negative correlation between N allocation to cell walls and to Rubisco (Fig. 4f). Assuming that Rubisco constitutes about a half of photosynthesis-related leaf N (Evans & Seemann, 1989), the summed N allocation to photosynthetic proteins and cell walls was on average 46% (90%QR 30–70%). This means that photosynthetic proteins and cell walls are major players in the leaf N economy, and it would not be surprising that there is a trade-off between N allocation between these two components. On the other hand, this also means that c. 50% of leaf N consists of materials

that are neither photosynthetic proteins nor cell walls, and there was rather large variation in the summed N allocation to the two components across species. These results suggest that there is broad flexibility in N allocation across species and may be a reason why some previous studies across fewer species did not find a negative relationship between N allocation to Rubisco and to cell walls. Our view is that this is a glaring and as yet unanswered, fundamental question in plant ecology-physiology. So then, what are the other key locations of N in leaves?

N is allocated to several other pools besides proteins associated with photosynthesis and cell walls. Nucleic acids may account for c. 10–15% of leaf N (Chapin & Kedrowski, 1983; Evans & Seemann, 1989). There are amino acids and numerous proteins in the cytosol and mitochondria not directly related to photosynthesis which have not been adequately quantified. Some arid plants may have high concentrations of glycine betaine or proline (Ashraf & Foolad, 2007) which may account for up to 10% of leaf N (Erskine et al., 1996). There are also some species that have N-rich defensive compounds such as alkaloids and cyanogenic glycoside which account for up to 15% of leaf N (Miller & Woodrow, 2008). Nitrate could be accumulated up to 30% of leaf N in some herbaceous species especially when soil N availability is high (Evans & Poorter, 2001). N_2-fixing species may have more non-photosynthetic N than non-N_2 fixing plant species as they often have higher N_{area} but lower PNUE (Adams et al., 2016).

Anatomy and mesophyll conductance

High-LMA leaves had both high mesophyll surface area (S_m) and thicker cell walls (T_{CW}) (Fig. 5c,d; Eqn 7), which can have positive and negative effects respectively on g_m and therefore on A_{area}. However, the positive effect due to larger S_m may be marginal because chloroplast surface area (S_c) did not increase with LMA despite their higher N_{area}. The lack of overall correlation between N_{area} and S_c across species suggests that N allocation to

chloroplasts may be low in high-LMA leaves, which also supports the first hypothesis. On the other hand, thicker mesophyll cell walls were strongly and negatively correlated with g_m/S_c, which supports the second hypothesis. The log–log scaling SMA slope between g_m/S_c and T_{CW} was -2.41, indicating that g_m/S_c decreased disproportionately more with increase in cell wall thickness. This may suggest that thicker cell walls have lower cell wall porosity (Evans et al., 2009; Tosens et al., 2012). There was some variation in g_m/S_c for a given cell wall thickness, suggesting that variation in other physiological processes could also be important (Terashima et al., 2006; Flexas et al., 2008; 2013); e.g. CO_2 diffusion related to aquaporin (Hanba et al., 2004; Flexas et al., 2006), carbonic anhydrase activity (Coleman, 2000), chloroplast movement (Tholen et al., 2008), and mitochondrial CO_2 release (Tholen et al., 2012).

Regardless of the detailed causes of lower mesophyll conductance, an important ecological question is why thicker mesophyll cell wall is a feature of high-LMA leaves, at the expense of photosynthetic rate, when one may think that thicker epidermal cell walls and cuticles are enough to protect leaves, and thinner mesophyll cell walls should be always better for photosynthesis and water use efficiency (Flexas et al., 2013). We considered at least two advantages for thicker mesophyll cell walls. First, long-lived leaves are more likely to experience strong drought stress at some time during their life, therefore rigid mesophyll structures may be required to maintain the mesophyll anatomy and consequently the photosynthetic activity over a long period. This notion may be supported by the evidence that high-LMA leaves had higher bulk elastic modulus measured by pressure chamber techniques (Niinemets, 2001). Second, thicker mesophyll cell walls may be important in defense against herbivores especially leaf miners. Leaf miners preferentially feed on mesophyll tissues, which cannot be prevented by stronger epidermal tissues once leaf miners get into the inner leaf lamina tissue (Kimmerer & Potter, 1987; Sinclare & Hughes, 2010), therefore long-lived leaves may in some sense require thicker mesophyll cell walls.

Synthesis

The scaling slope of N allocation to Rubisco–LMA relationship was less steep than that of the g_m/S_c–LMA relationship (- 0.59 vs -1.20), suggesting that the gas diffusion limitation may contribute more to the lowering of PNUE in high-LMA leaves. However, it is still too early to conclude the relative importance because there is no single study that compared the relative importance of N allocation trade-off and CO_2 diffusion limitation in relation to LES on the same set of species. Furthermore, there are inter-correlations among the components of PNUE in Eqn 9, which requires careful consideration in interpreting the relative importance of each component. One of the remaining components of PNUE, C_i–C_c, was correlated positively with LMA (slope = 0.57; see also Evans et al., 2009; Niinemets et al., 2009), which partly counteracted the effects of N allocation to Rubisco and g_m/S_c on PNUE. Larger C_i–C_c should be associated with higher carboxylation capacity (i.e. Rubisco content and/or activity) (Wright et al., 2003) and/or lower g_m (Niinemets et al., 2009), therefore the relative importance of N allocation to Rubisco and g_m/S_c may depend on the strength of inter-correlations among these components. While we do not have enough data to examine the strength of these inter-correlations, the range of variation of C_i-C_c (approx. seven-fold) was smaller than the ranges of variations in N allocation to Rubisco (11-fold) and in g_m/S_c (68-fold) across species. This suggests that N allocation to Rubisco and g_m/S_c are both key components contributing to the negative PNUE–LMA relationship even if C_i–C_c is taken into account. Available knowledge for the fourth component of PNUE, S_c/N_p, is limited, ranging from 5.4 to 12.5 m^2 g^{-1} Rubisco for three herbaceous species (Evans et al., 1994; Miyazawa & Terashima, 2001; Oguchi et al., 2003). Since the sum of the earlier-mentioned three scaling slopes was -1.23 and similar to the slope of the PNUE–LMA relationship (-0.96, Fig. 6d), S_c/Photo-N_{area} may not correlate strongly with LMA.

Shipley et al. (2006) proposed that variation among species in a single unmeasured

trait 'the ratio of cell volume to cell wall volume' is responsible for generating the LES. Their hypothesis is partly similar to our hypothesis in that cell walls play a major role in LES. However, their hypothesis was different from ours in how cell walls were assumed to be linked with photosynthesis. They considered cell walls as the dry mass cost for long-lived leaves, but they did not explicitly consider how cell walls affect the key physiological process of photosynthesis through their effects on mesophyll diffusion conductance and nitrogen allocation. This may be the reason why they did not detect a link between photosynthesis and N despite N being an essential resource for photosynthesis.

Concluding remarks

In this study we studied physiological processes underpinning the leaf economics spectrum – how traits required for long leaf lifespan influenced photosynthetic processes. By summarizing the current available data, we showed that cell walls are a major constituent of leaves that is tightly linked with leaf lifespan and photosynthetic rate. Cell walls accounted for 18–70% of leaf mass, with the proportion increasing as LMA increased. We showed how the cell wall fraction influenced photosynthesis via two processes; first, higher cell wall fraction in high-LMA leaves was accompanied by higher fractional N allocation to cell walls which, in turn, reduced N fractional allocation to photosynthetic proteins. Second, thicker mesophyll cell walls in high LMA leaves reduced CO_2 diffusion to the sites of carboxylation. The lower efficiency of photosynthesis in long-lived leaves is complemented by higher N_{area}, which largely equalizes A_{area} across LMA (Fig. 2a). It could be argued that the potential A_{area} in sunlit leaves is roughly invariant across LMA and thereby leaf lifespan because the maximum solar energy, which determines the potential for photosynthetic rate, is independent from disturbance regimes, whereas leaf lifespan varies extensively with disturbance regimes, such that short-lived species are favored in frequently disturbed environments while long-lived species are favored in stable environments. These tendencies

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may result in larger interspecific variation in leaf lifespan (22-fold for the 90%QR of leaf lifespan, n = 749; Wright et al., 2004) than in A_{area} (eight-fold for the 90%QR of A_{area}, n = 2400; Maire et al., 2015). High LMA and high cell wall mass are required for long leaf lifespan, which reduce the efficiency of photosynthesis due to the fundamental trade-offs. However, the lower efficiency of photosynthesis can be complemented by greater N_{area}, thereby largely equalizing A_{area} across species with different LMA and leaf lifespan.

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Author contributions

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Table S1 The list of references that were used to make Figs 3–6

Table S2 Cell wall dataset including species information, site locations, LMA, CW_{area}, CW_{mass} and references

Table S3 Leaf N allocation dataset including species information, growth conditions, LMA, N_{area}, N_{mass}, CW_{mass}, CW_{area}, N_{CW}, Rubisco, A_{area} and references

Table S4 Leaf anatomy and conductance dataset including species information, growth conditions, LMA, g_s, g_m, C_a, C_i, C_c, S_m, S'_m, S_c, T_{CW}, N_{area}, A_{area} and references

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Fig. 1 The conceptual framework underpinning this study, illustrating how major traits in the leaf economics spectrum (in red letters) are putatively linked via the underlying physiological/anatomical traits. The inset shows the overarching scheme, where A_{area} is the product of leaf nitrogen (N) **[Author, please confirm inserted text 'nitrogen' is appropriate]** content per unit leaf area (N_{area}) and photosynthetic N use efficiency (PNUE), which themselves are positively and negatively associated with leaf mass per area (LMA), respectively. The main figure illustrates presumed linkages between the physiological and anatomical traits (pink background) that together determine PNUE. Traits in grey boxes are expressed per unit leaf area, and double-headed arrows indicate hypothetical trade-offs corresponding to the hypotheses 1 and 2 (H1 and H2, see text for detail). Black and blue arrows denote hypothetical positive and negative relationships respectively. C_a, ambient CO_2 concentration; C_c, chloroplast CO_2 concentration; C_i, intercellular CO_2 concentration; g_m, mesophyll conductance; g_s, stomatal conductance; LL, leaf longevity; N_{CW}, cell wall-N;

N_p, photosynthetic-N; N_{Rub}, Rubisco-N; S_c, chloroplast surface area per unit leaf area; S_m, mesophyll surface area per unit leaf area; T_{CW}, thickness of mesophyll cell wall.

Fig. 2 Relationships between some major leaf traits and leaf mass per area (LMA). (a) Leaf photosynthetic rate per unit area (A_{area}); (b) leaf nitrogen (N) **[Author, please confirm inserted text 'nitrogen' is appropriate]** content per unit area (N_{area}); (c) photosynthetic N use efficiency (PNUE) (= A_{area}/N_{area}) and (d) leaf longevity. The data compiled in this study are shown in the closed symbols with black margin, and the data from previous studies are shown in half-transparent symbols (a–c, Maire et al., 2015; d, Wright et al., 2004). Standardized major axis (SMA) slopes are fitted to the data of this study except for (d). SMA equations (the slopes being the exponents): (a) $y = 549x^{-0.899}$, $R^2 = 0.13$, P < 0.001, n = 293 for this study and $y = 0.126x^{0.936}$, $R^2 = 0.004$, P = 0.006, n = 1964 for Maire et al., (2015); (b) $y = 0.0889x^{0.655}$, $R^2 = 0.141$, P < 0.001, n = 217 for this study and $y = 0.0495x^{0.774}$, $R^2 = 0.43$, P < 0.001, n = 1665 for Maire et al., (2015); (c) $y = 439x^{-0.958}$, $R^2 = 0.49$, P < 0.001, n = 213 for this study and $y = 369x^{-0.924}$, $R^2 = 0.26$, P < 0.001, n = 1665 for Maire et al., (2015); (d) $y = 0.0031x^{1.71}$, $R^2 = 0.42$, P < 0.001, n = 678 (Wright et al., 2004).

Fig. 3 Relationships between leaf structural traits and leaf mass per area (LMA). (a) Cell wall mass per unit leaf area (CW_{area}). (b) Force to punch (maximum force required for the punch rod to penetrate a leaf lamina, expressed per unit circumference of the punch rod). (c) The fraction of leaf mass in cell walls (CW_{mass}). Standardized major axis (SMA) slopes were fitted when correlations were deemed significant (P < 0.01); (a) $y = 0.0643x^{1.37}$, $R^2 = 0.79$, n = 416; (b) $y = 2.78e{-}05x^{2.07}$, $R^2 = 0.30$, n = 561; (c) $y = 1.69x^{0.648}$ $R^2 = 0.19$, n = 392 for data without graminoid species (black line) and $y = 1.67x^{0.661}$, $R^2 = 0.10$, n = 416 for all data (dashed line). The references used to make these figures are summarized in Supporting Information Table S1.
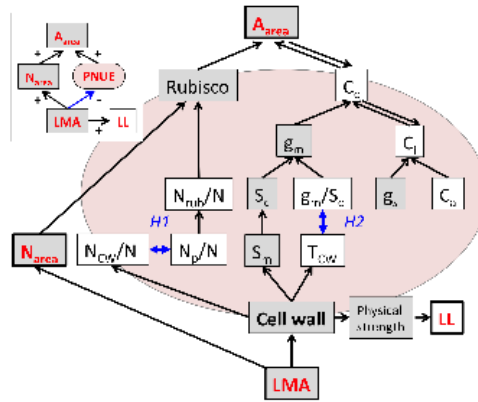
Fig. 4 Leaf nitrogen (N) **[Author, please confirm inserted text 'nitrogen' is appropriate]** allocation traits and leaf mass per area (LMA). (a) N concentration in cell walls (n_{CW}), (b) cell wall-N per unit leaf area (N_{CW}), (c) cell wall N per unit leaf nitrogen (N_{CW}/N), (d) Rubisco mass per unit leaf area (N_{Rub}), (e) Rubisco N per unit leaf nitrogen (N_{Rub}/N) and (f) relationship between the fractions of N allocated to cell walls and Rubisco. Standardized major axis (SMA) slopes were fitted when correlations were deemed significant ($P < 0.01$); (a) no correlation, $n = 67$; (b) $y = 0.000355x^{1.39}$, $R^2 = 0.55$, $n = 94$; (c) $y = 28.7\log_{(x)}-41.5$, $R^2 = 0.37$, $n = 94$; (d) no correlation, $n = 138$; (e) $y = -22.1\log_{(x)}+58.3$, $R^2 = 0.27$, $n = 103$; (f) $y = -0.685x + 25.4$, $R^2 = 0.10$, $n = 75$ for all data and $y = -0.832x + 26.1$, $R^2 = 0.29$, $n = 72$ excluding three conifer data. The references used to make these figures are summarized in Supporting Information Table S1.

Fig. 5 Leaf traits associated with CO_2 diffusion. (a) Stomatal conductance (g_s), (b) mesophyll conductance (g_m), (c) mesophyll cell wall thickness (T_{CW}), (d) mesophyll surface area exposed to intercellular airspace per unit leaf area (S_m), (e) chloroplast surface exposed to intercellular airspace area per unit leaf area (S_c), (f) the ratio of S_c to S_m, are plotted against leaf mass per area (LMA). (g) S_c is plotted against leaf nitrogen (N) **[Author, please confirm inserted text 'nitrogen' is appropriate]** content per unit area (N_{area}), and mesophyll conductance (g_m) is plotted against mesophyll cell wall thickness. Standardized major axis (SMA) slopes were fitted when correlations were deemed significant ($P < 0.01$); (a) $y = 47046x^{-1.24}$, $R^2 = 0.07$, $n = 942$; (b) $y = 27005x^{-1.2}$, $R^2 = 0.11$, $n = 153$; (c) $y = 0.033x^{0.472}$, $R^2 = 0.41$, $n = 74$; (d) $y = 2.83x^{0.458}$, $R^2 = 0.28$, $n = 91$; (e) no correlation, $n = 91$; (f) $y = 5.0x^{-0.468}$, $R^2 = 0.11$, $n = 89$; (g) no correlation, $n = 64$; (h) $y = 0.307x^{-2.41}$, $R^2 = 0.50$, $n = 92$. The references used to make these figures are summarized in Supporting Information Table S1.
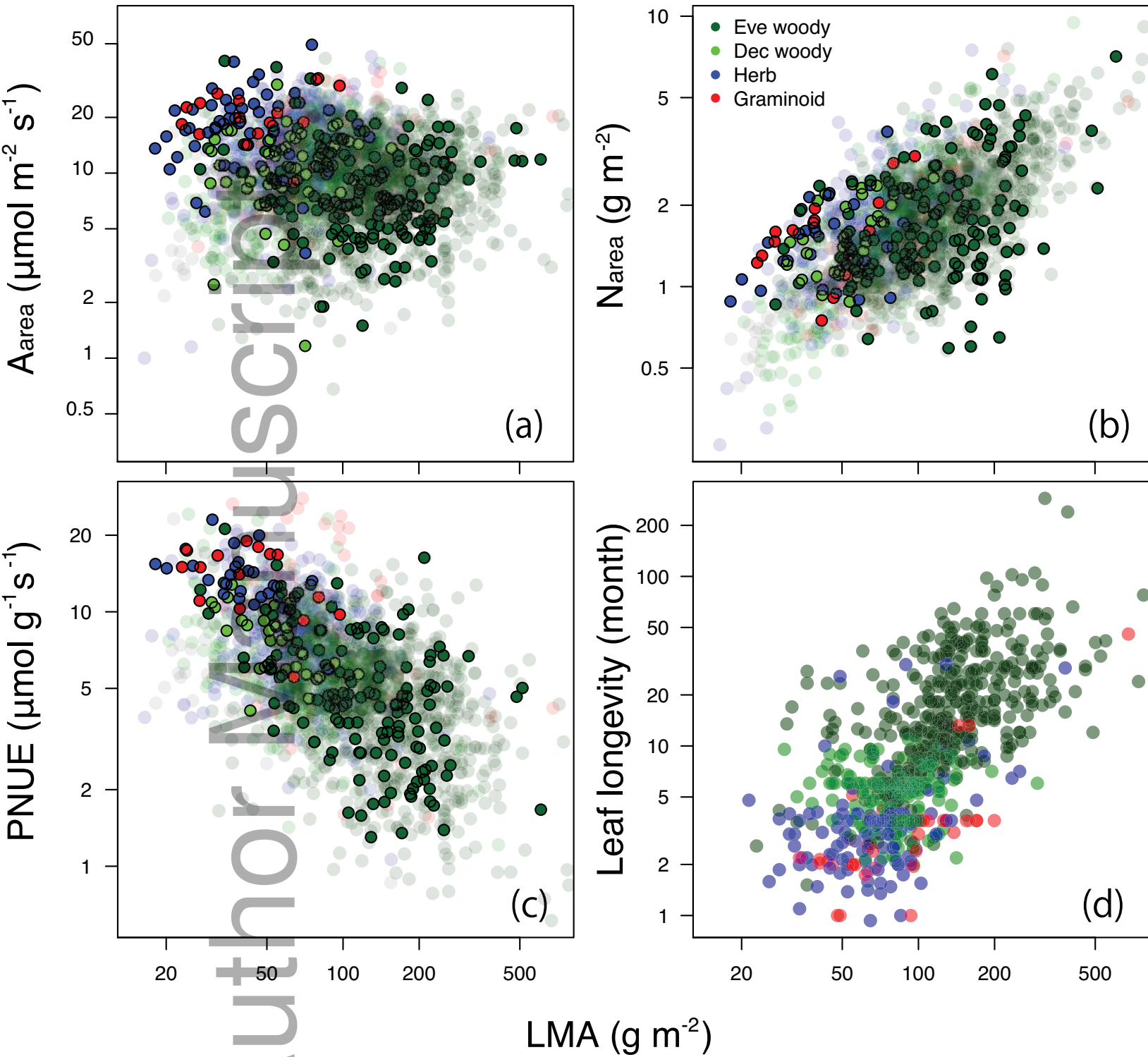
Fig. 6 The underlying components of photosynthetic nitrogen (N) **[Author, please confirm inserted text 'nitrogen' is appropriate]** use efficiency (PNUE) in relation to leaf mass per area (LMA). (a) Percentage of leaf N allocated to Rubisco (N_{Rub}/N), (b) mesophyll conductance per unit chloroplast area (g_m/S_c), (c) difference in CO_2 concentration between intercellular airspace and the sites of carboxylation (C_i–C_c) and (d) summary table of these scaling slopes and their 95% confidence intervals. (a) Fig. 4(e) replotted with the Y-axis log-scaled, so that the scaling slopes of different relationships can be compared (see main text for detail). Standardized major axis (SMA) slopes: (a) $y = 204x^{-0.595}$, $R^2 = 0.28$, $P < 0.001$, n = 103; (b) $y = 1709x^{-1.20}$, $R^2 = 0.20$, $P < 0.001$; n = 85; (c) $y = 6.11x^{0.569}$, $R^2 = 0.12$, $P < 0.001$, n = 120.

Table 1 A list of abbreviations used in this study

Abbreviation	Definition	Unit
A_{area}	Net assimilation rate per unit leaf area	$\mu mol\ m^{-2}\ s^{-1}$
A_{mass}	Net assimilation rate per unit leaf drymass	$nmol\ m^{-2}\ s^{-1}$
C_a	Ambient CO_2 concentration	$\mu mol\ mol^{-1}$
C_c	Chloroplast CO_2 concentration	$\mu mol\ mol^{-1}$
C_i	Intercellular CO_2 concentration	$\mu mol\ mol^{-1}$
CW_{area}	Cell wall mass per unit leaf area	$g\ m^{-2}$
$CW_{m,area}$	Cell wall mass of mesophyll per unit leaf area	$g\ m^{-2}$
CW_{mass}	Cell wall mass per unit leaf mass	$\%$
g_m	Mesophyll conductance for CO_2	$mmol\ m^{-2}\ s^{-1}$
g_s	Stomatal conductance for CO_2	$mmol\ m^{-2}\ s^{-1}$
LES	Leaf economics spectrum	
LL	Leaf lifespan	month
LMA	Leaf mass per area	$g\ m^{-2}$
N_{area}	Leaf nitrogen (N) content per unit leaf area	$g\ m^{-2}$
N_{CW}	Cell wall N content per unit leaf area	$g\ m^{-2}$
n_{CW}	N concentration in cell walls	$\%$
N_{mass}	Leaf N concentration	$\%$
N_o	Other cytosolic N (excluding photosynthetic N) content	$g\ m^{-2}$
N_p	Photosynthetic N content	$g\ m^{-2}$
N_{Rub}	Rubisco N content per unit leaf area	$g\ m^{-2}$
PNUE	Photosynthetic N use efficiency measured at saturating light intensity ($=A_{area}/N_{area}$)	$\mu mol\ [CO_2]\ g[N]^{-1}\ s^{-1}$
ρ_{CW}	Specific gravity of mesophyll cell walls	$g\ m^{-3}$
S_c	Surface area of chloroplasts exposed to intercellular airspace per unit leaf area	$m^2\ m^{-2}$
S_m	Surface area of mesophyll exposed to intercellular airspace per unit leaf area	$m^2\ m^{-2}$
S'_m	Surface area of mesophyll cells per unit leaf area	$m^2\ m^{-2}$
T_{CW}	Cell wall thickness of mesophyll	m

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Legend (panel b):
- Eve woody
- Dec woody
- Herb
- Graminoid

(a) A_{area} ($\mu mol\ m^{-2}\ s^{-1}$) vs LMA

(b) N_{area} ($g\ m^{-2}$) vs LMA

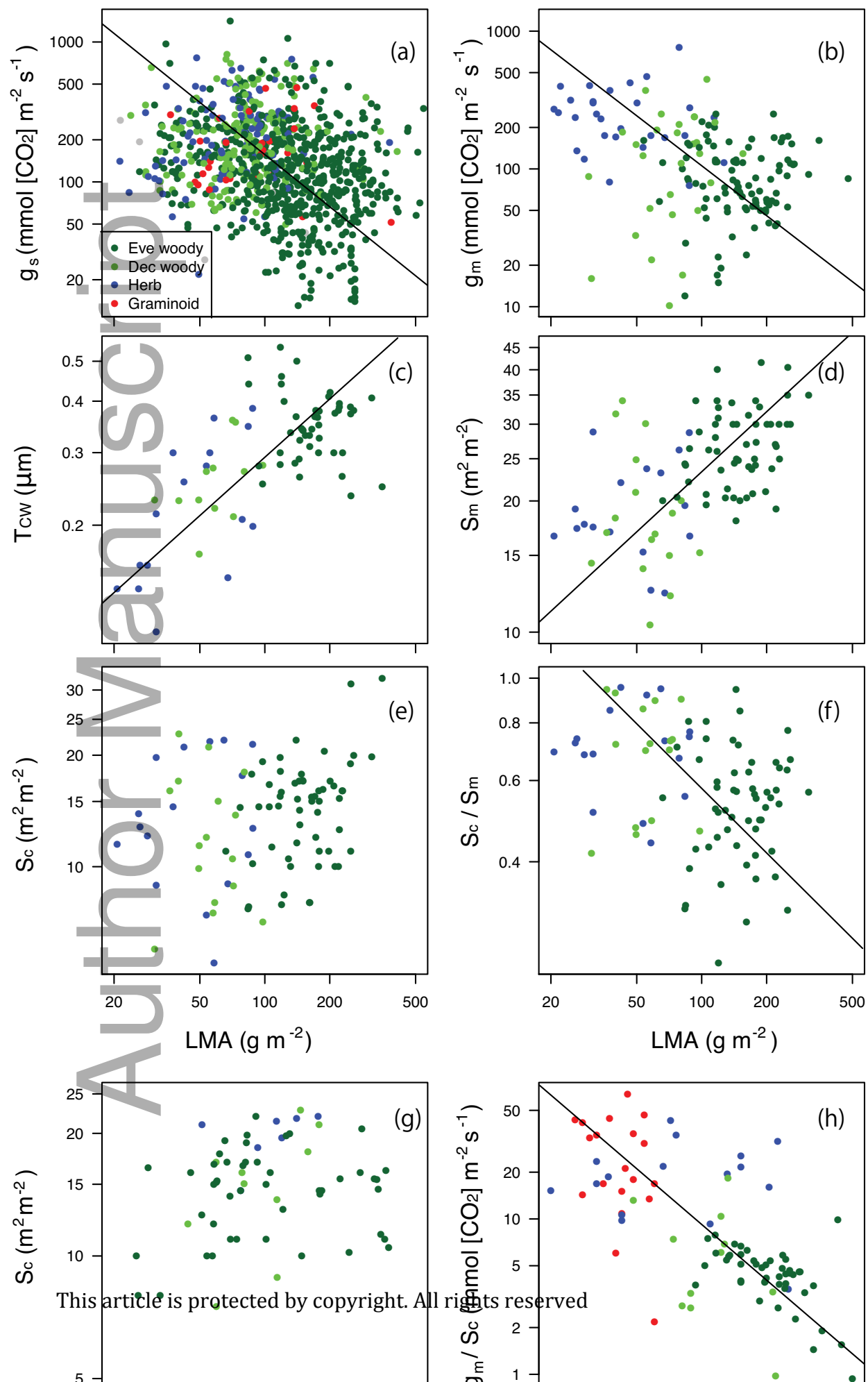(c) PNUE ($\mu mol\ g^{-1}\ s^{-1}$) vs LMA

(d) Leaf longevity (month) vs LMA

LMA ($g\ m^{-2}$)
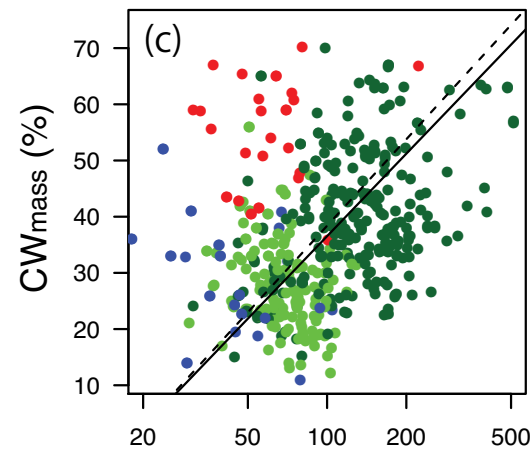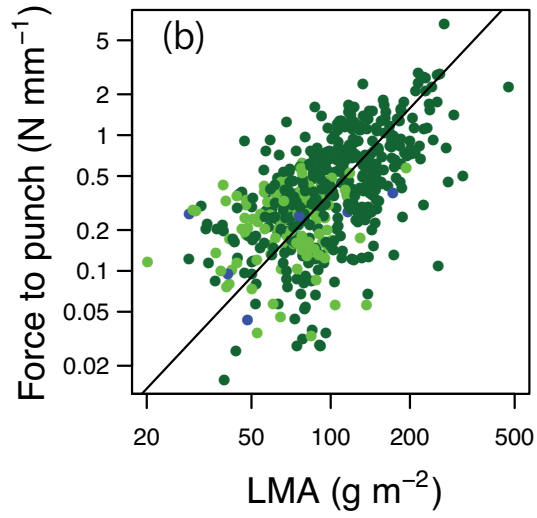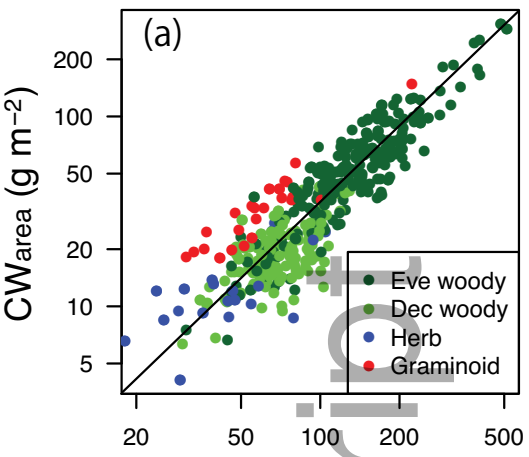
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(a) — N_Rub / N_area (%) vs LMA (g m⁻²)
Legend: Eve woody, Dec woody, Herb, Graminoid

(b) — g_m / S_c (mmol [CO₂] m⁻² s⁻¹) vs LMA (g m⁻²)

(c) — C_i–C_c (µmol mol⁻¹) vs LMA (g m⁻²)
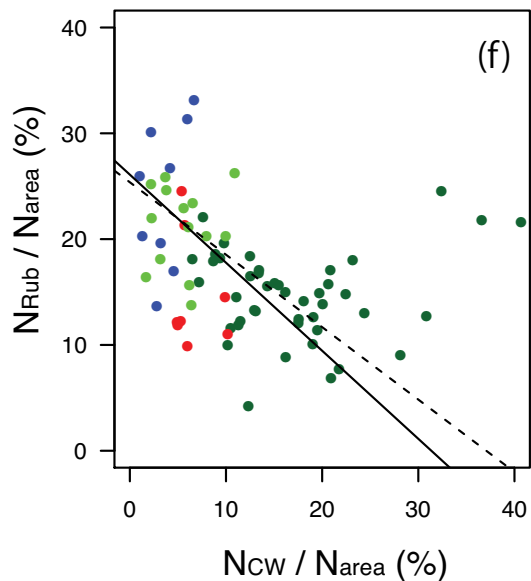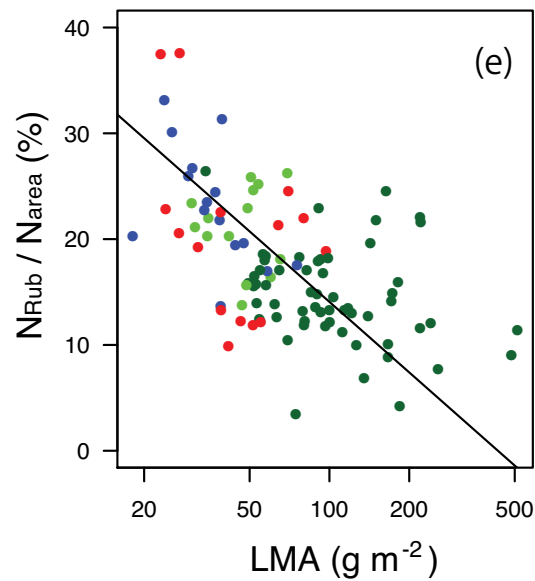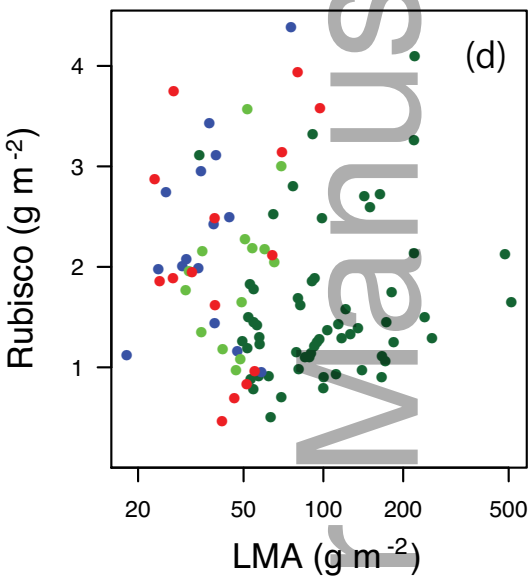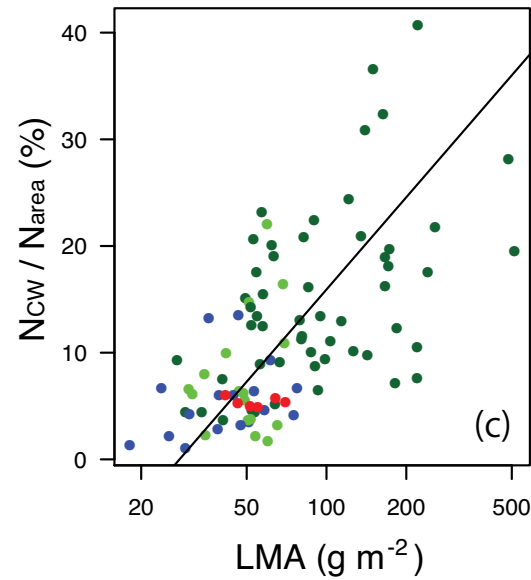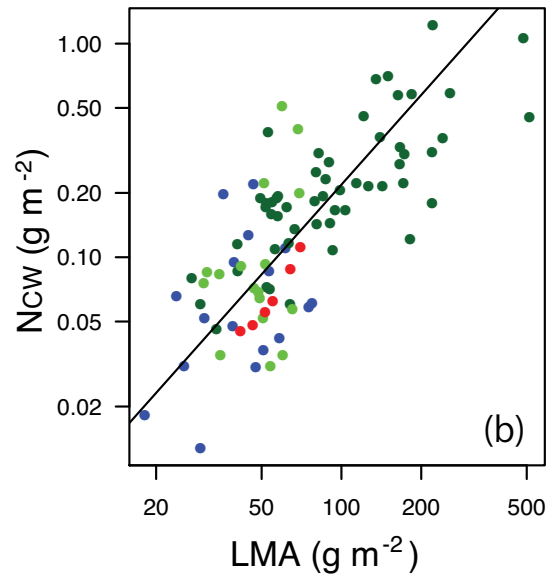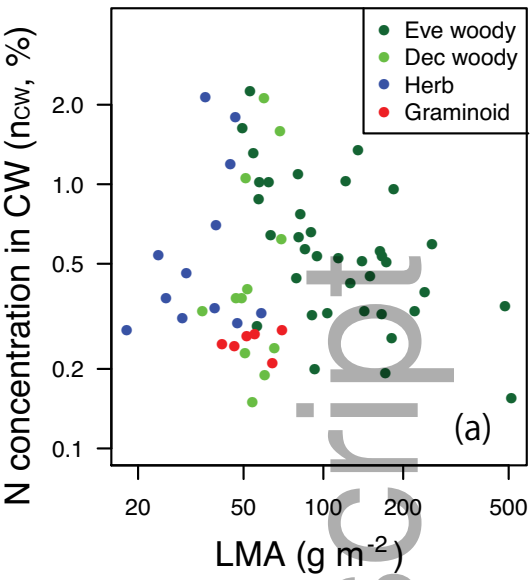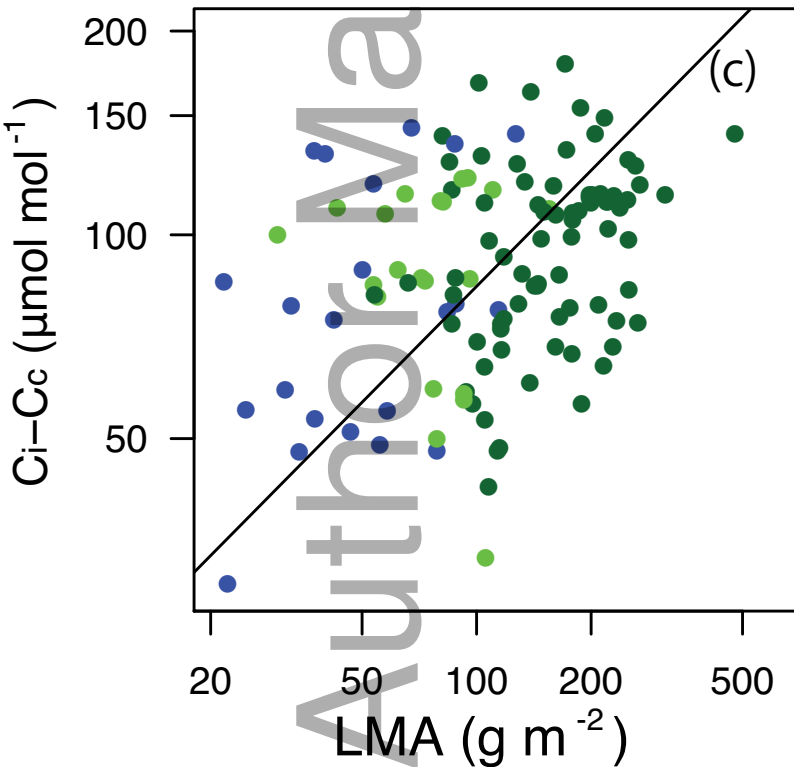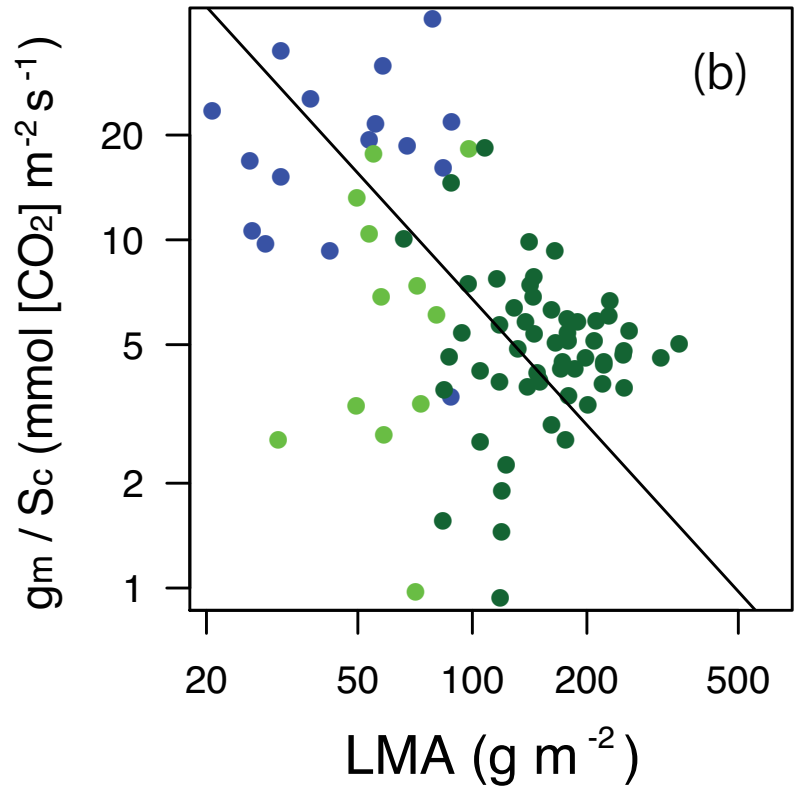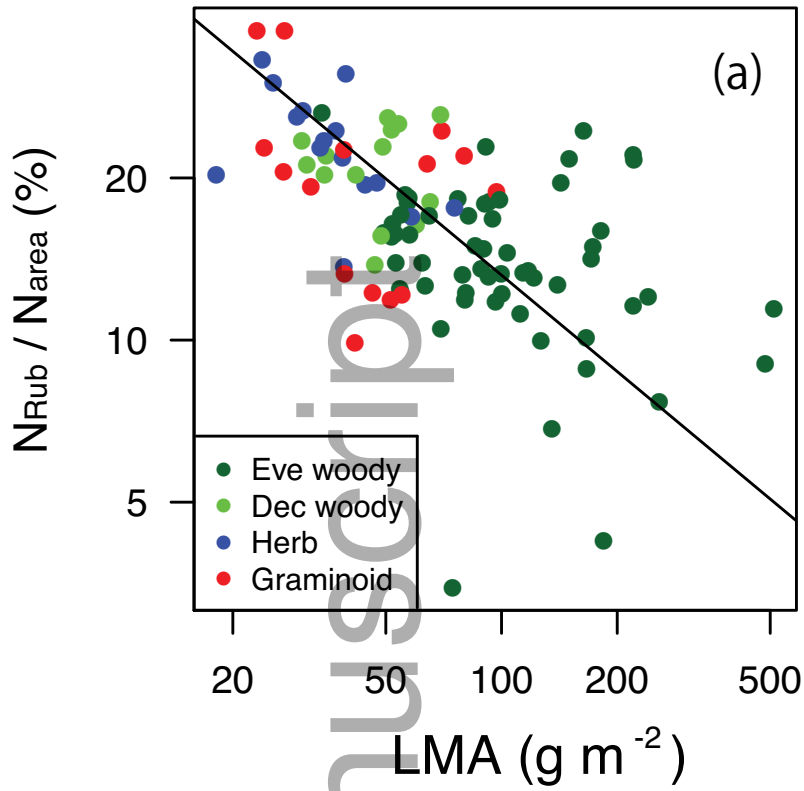
(d)

	Slope	(95%CI)
PNUE	-0.96	(-1.06, -0.86)
N_{Rub}/N_{area}	-0.59	(-0.70, -0.50)
g_m/S_c	-1.20	(-1.46, -0.99)
$C_i–C_c$	0.57	(0.48, 0.67)

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