Assessing the causes and scales of the leaf economics spectrum using venation networks in *Populus tremuloides*

Benjamin Blonder[1,2,3]*, Cyrille Violle[1,4] and Brian J. Enquist[1,2,5]

[1]*Department of Ecology and Evolutionary Biology, University of Arizona, 1041 E Lowell St., Tucson, AZ 85721, USA;* [2]*Rocky Mountain Biological Laboratory, PO Box 519, Crested Butte, CO 81224, USA;* [3]*Center for Macroecology, Evolution, and Climate, University of Copenhagen, Universitetsparken 15, København Ø DK-2100, Denmark;* [4]*Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175, CNRS, Montpellier, France; and* [5]*The Santa Fe Institute, 1399 Hyde Park Road, Santa Fe, NM 87501, USA*

Summary

1. The leaf economics spectrum (LES) describes global interspecific correlations between leaf traits. Despite recent theoretical advances, the biological scale at which LES correlations emerge and the physiological and climatic causes of these correlations remains partially unknown.

2. Here, we test an extant theory based on universal trade-offs in leaf venation networks that predicts that (i) the LES primarily originates within individuals; (ii) minor vein density drives LES trait correlations; and (iii) between individuals, LES correlations reflects variation in minor vein density driven by water availability. To test these predictions, we sample leaves within and between clones of *Populus tremuloides* across a wide climate gradient.

3. We show that predictions i) and iii) are supported but ii) is only partially supported. To account for this discrepancy, we develop a more general venation theory. This theory describes linkages between vein density, leaf area and leaf thickness that can modulate LES correlations across scales. This theory helps to identify multiple selective pressures that can drive trait covariation underlying the LES.

4. *Synthesis.* Our results broaden the range of biological scales at which the leaf economics spectrum (LES) is found and highlight the complex causal roles of venation networks in LES correlations. This study points to the need to better understand the coupling between venation networks, leaf size and climate to fully understand the LES.

Key-words: aspen, clone, ecophysiology, functional trait, leaf economics spectrum, *Populus tremuloides*, structural equation modelling, vein density, venation network

Introduction

The leaf economics spectrum (LES) describes a nexus of interspecific correlations between the traits that control carbon, nitrogen and water usage in all vascular plants (Reich, Walters & Ellsworth 1997; Wright *et al.* 2004). LES traits include the rate of carbon assimilation per unit mass, mass per unit area, lifespan and nutrient content. Studies have mainly focused on interspecific LES correlations found within and between diverse biomes world-wide (Reich, Walters & Ellsworth 1997; Reich *et al.* 1999). Over 80% of observed total global variation in the above traits can be explained by the first principal component axis of variation (Wright *et al.* 2004, 2005), raising the question: what generates such striking convergence in leaf form and function across the planet?

There are three questions that are relevant to understanding the origin of the LES. First, what is the biological scale at which the LES emerges? There is already evidence that LES correlations hold between closely related species (Edwards 2006; Dunbar-Co, Sporck & Sack 2009; Santiago & Kim 2009; Milla & Reich 2011) and between individuals within species (Martin, Asner & Sack 2007; Bonser *et al.* 2010; Vasseur *et al.* 2012) although perhaps not within local species pools exposed to different resource availability treatments (Wright & Sutton-Grier 2012). It also has been unclear whether the LES is also found between leaves within individuals. If this latter pattern should hold, it would indicate that the physiological cause of trait correlations (Donovan *et al.* 2011) could be found without invoking mechanisms applicable to the between-individual or between-species scale.

Secondly, what fundamental trade-offs cause the LES at the relevant biological scale? Several physiological theories have

been advanced to explain the origin of the LES (Shipley *et al.* 2006; Blonder *et al.* 2011; McMurtrie & Dewar 2011). Here, we focus on our recent publication (Blonder *et al.* 2011) in which variation in leaf venation network geometry implies physiological trade-offs that then generate LES correlations (hereafter, 'venation theory'). This theory predicts that per mass carbon assimilation rate (A_m) increases with increases in minor vein density (VD) because leaf water loss and carbon gain are both determined by the capacity to supply water through minor veins. Thus, a leaf characterized by a higher density of minor veins should also have an increased ability to supply water and a higher maximum carbon assimilation rate. Similarly, leaf mass per area ratio (LMA) should decrease with increasing minor vein density, assuming fixed leaf mass density. This relationship arises as follows from an assumed optimal coupling between leaf thickness and vein density. As vein density increases, the spacing between veins decreases. If leaf thickness does not change, then water diffusion from the veins reaches some mesophyll cells quickly, while others in the axial direction are supplied more slowly. Alternatively, if leaf thickness is proportional to vein spacing, then water supply is more uniform throughout the leaf. Then because increased leaf thickness leads to higher LMA, it follows that higher VD leads to lower LMA. However, note that leaf mass density may also be variable, confounding this argument (Niinemets 2001). Finally, leaf area is developmentally coupled to vein density in such a way that we expect higher VD to be associated with lower leaf area (LA). This argument follows based on simple geometrical scaling of a planar object (Carins Murphy, Jordan & Brodribb 2012; Sack *et al.* 2012). To summarize, venation theory predicts that higher VD should simultaneously lead to lower LA, lower LMA and higher A_m, thereby explaining the observed negative relationship between LMA and A_m (Fig. 4a).

As these venation and hydraulic constraints apply to all leaves where venation network traits can covary (i.e. all megaphylls), this theory should be able to explain the LES at all biological scales (between leaves within an individual, between individuals within species, between species). This venation theory is supported by several studies (Roth-Nebelsick *et al.* 2001; Sack *et al.* 2003; Sack & Holbrook 2006; Brodribb, Feild & Sack 2010; Boyce & Zwieniecki 2012; Simonin, Limm & Dawson 2012) that link venation networks to multiple leaf traits. However, predictions of the theory have only been directly tested at the interspecific scale (e.g. Blonder *et al.* 2011).

Thirdly, what are the climatic causes of the position of a leaf or species on the LES? While the scaling slopes between traits for the interspecific LES are largely independent of climate (Reich, Walters & Ellsworth 1997; Reich *et al.* 1999; Wright *et al.* 2005), it has been unclear what factors segregate leaves (species) along the spectrum. This is especially true given the fact that the lifetime carbon gain of a leaf may be an approximately invariant quantity (Kikuzawa & Lechowicz 2006) but see (Falster *et al.* 2011). To answer this question, we propose a 'use-it-or-lose-it' hydraulic hypothesis and to more formally link how the LES is expressed as a function of climate. Building upon earlier arguments, we hypothesize that

as available water increases, competition between plants for a limited resource will select for strategies that result in the fastest rates of resource use and growth rate given the local environment (Grime & Hunt 1975). As a result, with increasing water availability, local communities will consist of individuals with leaves that will increasingly be able to transpire water at higher rates, and so these plants will have higher growth rates. Selection to increase rates of transpiration would then be reflected in higher minor vein density to supply this higher water demand. Independently, higher vein density also may be necessary for rapid phloem loading associated with high rates of photosynthesis (Amiard *et al.* 2005). As predicted by venation theory (Blonder *et al.* 2011), any variation in minor vein density should be reflected in variation in several LES traits. This hypothesis is supported by previous work that shows a coordination of leaf hydraulic supply with local environmental water supply (Givnish *et al.* 2005; Boyce *et al.* 2009; Brodribb & Jordan 2011) – while instantaneous water fluxes may be regulated by stomata to match environmental demand, maximum leaf water supply is matched to environmental supply (Carins Murphy, Jordan & Brodribb 2012; Héroult *et al.* 2012). However, this overall hypothesis has not yet been comprehensively tested across wide climate gradients [but see (Uhl & Mosbrugger 1999) for a paleoclimate application].

Here, we assess the biological scale and theory proposed for the causes of the LES. We use observational data from *Populus tremuloides* growing across a precipitation gradient in Colorado (Fig. 1). This clonal tree species is common and has a broad geographical range spanning northern Alaska to central Mexico. Clones (individuals) can persist for tens of thousands of years, can cover hundreds of thousands of square metres of forest and can acclimate to environments that may vary over space by hundreds of metres of elevation and over times that may span glaciation events (Mitton & Grant 1996). We focused on the A_m-LMA relationship as a key representation of resource acquisition–storage trade-offs underlying the LES.
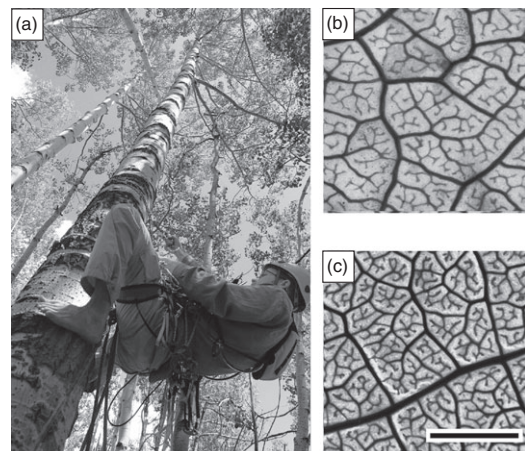

Fig 1. Leaf traits: from field to laboratory. (a) We studied the clonal tree quaking aspen (*Populus tremuloides* Michx.), a common North American species, along a natural climate gradient. Vein density (VD) is highly variable even between leaves of the same clone: (b) a leaf with VD of 10.6 mm mm^{-2}; (c) 13.7 mm mm^{-2}. Scale of both panels is the same; scale bar, 1 mm.

We tested the following predictions from the preceding three questions: (i) within clones, the same trait correlations are observed as for the interspecific LES, (ii) within clones, the LES is caused by variation in VD consistent with venation theory and (iii) between sites and clones, VD increases with growing season precipitation. We find that while predictions i) and ii) are supported by data, prediction ii) is not fully supported and challenges extant theory. In the light of these results, we also extend venation theory (Blonder *et al.* 2011) to more fully link the intraspecific LES and the interspecific LES. This revised theory more accurately identifies measureable traits that can assess biological scale dependencies of the LES and more robustly falsify fundamental assumptions.

Materials and methods

FIELD SITES

We studied venation networks in 27 naturally established *P. tremuloides* clones at sites in the Colorado Rocky Mountains (Fig. S1 in Supporting Information). Clones were chosen to span a range of climate variability (Table S1 in Supporting Information). Of these clones, six from sites in the Gunnison Valley were designated as focal clones on which measurements of A_m and LMA were also made. Within each site, we assumed that all sampled leaves belonged to a single clone. Clone identity was determined by observations of non-vegetated gaps (> 100 m) between clusters of stems and by the within-clone synchrony of autumn leaf colour change. Previous morphological studies have shown that clones often show strong differences in appearance (Barnes 1969), while genetic studies have shown that spatially disjunct stands almost certainly represent distinct genotypes and very few genotypes are represented within stands (Mock *et al.* 2008). However, because we did not perform any genetic analyses, we cannot absolutely rule out the possibility that leaves sampled from different stems represent different genotypes. Nonetheless, the large spatial distance between clones and the morphological differences between sampled clones strongly suggest that we sampled from genetically distinct clones. Sites were sampled between June and August of 2010 and 2011 when leaves were mature and fully expanded. Day of year had a non-significant effect on each clone-mean log-transformed trait (ANOVA; all $P > 0.09$), so we conducted all analyses without further considering growing season effects.

CLIMATE DATA

Mean summer temperature (°C) and precipitation (mm/mo) were determined at each site from June, July and August 1971–2000 PRISM climate data (30 arcsecond resolution data) (http://prism. oregonstate.edu/). Mean summer potential evapotranspiration (PET; mm/mo) was estimated at each site using the CGIAR-CSI Global-PET data set's 1950–2000 mean monthly values for June, July, and August (30 arcsecond resolution data) (Zomer & Trabucco 2009). Elevation (metres above sea level) was determined from the National Elevation Data set (1/3 arcsecond resolution data; ned.usgs.gov). Values were extracted from climate layers using GDAL (www.gdal.org) and the MATLAB Mapping Toolbox (MathWorks).

LEAF SAMPLING

At each site, we obtained mature undamaged leaves from several ramets. We removed lower leaves by hand and higher leaves by cutting branches with pruning shears after climbing ramets (Fig. 1a). For some smaller clones (e.g. high elevation Krummholz), only sun leaves were available. However, there was no significant effect of strata on each clone-mean log-transformed LES trait, including VD (ANOVA; all $P > 0.07$), so we did not treat sun and shade leaves differently. We measured venation traits and leaf area (LA) of each leaf. We obtained a total of 337 leaves, with most leaves being collected at six focal clones for which A_m and LMA were also measured. Full data on collections are found in Table S1.

At six focal clones, we measured A_m and LMA ($n = 259$ leaves). We determined peak per area photosynthetic rate (A_a) using a Li-Cor 6400XT portable photosynthesis system. Measurements were made on sunny days before 1100 h. Leaf conditions were standardized at 25 °C, 400 ppm CO_2 and 2000 µmol m^2 s^{-1} PAR. We had to discard A_m values for 10 leaves due to lack of stabilization of leaf chamber conditions. Each leaf was then removed from the tree and stored it in a plastic bag on ice for 1–3 h. We did not rehydrate leaves because average LMA values were not low enough to warrant it (Cornelissen *et al.* 2003). For all leaves, we determined fresh LA by scanning each leaf at 300 dpi and then applying an Otsu threshold to the resulting images. Each leaf was then dried at 60 °C for at least 72 h before its dry mass (M) was determined. These measurements enabled us to calculate LMA (=M/LA) and $A_m = (A_a /LMA)$ for each leaf following established protocols (Cornelissen *et al.* 2003).

VENATION TRAITS

We made images of the venation network of a 6–30 mm^2 region taken from the edge of the leaf lamina midway between the leaf base and apex. Regions were selected to avoid major veins. Leaf samples were soaked in a 5% (w/v) sodium hydroxide:water solution for several days before being rinsed in water, bleached in 5% (w/v) sodium hypochlorite:water solution for 5 min. After a subsequent rinse, leaves were transferred via an ethanol dehydration series (50%, 100%) to a 0.1% (w/v) safranin:ethanol staining solution for 1 h before being destained in ethanol for 2 h. Leaves were then transferred via series (50% toluene, 50% ethanol) to 100% toluene and permanently mounted on glass slides using Fisher Permount medium. Images of each leaf were obtained using an Olympus SZX-12 dissecting microscope (Olympus, Tokyo, Japan) and a Canon T2i camera body (Canon, Tokyo, Japan). We enhanced image contrast by keeping only the green channel of each image and then performing a contrast limited adaptive histogram equalization. Final image scale was 430 pixels per millimetre. We did have to discard vein data from 31 leaves where contaminated chemicals yielded low-quality images in which minor veins were not visible (Table S1 and Data S1 in Supporting Information).

We hand-traced all veins in each usable image using GIMP (www. gimp.org). All images and tracings are available as supporting information. We determined values for *VD* (mm mm^{-2}) on skeletonized images using previously published MATLAB code (Blonder *et al.* 2011).

STATISTICAL ANALYSIS

All analyses were conducted within the R environment (www.r-project.org). Variance partitioning of LES traits was carried out with 'lme' in the *nlme* package and 'varcomp' in the *ape* package. Standardized major axis (SMA) regressions were used for assessing the

A_m-LMA and VD-LA relationships using 'sma' from the *smatr* package. Structural equation models were fit with the *lavaan* package as follows. Using data from the six focal clones, we compared models that involved causal relationships between four functional traits (LA, VD, A_m, LMA). All variables were $\log_{10}$-transformed prior to analysis to improve normality. Because of the grouped structure of the data (multiple clones), we performed a multi-group analysis, which provides unified fit statistics but also estimate parameters for each group. A multi-group analysis would also be appropriate because variation within groups (Fig. 3) may have an equally large effect on trait values as variation between groups. Because we only had access to site-mean climate values, leaf-scale microclimate could not be included as a causal variable in these models.

We first determined whether the data were better explained by causal relationships between the observed variables or instead by an unobserved (latent) variable. The latter option would be possible if both 1) the undirected dependency graph between all observed variables is fully connected and 2) at least one of the three tetrads describing correlations between the observed variables is not significantly different from zero (Shipley 2000). To test this possibility, we applied the Fast Causal Inference algorithm to each focal clone (Spirtes, Glymour & Scheines 2000). For every clone, even at the liberal α = 0.5 level, no undirected dependency graph was fully connected. We therefore did not further consider the possibility of latent variables and restricted our analysis to measured variables. We then proceeded by model enumeration. Each pair of variables can have one of four relationships (null, ↔, →, ←), resulting in a total of $4^{\binom{n}{2}}$ possible graphs (Shipley 2000)[1]. In this study, $n = 4$ variables yielded 4096 possible graphs. Next, we evaluated each of these 4096 possible structural equation models. We chose to take this exploratory approach to SEM (rather than a confirmatory approach that would rule out a small set of candidate models) because we then were able to identify all of the causal hypotheses consistent with the observed data, rather than being able to only reject a single candidate hypothesis. This approach allows for causality to operate in multiple directions, so that it is possible for any functional trait to cause variation in any other function trait. By 'cause', we mean that if A causes B, then variation in A is linked to variation in B, while variation in B may not always be linked to variation in A. This type of causation does not necessarily identify developmental processes but does identify statistically independent drivers of correlational patterns.

Modelling was implemented using the 'sem' command in *lavaan*, grouping by site. We retained a subset of models that had positive degrees of freedom and convergent fits. These models then were ordered by relative goodness-of-fit using Bayesian Information Criterion (BIC) values. All models were also evaluated with a lack-of-fit *P*-value (large values indicate that the proposed causal model cannot be rejected based on the data). We used a Satorra–Bentler test statistic, which is an appropriate approach for generating *P*-values from data sets with low sample sizes and potentially non-normal data (Satorra & Bentler 2001).

Results

THE LES IS FOUND WITHIN INDIVIDUALS

In four of the six clones, there was a significant negative relationship between $\log_{10} A_m$ and $\log_{10}$ LMA (Fig. 2). The within-

[1] Note that the enumeration approach becomes less practical with more variables. For example, when $n=6$ there are 1,073,741,824 possible graphs to be evaluated.

clone slopes (mean of within-clone slopes, −1.1) were not significantly different from the global interspecific LES slope of −1.3 (Wright *et al.* 2004) in four of the six clones (all $P > 0.2$). Using a variance partitioning analysis, we found that anywhere from 28–69% of the total observed variance in A_m, LMA and VD occurred within individual clones, rather than between clones (Fig. 3). Together, these findings indicate that a large fraction of variation in LES traits can reside within individuals. We also assessed the overall magnitude of this trait variation by comparing ranges with global interspecific trait ranges (Wright *et al.* 2004; Boyce *et al.* 2009). In *P. tremuloides*, A_m ranged from 107 to 313 nmol g^{-1} s^{-1} (global range 5–660); LMA from 37 to 119 g m^{-2} (global range 14–1500); and VD from 7 to 19 mm mm^{-2} (global range 0.5–25). Indeed, observed intraspecific trait ranges span a reasonable fraction of global interspecific trait ranges, indicating that this intraspecific variation in the LES is comparable with the interspecific variation in the LES. Such a finding is consistent with previously reports of comparing intraspecific variation of LES traits with global values (Albert *et al.* 2010; Kattge *et al.* 2011).

WITHIN CLONES, THE COVARIATION OF A_M-LMA IS LINKED TO VENATION NETWORK GEOMETRY

We next tested the hypothesis that covariation between A_m and LMA is directly linked to variation in VD and is also modulated by variation in LA. We proceeded by model enumeration and identified the best model via BIC (ΔBIC = 6.93 relative to the next best model; approximately $e^{6.93} \approx 1000$ times more likely). This model could not be rejected based on the data ($\chi^2 = 17.8$, d.f. = 12, $P = 0.12$ using a Satorra–Bentler correction).

The best model was partially consistent with venation theory predictions (Fig. 4). We successfully found the predicted negative path from VD to LMA, and a predicted negative path between VD and LMA, although its causal orientation could not be resolved by data. However, we also found a positive path from LA to A_m, and an unresolved covariance between A_m and
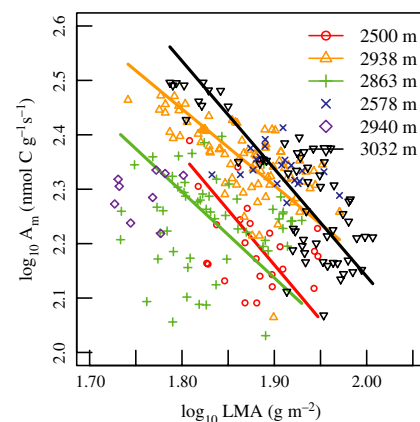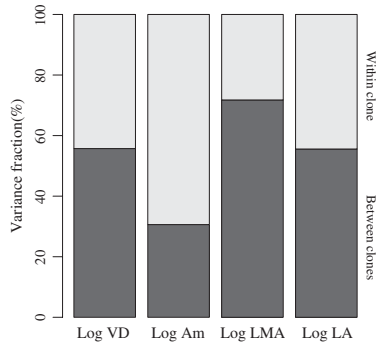

Fig. 2. The LES relationship between the per mass carbon assimilation rate (A_m) and leaf mass per area ratio (LMA). Each point represents an individual leaf; SMA regression lines are shown when significant.

Fig. 3. Variance partitioning analysis for leaf traits. A large fraction of the total variation in each trait is found within clones (light grey) vs. between clones (dark grey).

LMA, which were not predicted by venation theory. It appears that the observed negative correlation between A_m and LMA is due to variation from VD and LA in ways that are not fully explained by extant theory.

BETWEEN CLONES, VEIN DENSITY IS MEDIATED BY SITE CLIMATE

We then assessed the hypothesis that variation in venation traits is driven by variation in climate (Fig. 5) using clone-mean values of VD to compare with site-mean climate values. Using linear regression models, we found that $\log_{10}VD$ was significantly negatively correlated with summer temperature and potential evapotranspiration, and positively correlated with elevation and precipitation (all $P < 0.04$, all $R^2 > 0.16$). Summer precipitation showed the strongest signal ($R^2 = 0.27$). However, these climate variables represent only one dimension of climate (93% of variation accounted for by the first principal component of these four variables) because of the strong altitudinal control of climate in the Rocky Mountains.

Discussion

EMPIRICAL RESULTS

We showed that the LES emerges within clones, demonstrating a new level of generality for previously known between-species and between-individual relationships. These results also

indicate that within-clone variation in VD is as large as between-clone variation, consistent with studies of other functional traits (Albert *et al.* 2010). We also showed that VD is causally associated with within-clone variation in A_m and LMA, but not always as predicted by extant theory and that between-clone variation in VD is associated with variation in water availability. Thus, the high levels of plasticity observed in VD may reflect multiple functional and environmental linkages. Based on these results, we argue that correlations in the traits that describe the LES are empirically linked to more fundamental traits that define the venation networks and that these venation traits are associated with local climate conditions.

Currently, it is not clear whether theories for the LES based on venation networks can provide more predictive power than other theories for the LES based on other aspects of plant physiology (Shipley *et al.* 2006; McMurtrie & Dewar 2011). However, the predictions of one alternate theory for the LES (Shipley *et al.* 2006) may be consistent with venation theory. Specifically, the ratio of cell wall to cytoplasm, a key variable for this theory, could change with venation development and thus should increase with increasing VD. However, this ratio (or 1/LDMC, a proxy for this ratio) was not measured in our study. Comparison with the McMurtrie & Dewar (2011) theory would require more detailed paired measures of a suite of other leaf, plant and environment traits. We are presently unaware of any data sets that would make this possible.

Additionally, our 'use it or lose it' hypothesis has not been conclusively tested. While we found evidence consistent with this hypothesis via the linkage between vein density and precipitation, our study was not able to disentangle the effects of multiple environmental or physiological variables that were also correlated with precipitation. The linkage between VD and precipitation is initial confirmatory evidence for our hypothesis. However, other aspects of leaf physiology are also known to respond to climate. For example, precipitation has a strong effect on leaf mass density, while temperate and light availability may impact leaf thickness (Niinemets 2001). Thus, leaf functional traits may show a climate signal from more variables than only vein density. Measurements of these traits over wider climate gradients will be necessary to assess the generality of our result. Another important (and here unmeasured) source of environmental variation could be soil fertility, which is known to strongly impact multiple leaf traits (Ordoñez *et al.* 2009;
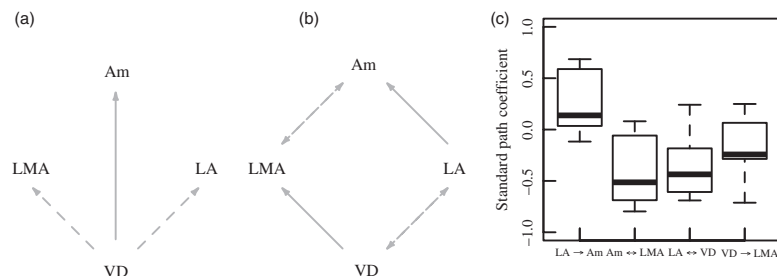

Fig. 4. Structural equation models for the causal origin of the leaf economics spectrum. (a) Predictions based on venation theory. (b) Observed results based on the best model identified via model selection. Arrows have solid lines if standardized path coefficients are positive; dashed if negative. Bidirectional arrows represent unresolved covariances, while one-way arrows represent causal relationships. (c) Distribution of standardized path coefficients across groups (clones) for the observed results summarized in B.
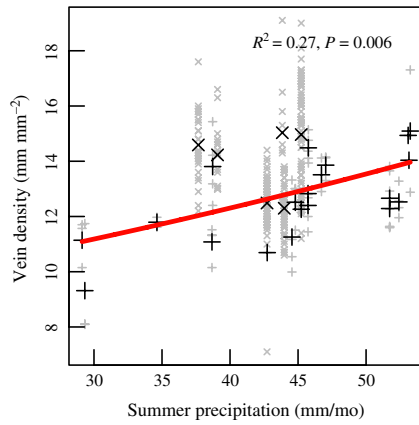
Fig. 5. Venation network geometry is linked to climate. Here, the relationship between VD and summer precipitation is shown. Individual leaves are shown in grey; clone means in black, for focal (×) and non-focal (+) clones. The regression line is fit to all clone-mean $\log_{10}$ VD values. Other climate variables also showed significant but collinear relationships in this data set.

Hodgson *et al.* 2011). However, soil fertility is often associated with moisture availability (Jenny & Leonard 1934; Chapin 1980), and leaf development is known to be very sensitive to the hydraulic environment (Pantin *et al.* 2011).

Our structural equation modelling results challenge extant venation theory (Blonder *et al.* 2011) and require further explanation. In particular, we failed to find the predicted positive relationship between VD and A_m, but did find an unexpected positive relationship between LA and A_m. A key component of the theory is that hydraulic path length (a function of both VD and leaf thickness, LT) is a more primary variable driving A_m than variation in VD (Brodribb, Feild & Jordan 2007). The extant venation theory assumes, based on optimality arguments, that leaf thickness (a proxy for mesophyll depth) is inversely proportional to vein density (Noblin *et al.* 2008). Additionally, this theory does not explicitly formalize the relationship between VD and LA, which may be variable. For example, the local light environment may interact with VD and LA (Granier & Tardieu 1999; Sack *et al.* 2012), resulting in conflicting selection pressures on VD and LA due to the different demands of water transport and heat dissipation or cavitation (Sack & Frole 2006; Brodribb & Jordan 2011; Peppe *et al.* 2011; Scoffoni *et al.* 2011). Indeed, much intra-canopy variability in leaf functioning can be explained by variation in light availability (Prieto *et al.* 2012).

EXTENDING VENATION THEORY FOR THE ORIGIN OF THE LES

To account for these observed results, we returned to the venation theory to relax the assumptions linking VD, LA and LT. In doing so, we advance a more general revised theory for the origin of LES correlations at different levels of biological organization. It is possible that the strength of the covariation between VD, LA and LT is scale-dependent, taking one form within species and a different form between species. Such findings would provide a proximate explanation for the

observation that between species, the A_m-LMA relationship is negative (Wright *et al.* 2004), but within species, the A_m-LMA relationship can also be null (as we found here in two of six clones) or positive [e.g. sun leaves often have higher A_m but are also smaller and thicker with higher LMA than shade leaves (Atkinson *et al.* 2010; Lusk *et al.* 2008)]. Our extension of venation theory can model these effects and identify variables that could be measured in the future to verify the existence of such VD-LA and VD-LT couplings. For simplicity, we focus again on the A_m-LMA relationship.

We model leaf mass per area as a partitioning between vein mass and lamina mass as

$$LMA = \pi \cdot r_v^2 \cdot (\rho_v - \rho_l) \cdot VD + \rho_l \cdot LT \qquad \text{eqn 1}$$

where r_v is the mean radius of a minor vein and ρ_v and ρ_l are the mass density (mass/volume) of minor veins and lamina, respectively. This equation is identical to that presented in (Blonder *et al.* 2011) except it is written in terms of leaf thickness instead of leaf half-thickness. As in the extant theory, the differential contribution of major veins is not accounted for here, although the relationship can be variable (Sack *et al.* 2012).

We next model per mass carbon assimilation rate as

$$A_m = \frac{A_0}{(LT^2 + 4VD^{-2})^{1/2}} \cdot \frac{1}{LMA} \qquad \text{eqn 2}$$

where A_0 is a constant and the denominator reflects the hydraulic path length for diffusion of water away from the veins. The value of the exponent in the denominator may not exactly be 1/2 (Brodribb, Feild & Jordan 2007), but such variation does not change the overall conclusions described below. This equation is a simplification of extant theory that places many variables (not of interest here) into the A_0 constant.

Next, we describe the potential couplings between VD, LT, and LA. Specifically, we have

$$LT = \delta_0 \cdot VD^\Gamma \qquad \text{eqn 3}$$
$$VD = \sigma_0 \cdot LA^B \qquad \text{eqn 4}$$

where δ_0 and σ_0 are leaf-specific constants which may or may not vary across scales. These constants set the overall magnitude of couplings and B and Γ are unknown exponents.

Values of B and Γ quantify the strength of covariation of VD, LT and LA. We expect that at each scale (e.g. between species, within a species) or environment (e.g. high or low precipitation), selection will yield different values of B and Γ that indicate different evolutionary trajectories, and presumably selection along certain axes of variation. We highlight some of these biologically relevant scenarios below. Selection for optimal water supply to all cells would imply $\Gamma < 0$, so that leaf thickness decreases as vein density increases. This arises because if the spacing between veins (inversely proportional to the vein density) scales linearly with the thickness of the leaf, no portion of mesophyll is over- or under-supplied with water via diffusion (Noblin *et al.* 2008). When $\Gamma=0$, vein density and leaf thickness can vary independently. When $\Gamma > 0$, leaf thickness increases as vein density increases, potential reflecting (i) selection for sclerophylly, possibly due

to changes in development of parenchyma cells or (ii) selection for variable light interception strategies in sun vs. shade leaves, that is, variation in the proportion of palisade and spongy mesophyll cells (Lichtenthaler *et al.* 1981). When $B < 0$, vein density decreases as leaf area increases, reflecting the sun-shade gradient – sun leaves are often smaller and also have higher vein density (Tumanow 1927). A null expectation is $B = -1/2$, consistent with geometrical effects of leaf expansion (Brodribb, Feild & Sack 2010; Sack *et al.* 2012). When $B = 0$, vein density and leaf area are independent, as in the extant theory. When $B > 0$, vein density increases as leaf area increases, potentially reflecting an increased demand for water supply when major veins become more distantly spaced (McKown, Cochard & Sack 2010; Scoffoni *et al.* 2011). These exponents might also change depending on biological scale (e.g. $\Gamma = 0$ when estimated between species, but $\Gamma < 0$ within species), leading to different consequences for the LES.

We next investigated theoretical predictions for how the LES scaled with parameters including leaf area (LA), vein density (σ_0) and leaf thickness (δ_0), conditional on the values of B and Γ. To facilitate analysis, we eliminated parameters whose only effect was on the overall magnitude of A_m and LMA (achieved by rescaling; $1 = A_0 = r_v = \pi$) and also assumed that variation in mass density was small ($1 = \rho_v = \rho_l$). While this latter assumption is false (Castro-Diez, Puyravaud & Cornelissen 2000; Niinemets 2001; Poorter *et al.* 2009), its violation qualitatively changes results only for very high values of VD but greatly simplifies the analytic interpretation of the theory. These simplifications lead to the following equations for the LES:

$$\text{LMA} = \delta_0(LA \cdot \sigma_0)^\Gamma \qquad \text{eqn 5}$$

$$A_m = \frac{(\text{LA}^B \cdot \sigma_0)^{2-\Gamma}}{2 \cdot \delta_0 \cdot \left(4 + \delta_0^2(\text{LA}^B \cdot \sigma_0)^{2(1+\Gamma)}\right)} \qquad \text{eqn 6}$$

Equations 5 and 6 can yield a wide range of predictions for the LES, as seen in Table 1. Due to the complexity of these equations, we have also provided plots of the theory's predictions as functions of σ_0, δ_0 and LA, which are shown in Figs S2–S4. To guide the interpretation of these figures, we highlight two relevant cases. First, the combination $\Gamma = -1$, $B = 0$ corresponds to the original assumptions of venation theory. Here, increase in vein density (σ_0) also increase A_m and decrease LMA, leading to the expected negative correlation between A_m and LMA. However, changes in leaf area have no effect on the LES. Alternatively, in the case where $\Gamma = 1$, $B = -1$, any increases in σ_0 would then yield a decrease in A_m but an increase in LMA. A recent study found such a positive VD-LMA relationship in some species (Nardini, Pedà & Rocca 2012). Negative covariation would then lead to a negative correlation between A_m and LMA, but for a different reason. However, for fixed values of σ_0, increases in LA lead to increases in both LMA and A_m which yields a positive correlation between LMA and A_m. Such a relationship is found between large (shade) and small (sun) leaves within some species (Carins Murphy, Jordan & Brodribb 2012).

A full visual summary of the theory's predictions for the A_m-LMA relationship, conditional on each value of B and Γ, is provided for σ_0 (Fig. S2), LA (Fig. S3), and δ_0 (Fig. S4). Broadly, variation in σ_0 and δ_0 always leads to a negative A_m-LMA correlation regardless of B and Γ, while variation in LA can lead to either positive or negative A_m-LMA correlations depending on the values of B and Γ. By measuring these exponents, it should be possible to gain insight into the selective forces generating the LES at each scale. Thus, the key insight from this revised theory is that the observed correlations in the LES can arise from a wide range of physiological scenarios that are all consistent with a primary role for venation networks.

We can begin to assess the plausibility of these different scenarios using our empirical data. Regression analysis appears to further support the structural equation modelling result of $B < 0$ (Fig. S5), although the positive path observed from LA to A_m would instead be most consistent with $B > 0$ (Fig. S3). This inconsistency may lead to incorrect selection of the 'best' model, whose identification can be sensitive to the maximum likelihood fitting procedure. Alternatively, the link between VD and A_m may not be as strong as predicted by venation theory, because many other factors [e.g. stomatal traits (de Boer *et al.* 2012) and leaf mass density and stoichiometry (Niinemets 2001)] may also drive variation in A_m. For example, VD and A_m may become increasingly uncoupled at high values of VD and in the low atmospheric CO_2 concentrations experienced in the present day relative to the Cretaceous (de Boer *et al.* 2012; Boyce & Zwieniecki 2012). If this were the case, then the venation theory would be significantly weakened. We suggest that future studies assessing the mechanisms

Table 1. Predictions of the extended venation theory for the leaf economics spectrum (LES) vary depending on the coupling between vein density and leaf thickness (Γ exponent) and the coupling between vein density and leaf area (B exponent). See Discussion for explanation of all symbols and interpretation. These relationships are plotted in Figs S2–S4

Γ	B	LMA	A_m
-1	-1	$\frac{\text{LA} \cdot \delta_0}{\sigma_0}$	$\frac{\sigma_0^3}{2 \cdot \text{LA}^3 \cdot (4\delta_0 + \delta_0^3)}$
-1	0	$\frac{\delta_0}{\sigma_0}$	$\frac{\sigma^3}{8\delta_0 + 2\delta_0^3}$
-1	1	$\frac{\delta_0}{\text{LA} \cdot \sigma_0}$	$\frac{\text{LA}^3 \cdot \sigma_0^3}{8\delta_0 + 2\delta_0^3}$
0	-1	δ_0	$\frac{\sigma_0^2}{8 \cdot \text{LA}^2 \cdot \delta_0 + 2\delta_0^3 \cdot \sigma_0^2}$
0	0	δ_0	$\frac{\sigma_0^2}{8 \cdot \delta_0 + 2\delta_0^3 \cdot \sigma_0^2}$
0	1	δ_0	$\frac{\text{LA}^2 \cdot \sigma_0^2}{8 \cdot \delta_0 + 2 \cdot \text{LA}^2 \cdot \delta_0^3 \cdot \sigma_0^2}$
1	-1	$\frac{\delta_0 \cdot \sigma_0}{\text{LA}}$	$\frac{\text{LA}^3 \cdot \sigma_0}{8 \cdot \text{LA}^4 \cdot \delta_0 + 2 \cdot \delta_0^3 \cdot \sigma_0^4}$
1	0	$\delta_0 \cdot \sigma_0$	$\frac{\sigma_0}{8 \cdot \delta_0 + 2 \cdot \delta_0^3 \cdot \sigma_0^4}$
1	1	$\text{LA} \cdot \delta_0 \cdot \sigma_0$	$\frac{\text{LA} \cdot \sigma_0}{8 \cdot \delta_0 + 2 \cdot \text{LA}^4 \cdot \delta_0^3 \cdot \sigma_0^4}$

underlying the LES make detailed measurements of *B* and Γ within and across other species, as well as to compare with alternate theories such as that of Shipley *et al.* (via measurements of LDMC, tissue density, and other traits), before rejecting or maintaining the venation hypothesis.

By relaxing assumptions that strictly link VD, LA and LT, we can advance a quantitative theory for leaf form and function. In doing so, our theory points to how differing selective pressures then drive variation in different leaf traits that ultimately underlie the LES between and within species. Indeed, understanding the origins of scale-dependent variation in Γ and *B* will also be important to fully understand the mechanisms that generate the LES. A strong test of venation theory would measure exponents both within and across species, and assess whether observed LES correlations at each scale are consistent with theoretical assumptions and predictions at that scale.

Recently it has been argued that the negative relationship between A_m and LMA may be a statistical artifact of dividing random variables, one of which is a ratio that includes the other (leaf mass) (Lloyd *et al.* 2013, Osnas *et al.* 2013). According to these studies, area-normalization is more appropriate for identifying mechanisms generating trait correlations, if any do exist. While we agree with these statistical arguments, these studies do not advance a clear hypothesis for why leaf economic spectrum traits should covary. Moreover, these arguments can be reversed to instead argue that area-normalized leaf traits obscure mechanisms that are relevant on a mass-normalized basis. Thus the circularity of this logic leads us to believe that such a line of reasoning does not bring us closer to understanding underlying biological mechanisms. In contrast, our study proposes potential mechanisms that do generate mass-based leaf trait correlations, and provides a set of equations based on physiological processes that do account for the effect of leaf area. These equations can be manipulated easily to make area-normalized predictions for the LES if such a basis is preferred.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Trait values for each leaf, and climate values for each site.

Data S1. Description of Data S1 with units and variable definitions.

Figure S1. (A) Distribution of *P. tremuloides* clones across elevations in Colorado (+). (B) Inset of outlined region in first panel, showing that focal clones ($\times$), on which A_m and LMA measurements were taken, were located within one environmentally heterogeneous valley.

Figure S2. Predictions for the A_m/LMA relationship based on Equation 5 and Equation 6.

Figure S3. Predictions for the A_m/LMA relationship based on Equation 5 and Equation 6.

Figure S4. Predictions for the A_m/LMA relationship based on Equation 5 and Equation 6.

Figure S5. The empirical relationship between vein density and leaf area.

Table S1. Summary of collections.