Photosynthesis at an extreme end of the leaf trait spectrum: how does it relate to high leaf dry mass per area and associated structural parameters?

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Abstract

Leaf dry mass per area (LMA) is a composite parameter relating to a suite of structural traits that have the potential to influence photosynthesis. However, the extent to which each of these traits contributes to variation in LMA and photosynthetic rates is not well understood, especially at the high end of the LMA spectrum. In this study, the genus Banksia (Proteaceae) was chosen as a model group, and key structural traits such as LMA, leaf thickness, and density were measured in 49 species. Based on the leaf trait variation obtained, a subset of 18 species displaying a wide range in LMA of 134–507 g m⁻² was selected for analyses of relationships between leaf structural and photosynthetic characteristics. High LMA was associated with more structural tissue, lower mass-based chlorophyll and nitrogen concentrations, and therefore lower mass-based photosynthesis. In contrast, area-based photosynthesis did not correlate with LMA, despite mesophyll volume per area increasing with increases in LMA. Photosynthetic rate per unit mesophyll volume declined with increasing LMA, which is possibly associated with structural limitations and, to a lesser extent, with lower nitrogen allocation. Mesophyll cell wall thickness significantly increased with LMA, which would contribute to lower mesophyll conductance at high LMA. Photosynthetic nitrogen use efficiency and the nitrogen allocation to Rubisco and thylakoids tended to decrease at high LMA. The interplay between anatomy and physiology renders area-based photosynthesis independent of LMA in Banksia species.

Key words: Gas exchange, leaf density, LMA, leaf internal conductance, leaf thickness, mesophyll conductance, photosynthesis, sclerophyll.

Introduction

In multispecies analyses, the area-based photosynthetic rate correlates poorly with dry mass per unit leaf area (LMA), whereas mass-based photosynthesis shows a clear decline with increasing LMA (Reich et al., 1997; Wright et al., 2004). While the second observation may be explained by the greater proportion of structural (non-photosynthetically
active) tissue per unit leaf dry mass, which is also expressed as lower mass-based nutrient concentrations (Chapin, 1980), it is less clear how high-LMA leaves are able to fix CO₂ at rates that are similar to those of low-LMA leaves that are usually found on fast-growing plants.

LMA is a key structural trait that measures the investment of dry mass per unit of light-intercepting leaf area and is widely used as an indicator of plant ecological strategies (Westoby et al., 2002; Wright et al., 2004). High LMA can be due to a thick leaf or high leaf density, or both (Witkowski and Lamont, 1991). High-LMA leaves are often hard, and referred to as sclerophylls (Turner, 1994), although succulent species can also display high LMA values due to high leaf thickness (Poorter et al., 2009). In the present study, LMA and its relationship with photosynthesis is discussed in the context of hard, thick, and dense leaves of a wide range of LMA, with robust construction, which confers long lifespans.

Despite the general anatomical organization of high-LMA leaves, which are thick and/or dense, fibrous, and often hairy, at least on the abaxial surface (Turner, 1994; Read et al., 2000; Mast and Givnish, 2002), the structural traits at the tissue and cell level that contribute to high LMA are particularly diverse and include bundle fibre caps, lignified bundle sheaths, vascular bundle extensions, lignified leaf margins, very thick cuticles, lignified hypodermal structures associated with the adaxial and/or abaxial surfaces, sclereids within the mesophyll, sclereids associated with vein endings, and thick cell walls (Dillon, 2002; Terashima et al., 2006). It must be noted that some of these characters are not restricted to high-LMA leaves, and not all high-LMA species possess all of these characters (Read et al., 2000). In other words, different combinations of the above leaf traits can result in high LMA (Read et al., 2000; Read and Sanson, 2003), and this explains the great variation in this trait that is usually found among hard leaves (Read et al., 2000), even within the same genus (Hassiotou et al., 2009a). While it is clear that variation in leaf thickness and density is due to the number of cell layers (photosynthetic or not) and the relative amount of cell types, respectively, the relative importance of these structural traits in determining thickness, density, and LMA is not well understood.

High LMA has been associated with low conductance to CO₂ diffusion from the substomatal cavity to the chloroplasts (mesophyll conductance, gₘ), which can restrict the rate of CO₂ assimilation (Loreto et al., 1992; Evans et al., 1994; Parkhurst, 1994; Evans and von Caemmerer, 1996; Evans and Loreto, 2000; Terashima et al., 2006; Hassiotou et al., 2009a). Moreover, surface properties of high-LMA leaves, including wax layers, epidermal cell shape, cuticular thickening, trichomes, and stomatal crypts, as well as specific scleromorphic structures, such as sclereids, can alter leaf optical properties (Myers et al., 1994; Baldini et al., 1997) and thus influence gas exchange. High-LMA leaves have low concentrations of key nutrients such as nitrogen, but whether this is simply due to ‘dilution’ by the presence of more structural tissue, or also applies to the photosynthetically active mesophyll, is not known. In fact, it is unclear whether the photosynthetically active mesophyll cells of high-LMA leaves differ from those in lower LMA leaves and, if so, whether this is because of the conditions in which they operate (CO₂, light) or because they are structurally and/or physiologically different.

To advance our understanding of the physiological consequences of leaf structure, the genus Banksia L.f. (Proteaceae), being predominantly endemic to Australia, was used as a model group on the basis of the great leaf structural diversity that it displays (LMA=134–507 g m⁻²; Hassiotou et al., 2009a). Key leaf structural traits such as LMA, leaf thickness, and density were examined in 49 Banksia species. Subsets of this large group representative of the diversity found in this genus were subsequently selected to investigate inter-relationships between leaf structure and photosynthesis. The following questions were asked:

(i) How much of the variability in LMA is due to variability in leaf thickness and how much to variability in leaf density in Banksia, and which anatomical parameters correlate most strongly with leaf thickness and density?
(ii) How does the light-saturated rate of photosynthesis at ambient CO₂ relate to leaf structural parameters at the high end of the LMA spectrum? If, as in previous studies, area-based photosynthetic rate does not correlate with LMA, is that because high-LMA leaves do not pack more photosynthetic tissue per unit leaf volume, or because this tissue is less efficient than that in low-LMA leaves?
(iii) How do chlorophyll and nitrogen content and the components of photosynthetic nitrogen use efficiency (PNUE) vary with LMA?

Materials and methods

Plant material and growth conditions

Three- to 5-year old plants of 49 broad-leaved (as opposed to needle-leaved) Banksia species were used (see Appendix). The plants, except for B. integrifolia L.f., B. paludosa R.Br., and B. serrata L.f., were grown from seed in 10.0 l pots containing a mixture of river sand and potting mix, in Peru (Australia), outdoors (with an average annual temperature and average daily solar exposure of 19 °C and 20 MJ m⁻², respectively; Australian Government, Bureau of Meteorology) until ~3 weeks before the measurements, when they were transferred to a controlled-temperature greenhouse (23 °C day/18 °C night). Mature plants of B. integrifolia, B. paludosa, and B. serrata were purchased from a nursery in Canberra (Australia). Upon purchase, the plants were re-potted into 10.0 l pots containing a mixture of grey sand and potting mix, and grown for 2 months prior to measurements in a greenhouse in Canberra (25 °C day/20 °C night). Key leaf traits, such as LMA, leaf thickness, and density, were measured in all 49 species. With the aim of always covering the wide range of LMA observed in the genus Banksia, subsets of this large group representative of the diversity observed across the genus were selected for further structural and physiological analyses. In all cases, the youngest fully expanded leaves were used. For a list of the traits obtained for each species see the Appendix.
Leaf morphology and anatomy

Three leaves per species, from different plants, were sampled early in the morning. Leaf lamina thickness \( (T_{\text{leaf}}) \) was measured with digital callipers at 5–10 different positions on each leaf. The midrib and petiole were removed prior to measuring the projected area of the lamina using a leaf area meter (LI-300A, Li-Cor, Lincoln, NE, USA). After drying at 80 °C for 3 d, leaf lamina dry mass was measured. Leaf dry tissue density \( (D_{\text{leaf}}) \) was computed from \( LMA \) and \( T_{\text{leaf}} \):

\[
D_{\text{leaf}} = \frac{LMA}{T_{\text{leaf}}} \tag{1}
\]

Based on the relationship between \( LMA \) and \( T_{\text{leaf}} \) and \( D_{\text{leaf}} \), subsets of species that covered the range of \( LMA \) of the 49 species were chosen for further analyses (Appendix).

In three leaves per species, for 14 species (Appendix), the fraction of the leaf volume filled with air \( (f_{\text{air}}) \) was measured by determining leaf buoyancy before and after vacuum infiltration of the leaf air spaces with water, using the method of Raskin (1983) and the equations modified by Thomson et al. (1990). In brief, fresh leaf volume \( (V_{\text{leaf}}) \), leaf gas volume \( (V_{\text{gas}}) \), and \( f_{\text{air}} \) were estimated as:

\[
V_{\text{leaf}} = \frac{M_{\text{leaf, in air}} - M_{\text{leaf, in water}}}{\rho} \tag{2}
\]

\[
V_{\text{gas}} = \frac{M_{\text{leaf, after}} - M_{\text{leaf, before}}}{\rho} \tag{3}
\]

\[
f_{\text{air}} = \frac{V_{\text{gas}}}{V_{\text{leaf}}} \tag{4}
\]

where \( M_{\text{leaf, in air}} \) and \( M_{\text{leaf, in water}} \) are the masses of the leaf in air and water before vacuum infiltration, respectively; \( M_{\text{leaf, after}} \) and \( M_{\text{leaf, before}} \) are the masses of the submerged leaf holder with the leaf after and before vacuum infiltration, respectively; and \( \rho \) is the density of water \( (1 \text{ mg mm}^{-2}) \) at 25 °C.

The density of the fresh leaf tissues excluding the gas volumes \( (\text{density corrected for porosity, } D_{\text{leaf}}^*) \) was calculated as:

\[
D_{\text{leaf}}^* = \frac{M_{\text{leaf}}}{(V_{\text{leaf}} - V_{\text{gas}})} \tag{5}
\]

where \( M_{\text{leaf}} \) is leaf dry mass.

Chemical composition

Nitrogen concentration \( (N_{\text{mass}}) \) was measured in the leaf blade (excluding the midrib) in 17 species (Appendix) using gas chromatography (Carlo Erba EA 1110). Analyses of 14 species were done at the Western Australian Biogeochemistry Centre (University of Western Australia, Perth). Samples from the other three species \( (B. \text{ integrifolia}, B. \text{ paludosa}, \text{ and } B. \text{ serrata}) \) were analysed at the Research School of Biology (Australian National University, Canberra). Finely ground leaf dry material was used from three leaves per species from three different plants, except for \( B. \text{ attenuata} \) and \( B. \text{ ilicifolia} \) where one leaf was analysed. \( N_{\text{area}} \) was subsequently calculated \( (N_{\text{area}} = N_{\text{mass}} \times LMA) \).

The fraction of nitrogen allocated to Rubisco \( (R_{\text{N}}/N) \) was estimated (Appendix) as:

\[
R_{\text{N}} = \frac{V_{\text{c}}}{300} \frac{M_{\text{rubisco}}}{N_{\text{area}}} \tag{6}
\]

where \( V_{\text{c}} \) is the rate of carboxylation, computed using the spreadsheet published by Sharkey et al. (2007), but using chloroplastic CO\(_2\) concentration \( (C_o) \) calculated by combined gas exchange and chlorophyll fluorescence \( (\text{Hassiotou et al., 2009a};) \); \( M_{\text{rubisco}} \) is the molecular mass of Rubisco \( [0.55 \text{ g of Rubisco} \text{ (m\( \mu \text{mol Rubisco})^{-1}};] k_{\text{cat}} \) is the catalytic turnover number at 25 °C \( [3.5 \text{ mol CO}_2 \text{ (mol Rubisco sites)}^{-1} \text{s}^{-1}; \text{von Caemmerer et al., 1994}]; \text{and } n_R \text{ is the number of catalytic sites per mole of Rubisco} \text{ [8 mol Rubisco sites (mol Rubisco)}^{-1};] N_R \text{ is the nitrogen concentration of Rubisco} \text{ [11.4 mmol N (g Rubisco)}^{-1};]\text{ and } N_{\text{area}} \text{ is the nitrogen content per unit leaf area} \text{ (mmol N m}^{-2}; \text{). It was assumed that } k_{\text{cat}} \text{ did not vary between } Bankstia \text{ species or with LMA, but the absolute fraction of nitrogen present in Rubisco could differ if } k_{\text{cat}} \text{ or the activation state varied between the species. Equation 6 provides a minimum estimate of } R_{\text{N}}/N \text{ as it assumes full Rubisco activation (Harrison et al., 2009).}

Total chlorophyll content \( (Chl_{\text{area}}) \) was determined in 12 species (Appendix) using three leaves per species from three different plants, sampled early in the morning and analysed immediately. Leaf segments were excised and their areas were measured with a leaf area meter \( (\text{LI-300A, Li-Cor, Lincoln, NE, USA}). \) Within 5 min of sampling, the leaf segments were finely ground with liquid nitrogen using a cold mortar and pestle and were subsequently extracted with \text{100% cold methanol. The extract was clarified by centrifugation at 1600 g (Beckman, Avantivm J-25 Centrifuge, USA)} for 20 min at 4 °C. To avoid condensation on the cuvette whilst taking measurements, the samples were stored in the dark at room temperature for 5 min. Absorbance was measured with a spectrophotometer \( (\text{Graphicord UV-240, Shimadzu, Kyoto, Japan)} \) at three wavelengths \( (710, 665.2, \text{ and } 652.4 \text{ nm}) \) and the equations of Wellburn (1994) were used to calculate \( \text{Chl a, Chl b, total chlorophyll per unit leaf area} \text{ (Chl}_{\text{area}}) \text{, and dry mass} \text{ (Chl}_{\text{mass}}) \text{. The fraction of nitrogen allocated to thylakoids} \text{ (Chl/N)} \text{, including pigment–protein complexes, the components of electron transport, and ATPase, was estimated from Chl}_{\text{area}} \text{ and N}_{\text{area}} \text{ assuming 50 mol of thylakoid nitrogen per mol of chlorophyll} \text{ (Evans, 1989).}

Microscopy

Cryo-scanning electron microscopy (CSEM) and fluorescence microscopy \( (\text{Zeiss Axioskop2, Zeiss Axioscam with AxioVision, Zeiss Oberkocken, Germany}) \) were used to obtain transverse views of leaf laminae originating halfway from the leaf tip in samples from two leaves per species, from different plants. Analyses were done in Image J (Abramoff et al., 2004). Figure 1 shows diagrammatically how the anatomical measurements were made. Leaf thickness \( (T_{\text{leaf}}) \), mesophyll thickness \( (T_{\text{mesophyll}}) \), and the thickness of the adaxial \( (T_{\text{epidermis, A}}) \) and abaxial \( (T_{\text{epidermis, B}}) \) epidermis plus hypoderms \( (T_{\text{epidermis, A}}) \) were measured from fluorescence micrographs taken at the same magnification in a subset of 10 species (Appendix), and the mean of at least six measurements was used. These measurements were confirmed with CSEM.

Leaf lamina thickness and mesophyll thickness do not take into account the presence of stomatal crypts. Thus, micrographs of transverse leaf views obtained with fluorescence microscopy at the same magnification, were used to calculate leaf volume per area \( (LVA) \) and mesophyll volume per area \( (MVA) \) which exclude the volumes taken by crypt voids. The width of an areole \( (W_{\text{areole}}) \) and the cross-sectional area of non-photosynthetic tissue per areole \( (A_1) \) (including the adaxial and abaxial epidermal and hypodermal tissues as well as the vascular bundles and their sclerified extensions) and of mesophyll tissue per areole \( (A_2) \) (including photosynthetic cells and intercellular airspaces) were measured. A mean of at least four measurements for each of the above parameters was obtained. \( LVA \) and \( MVA \) were calculated as:

\[
LVA = \frac{A_1 + A_2}{W_{\text{areole}}} \tag{7a}
\]

\[
MVA = \frac{A_2}{W_{\text{areole}}} \tag{7b}
\]

The leaf tissue was analysed by considering five compartments: the epidermis/hypodermis, mesophyll, intercellular airspace, vascular tissue, and stomatal crypts. The fraction of the leaf...
Fig. 1. Diagrammatic representation of a single areole showing the leaf anatomical measurements made. Leaf lamina thickness ($T_{\text{leaf}}$) was measured microscopically as the vertical distance between the adaxial and abaxial cuticle. Mesophyll thickness ($T_{\text{mesophyll}}$) was measured as the distance between the adaxial and abaxial epidermis, between the crypt and the vein (i.e. at its maximum). The thicknesses of the adaxial ($T_{\text{epidermis},A}$) and abaxial ($T_{\text{epidermis},B}$) combined epidermis and hypodermis as well as the thickness (depth) of the crypts were measured at the points shown. Two cross-sectional areas were measured: $A_1$ (shown in dark grey), which represents the non-photosynthetic tissue of an areole, including the adaxial and abaxial epidermal and hypodermal tissues as well as the vascular bundles and their sclerified extensions; and $A_2$ (shown in light grey), which represents the mesophyll tissue of an areole, including photosynthetic cells and intercellular airspaces. The width of an areole ($W_{\text{areole}}$) was also measured as shown. From the above, leaf volume per area ($LVA$) and mesophyll volume per area ($MVA$) were calculated (Equations 7a and b, respectively).

cross-section occupied by epidermis ($f_{\text{epidermis}}$) was calculated as the sum of the fractions associated with the adaxial and abaxial epidermis including hypodermal layers. The adaxial epidermis fraction ($f_{\text{epidermis},A}$) was:

$$f_{\text{epidermis},A} = \frac{T_{\text{epidermis},A}}{T_{\text{leaf}}} \quad (8a)$$

The abaxial epidermis fraction ($f_{\text{epidermis},B}$) was:

$$f_{\text{epidermis},B} = \frac{T_{\text{epidermis},B}}{T_{\text{leaf}}} (1 - A_{\text{crypt}} D_{\text{crypt}}) \quad (8b)$$

where the second part of this formula accounts for the portion of the abaxial epidermis and hypodermis that is occupied by crypts, with $A_{\text{crypt}}$ the projected area of an average crypt and $D_{\text{crypt}}$ the number of crypts per unit projected area. $A_{\text{crypt}}$ and $D_{\text{crypt}}$ were measured in scanning electron micrographs of the adaxial leaf surface as described in Hassiotou et al. (2009b). The fraction of the leaf cross-section occupied by mesophyll ($f_{\text{mesophyll}}$) was calculated as:

$$f_{\text{mesophyll}} = \frac{A_2}{A_1 + A_2} = \frac{MVA}{LVA} \quad (8c)$$

The fraction of the leaf cross-section occupied by vascular tissue (including vascular bundle extensions), $f_{\text{vascular}}$, was obtained from $f_{\text{epidermis}}$ and $f_{\text{mesophyll}}$ based on the assumption that:

$$f_{\text{epidermis}} + f_{\text{mesophyll}} + f_{\text{vascular}} = 1 \quad (8d)$$

Although the crypts are external to the leaf and thus do not contribute to $LVA$, for ease of comparison, crypt volume is expressed as a fraction of the leaf volume:

$$f_{\text{crypt}} = \frac{A_{\text{crypt}} D_{\text{crypt}} T_{\text{crypt}}}{LVA} \quad (8e)$$

where $T_{\text{crypt}}$ is the depth of the crypt, using values from Hassiotou et al. (2009b).

Usually one layer, but sometimes locally two layers, of adaxial palisade mesophyll is present in Banksia leaves. The length of adaxial palisade cells ($L_{\text{palisade}}$) was measured as the mean of at least seven measurements in transverse views of five species (Appendix) obtained with CSEM at the same magnification.

Wall thickness of palisade and spongy mesophyll cells was measured in six species (Appendix) and mean mesophyll cell wall thickness was calculated ($T_w$). Leaves of these species were frozen in liquid nitrogen and high-magnification images of the cell walls were obtained with CSEM following McCully et al. (2004). Segments of the leaf lamina from the middle part of each leaf were excised under liquid nitrogen, mounted on stubs with low-temperature Tissue-Tek (OCT Compound cryostat specimen matrix, ProSciTech), planed flat in the paradermal and transverse direction using a diamond knife in a cryomicrotome (Cryo-system Oxford CT1500, Oxford Instruments Ltd, Eynsham, Oxford, UK) at −100 °C, etched in the column of the CSEM (Cambridge S360, Cambridge Instruments Ltd, Cambridge, UK) for 1−2 min at −90 °C to reveal cell outlines, sputter-coated with gold, and examined at 15 kV. Images were captured using Microsoft PhotoDraw and analysed in Image J (Abramoff et al., 2004).

Photosynthetic measurements

Gas exchange measurements were carried out for 18 species (Appendix) using three leaves per species from different plants, at a photosynthetic photon flux density of 1500 μmol quanta m$^{-2}$ s$^{-1}$, at 380 μmol CO$_2$ mol$^{-1}$ air and at 25 °C, with a LI-6400 open gas exchange system (LI-6400-40, Li-Cor, Lincoln, NE, USA). Leaves were kept in the gas exchange chamber at high irradiance (1500 μmol quanta m$^{-2}$ s$^{-1}$) and low CO$_2$ concentration (100 μmol CO$_2$ mol$^{-1}$ air) for at least 10 min before the commencement of the measurements, ensuring stomata were fully open and steady state was reached. At ambient CO$_2$ concentration, 4−10 measurements of gas exchange, at least 7 s apart, were recorded for each leaf, and the mean value of the net CO$_2$ assimilation rate was calculated and expressed on a leaf area basis ($A_{\text{mass}} = A_{\text{areal}} L_{\text{MA}}$, nmol g$^{-1}$ s$^{-1}$), per unit Chl ($A_{\text{Chl}} = A_{\text{areal}} / \text{Chl}$, μmol g$^{-1}$ s$^{-1}$), per unit mesophyll volume ($A_{\text{mass}} = A_{\text{areal}} / \text{MVA}$, μmol m$^{-2}$ s$^{-1}$) and per unit nitrogen ($PNUE = A_{\text{mass}} / N_{\text{mass}}$, nmol g$^{-1}$ s$^{-1}$).

Combined gas exchange and chlorophyll fluorescence measurements (Harley et al., 1992) were conducted and mesophyll conductance ($g_{\text{mm}}$) was calculated in seven species (Appendix) as described in Hassiotou et al. (2009b).

Statistical analyses

Following previous studies (e.g. Poorter et al., 2009), the aim was to identify the extent to which $T_{\text{leaf}}$ and $D_{\text{leaf}}$, the two determinants
of the key structural trait LMA, contributed to its variation across the 49 Banksia species (see Appendix).

Log–log scaling slope analysis is a method that has been used previously (e.g. Poorter and van der Werf, 1998; Poorter et al., 2009) to estimate the contribution of explanatory variables (such as \( T_{\text{leaf}} \) and \( D_{\text{leaf}} \)) to variation in a particular variable of interest (such as LMA). This method is based on the relationship \( \text{LMA} = T_{\text{leaf}} \times D_{\text{leaf}} \) and thus \( \log(\text{LMA}) = \log(T_{\text{leaf}}) + \log(D_{\text{leaf}}) \), which is exact in this case due to the fact that \( D_{\text{leaf}} \) was calculated from measured LMA and \( T_{\text{leaf}} \). If the log of an explanatory variable (in this case either \( T_{\text{leaf}} \) or \( D_{\text{leaf}} \)) is fitted as a linear model of the log of the variable of interest (in this case LMA), then a slope coefficient value of close to 1 is supposed to indicate that the particular explanatory variable used is largely responsible for variation in the variable of interest, whereas a value close to 0 indicates that the particular explanatory variable used is not responsible for much of the observed variation in the variable of interest (Poorter and van der Werf, 1998; Poorter et al., 2009). However, this method has potential problems when explanatory variables are positively or negatively correlated. This method was thus applied in the present study to enable comparison with previous literature, but the contribution of \( T_{\text{leaf}} \) and \( D_{\text{leaf}} \) to variation in LMA was also evaluated using a simple and more transparent alternative method.

This simple alternative method is based on the fact that \( \log(\text{LMA}) = \log(T_{\text{leaf}}) + \log(D_{\text{leaf}}) \), and thus \( \text{var}[\log(\text{LMA})] = \text{var}[\log(T_{\text{leaf}})] + \text{var}[\log(D_{\text{leaf}})] + 2 \times \text{cov}[\log(T_{\text{leaf}}),\log(D_{\text{leaf}})] \). If the contributing variables \( \log(T_{\text{leaf}}) \) and \( \log(D_{\text{leaf}}) \) are not correlated then the covariance component \( 2 \times \text{cov}[\log(T_{\text{leaf}}),\log(D_{\text{leaf}})] \) will be relatively small and thus contribute little to the observed variability in \( \log(\text{LMA}) \). If the contributing variables \( \log(T_{\text{leaf}}) \) and \( \log(D_{\text{leaf}}) \) are (positively or negatively) correlated then the covariance component will be relatively large (and positive or negative, respectively), and thus contribute substantially to the observed variability in \( \log(\text{LMA}) \). The respective contributions of the three components \( \text{var}[\log(T_{\text{leaf}})] \), \( \text{var}[\log(D_{\text{leaf}})] \), and \( 2 \times \text{cov}[\log(T_{\text{leaf}}),\log(D_{\text{leaf}})] \) to \( \text{var}[\log(\text{LMA})] \) were thus simply calculated. Note that these three contributions must sum to 100%. If the contribution of \( 2 \times \text{cov}[\log(T_{\text{leaf}}),\log(D_{\text{leaf}})] \) is small, then the variables are relatively uncorrelated, and it makes sense to compare the other two contributions to determine whether variability in LMA is due more to variability in \( T_{\text{leaf}} \) or \( D_{\text{leaf}} \), or whether they are contributing similarly. If the contribution of \( 2 \times \text{cov}[\log(T_{\text{leaf}}),\log(D_{\text{leaf}})] \) is large (positive or negative), then the variables are relatively correlated, and the interpretation must be much more cautious. The correlation coefficient between \( \log(T_{\text{leaf}}) \) and \( \log(D_{\text{leaf}}) \) was also calculated, for reference as a more commonly used measure of correlation. Note that in most cases, the results of the two methods would be expected to support each other, but in particular cases discrepancies between these methods could highlight issues that need further investigation (such as high correlation between explanatory variables). Note that both these approaches are not investigating which of \( T_{\text{leaf}} \) and \( D_{\text{leaf}} \) contribute most to LMA, but rather which contributes most to variation in LMA.

These two approaches were also used to examine the main determinants of the variation in \( D_{\text{leaf}} (D_{\text{leaf}*}) \) and \( f_{\text{air}} \) in 14 species (Appendix), using the equation:

\[
D_{\text{leaf}} = (1 - f_{\text{air}}) \times D_{\text{leaf}*}
\]

and using log transformations to make the relationship additive. This again describes an exact relationship, because of how \( D_{\text{leaf}*} \) was calculated. The two approaches were again used to examine the main determinants of the variation in \( T_{\text{leaf}} (T_{\text{mesophyll}}, T_{\text{epidermis,B}}, \text{and } T_{\text{epidermis,T}}) \) in 10 species (Appendix), but since the relationship between \( T_{\text{leaf}} \) and its components is additive rather than multiplicative \((T_{\text{leaf}} = T_{\text{mesophyll}} + T_{\text{epidermis,B}} + T_{\text{epidermis,T}})\), the methods were applied directly to the original values of the different thicknesses, without log transformation. Also, in this case the relationship was not exact as all thicknesses were measured independently. As there were three contributing variables involved, four contributions to variance were calculated, the three contributions due to variability in \( T_{\text{mesophyll}}, T_{\text{epidermis,B}}, \text{and } T_{\text{epidermis,T}} \) and the covariance contribution, which is equal to \( 2 \times \text{cov}(T_{\text{mesophyll}}, T_{\text{epidermis,B}}) \times \text{cov}(T_{\text{epidermis,T}}, T_{\text{epidermis,B}}) \times \text{cov}(T_{\text{epidermis,T}}, T_{\text{epidermis,B}}) \). The three correlation coefficients between \( T_{\text{mesophyll}}, T_{\text{epidermis,B}}, \text{and } T_{\text{epidermis,T}} \) were also calculated to complete the picture.

To help understand the variability in \( A_{\text{area}} \), two relationships were considered. The first relationship aimed at assessing if variation in \( A_{\text{area}} \) was due mostly to differences in the amount of mesophyll tissue or in the mesophyll’s photosynthetic activity:

\[
A_{\text{area}} = f_{\text{mesophyll}} \times A_{\text{mes}} \times LVA
\]

where \( f_{\text{mesophyll}} \) is the mesophyll volume fraction \((\text{m}^3 \text{m}^{-3})\), \( A_{\text{mes}} \) is the \( \text{CO}_2 \) assimilation rate per mesophyll \((\mu\text{mol m}^{-2} \text{s}^{-1})\) and \( LVA \) is leaf volume per area \((\text{m}^3 \text{m}^{-2})\). The second relationship considered for \( A_{\text{area}} \) aimed at assessing if variation in \( A_{\text{area}} \) was related more to differences in the amount of chlorophyll or in the photosynthetic rate per unit chlorophyll:

\[
A_{\text{area}} = C_{\text{chl}} \times A_{\text{Chl}} \times MVA
\]

where \( C_{\text{chl}} \) is the chlorophyll concentration per mesophyll volume \((\text{g} \text{m}^{-3})\), \( A_{\text{Chl}} \) is \( \text{CO}_2 \) assimilation rate per chlorophyll \((\mu\text{mol g}^{-1} \text{s}^{-1})\), and \( MVA \) is the mesophyll volume per unit leaf area \((\text{m}^3 \text{m}^{-2})\). These two relationships were converted from multiplicative to additive relationships by taking the log of the various variables. Both these relationships were exact, due to the fact that one of the variables in each of the equations had been calculated from the others, and both involved three contributing variables. All these above analyses were conducted using the R statistical program (R Development Core Team 2009).

To examine whether \( T_{\text{epidermis,T}} \) was significantly different from \( T_{\text{epidermis,B}} \), a paired t-test was carried out (Microsoft Excel© 2007, Microsoft Corporation).

**Results**

**LMA and its anatomical correlates**

Among the 49 broad-leaved Banksia species examined, LMA varied 4-fold (134–507 g m\(^{-2}\)), which was associated with a 4-fold variation in leaf lamina thickness \((T_{\text{leaf}}; 193–700 \mu\text{m})\) and a 3-fold variation in leaf density \((D_{\text{leaf}}; 0.41–1.17 \text{mg mm}^{-3})\). Both \( T_{\text{leaf}} \) and \( D_{\text{leaf}} \) were approximately equally good predictors of LMA, as indicated by both the variance partitioning and the log–log scaling slope analyses (Table 1). Some species had high LMA due to their high \( D_{\text{leaf}} \) and others due to their high \( T_{\text{leaf}} \), whilst in some high LMA was due to both (Fig. 2). For example, both B. coccinea and B. quercifolia had an LMA of 215 g m\(^{-2}\), but a \( T_{\text{leaf}} \) of 0.50 mm and 0.38 mm, and a \( D_{\text{leaf}} \) of 0.4 mg mm\(^{-3}\) and 0.6 mg mm\(^{-3}\), respectively.

Thicker leaves, with high volume per area (LVA), had thicker mesophyll \((T_{\text{mesophyll}})\), adaxial \((T_{\text{epidermis,B}})\), and abaxial \((T_{\text{epidermis,T}})\) epidermis and hypodermis, greater mesophyll volume per area \((MVA)\), and longer adaxial palisade cells \((L_{\text{palisade}})\) (Fig. 3, Table 1). Both statistical analyses used to examine the contributions of the variability in the thickness of the different leaf layers to the variability in \( T_{\text{leaf}} \) indicated that the \( T_{\text{mesophyll}} \) contributed most to the variability of \( T_{\text{leaf}} \), although variability in \( T_{\text{epidermis,T}} \) and \( T_{\text{epidermis,B}} \) also contributed to variability in \( T_{\text{leaf}} \) (Table 1).
Table 1. Results of analyses of the relative contribution of explanatory variables to measured structural and physiological variables: variance partitioning between contributing factors and covariance, correlation between contributing factors (r), and log-log scaling slope analysis (slope)

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>% due to variance</th>
<th>% due to covariance</th>
<th>r</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δleaf to LMA</td>
<td>49</td>
<td>63%</td>
<td>-38%</td>
<td>-0.27</td>
<td>0.43***</td>
</tr>
<tr>
<td>Tleaf to LMA</td>
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<td>-30%</td>
<td>0.63</td>
<td>0.57***</td>
</tr>
<tr>
<td>(Tleaf - Tmesophyll) to Tleaf</td>
<td>14</td>
<td>61%</td>
<td>30%</td>
<td>0.63</td>
<td>0.76***</td>
</tr>
<tr>
<td>(Δleaf - Tmesophyll) to Δleaf</td>
<td>14</td>
<td>9%</td>
<td>30%</td>
<td>0.24**</td>
<td></td>
</tr>
<tr>
<td>Tmesophyll to Tleaf</td>
<td>10</td>
<td>60%</td>
<td>27%</td>
<td>0.59, 0.26, 0.30</td>
<td>0.74***</td>
</tr>
<tr>
<td>Tepidermis,T to Tleaf</td>
<td>10</td>
<td>0.3%</td>
<td>-0.04</td>
<td>0.04*</td>
<td></td>
</tr>
<tr>
<td>Tepidermis,T to Δleaf</td>
<td>10</td>
<td>12%</td>
<td>-0.04</td>
<td>0.16 ns</td>
<td></td>
</tr>
<tr>
<td>ΔA mins to Aarea</td>
<td>10</td>
<td>424%</td>
<td>-697%</td>
<td>-0.76, -0.19, -0.30</td>
<td>1.27 ns</td>
</tr>
<tr>
<td>LVA to Aarea</td>
<td>10</td>
<td>271%</td>
<td>-0.38</td>
<td>0.11 ns</td>
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</tr>
<tr>
<td>fmesophyll to Aarea</td>
<td>10</td>
<td>100%</td>
<td>0.39</td>
<td>1.00 ns</td>
<td></td>
</tr>
<tr>
<td>ΔAChl to Aarea</td>
<td>9</td>
<td>561%</td>
<td>-105%</td>
<td>-0.45, -0.72, 0.18</td>
<td>0.09 ns</td>
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<tr>
<td>Chl to Aarea</td>
<td>9</td>
<td>401%</td>
<td>0.39</td>
<td>0.39 ns</td>
<td></td>
</tr>
<tr>
<td>MVA to Aarea</td>
<td>9</td>
<td>203%</td>
<td>-0.19</td>
<td>1.00 ns</td>
<td></td>
</tr>
</tbody>
</table>

n, species number; ***Ρ<0.001; **Ρ<0.01; *Ρ<0.05; ns, not significant; asterisks indicate the significance of the slope parameter, i.e. whether the explanatory variable contributes significantly to the response variable (†: calculated parameter).

Aarea, net CO₂ assimilation rate per unit leaf area; AChl, net CO₂ assimilation rate per chlorophyll; Amins, net CO₂ assimilation rate per unit mesophyll; Chl, chlorophyll concentration per mesophyll volume; Dleaf, leaf density; Dleaf*, leaf density corrected for porosity; fair, intercellular airspace fraction; fmesophyll, mesophyll fraction; LMA, leaf dry mass per area; Tepidermis,T, thickness of abaxial epidermis and hypodermis combined; Tepidermis,T, thickness of adaxial epidermis and hypodermis combined; Tleaf, leaf lamina thickness; Tmesophyll, mesophyll thickness.

Mesophyll tissue represented on average 74% of leaf lamina thickness (based on Tmesophyll/Tleaf) and 58% of leaf volume (based on MVA/LVA). High Tmesophyll/LVA, Tmesophyll, and MVA were associated with high LMA (P < 0.01) (data not shown). Tepidermis,T varied 3-fold among the examined species and was significantly higher than Tepidermis,B (P < 0.001), which varied 2-fold. Both Tepidermis,T and Tepidermis,B increased with increasing LMA, although this was significant (P=0.008) only for Tepidermis,B (data not shown).

Leaf density corrected for porosity (Dleaf*) tended to increase with increasing thickness of the different leaf layers, but none of these relationships were significant. The fraction of leaf occupied by air (fair) tended to decrease with increasing thickness of the different leaf layers, although only its relationship with Tleaf was significant (P=0.016). fair varied 3.5-fold among the species, ranging from 0.06 (in B. elderiana; Tleaf=0.63 μm) to 0.22 (in B. littoralis; Tleaf=0.22 μm) (Fig. 4B). fair was the only fraction of those examined that showed a significant (and negative) correlation with LMA (Fig. 4B). The mesophyll fraction (fmesophyll) was ~0.6, irrespective of LMA, as was also the crypt fraction (fcrypt) of 0.1–0.2 (Fig. 4A). The epidermal fraction (fepidermal) was 0.2–0.3 in all species examined (Fig. 4A) except for B. repens with a fraction of 0.1 and B. ilicifolia with a fraction of 0.4, which was indicative of the unusually thick adaxial epidermis and hypodermis of this species. Finally, the vascular tissue fraction (fvascular) was 0.1–0.2, similar to fcrypt, for most species (Fig. 4A), although B. repens and B. attenuata showed a higher fraction of 0.3.

Dleaf was positively correlated with leaf dry matter content (r²=0.28, P=0.023), which was similar but not quite significant for Dleaf*. Dleaf* contributed most to the variability of Dleaf (Table 1).

High-LMA leaves had significantly thicker mesophyll cell walls (Tw) (Fig. 5A). Thicker cell walls should impede CO₂ diffusion and were associated with lower mesophyll conductance (gma) (Fig. 5B). Doubling Tw was associated with a halving in gma.

Leaf structure, photosynthesis, and mesophyll conductance

The CO₂ assimilation rate per unit leaf area (Aarea) and leaf conductance (gleaf, which in the case of species with crypts

Fig. 2. Relationship between log10-transformed leaf lamina thickness (Tleaf, circles) or leaf density (Dleaf, squares) and leaf dry mass per area (LMA) in 49 Banksia species. Grey symbols show the seven species used for the measurement of mesophyll conductance, while crossed symbols represent the 10 species examined by microscopy (see Appendix for species names). [For the relationship between Tleaf and LMA, the slope is 0.57 and r² is 0.43 (P < 0.001); for the relationship between Dleaf and LMA, the slope is 0.43 and r² is 0.30 (P < 0.001).]

Fig. 3. Palisade cell length (lpalisade) against mesophyll thickness (Tmesophyll) in five Banksia species (see Appendix for species names).
comprises stomatal and crypt conductance) correlated poorly with LMA (Fig. 6A, B), leaf thickness, and density (data not shown) in 18 species. In the subset of seven species in which mesophyll conductance ($g_m$) was measured, $A_{area}$ and $g_{leaf}$ tended to decrease with increasing LMA, while $g_m$ strongly decreased with LMA (Fig. 6C). The decrease in $g_{leaf}$ with increasing LMA that was observed in seven Banksia species was better correlated with $D_{leaf}$ ($r^2=0.76, P=0.01$) than with $T_{leaf}$ ($r^2=0.34, P=0.17$). CO$_2$ assimilation rate per unit leaf mass ($A_{mass}$) showed a strong negative correlation with LMA (Fig. 7A), but not with $T_{leaf}$ or $D_{leaf}$ (data not shown). Nitrogen concentration ($N_{mass}$) varied 4-fold and decreased with increasing LMA ($r^2=0.49, P=0.0018$). As expected, $A_{mass}$ was positively associated with $N_{mass}$ ($r^2=0.49, P=0.0018$). No correlation was found between CO$_2$ assimilation rate per unit chlorophyll ($A_{Chl}$) and LMA (data not shown), while CO$_2$ assimilation rate per unit mesophyll ($A_{mes}$) decreased with increasing LMA (Fig. 8A). Factorizing $A_{area}$ into CO$_2$ assimilation rate per unit mesophyll ($A_{mes}$), LVA, and $f_{mesophyll}$ showed that $A_{mes}$ was more variable than the other parameters (Table 1). However, the large contribution of covariance, the negative correlations between $A_{mes}$, $LVA$, and $f_{mesophyll}$, and the non-significance of the slope analysis all indicate that further conclusions should not be drawn from these analyses. Factorizing $A_{area}$ into the product of CO$_2$ assimilation rate per chlorophyll ($A_{Chl}$), chlorophyll concentration per mesophyll volume ($Chl_{mes}$), and mesophyll volume per unit leaf area ($MVA$), showed that $A_{Chl}$ and $Chl_{mes}$ were more variable than $MVA$, but the results also indicate that further conclusions should not be drawn from these analyses (Table 1). However, CO$_2$ assimilation rate per unit nitrogen (PNUE) tended to decrease with increasing LMA (Fig. 8B). The fractions of nitrogen allocated to Rubisco and thylakoids tended to decrease with increasing LMA, although this was not significant (Fig. 9). Chlorophyll content per unit leaf area tended to be higher in high-LMA species with thicker mesophyll ($r^2=0.22, P=0.08$).

**Discussion**

Many comparative studies examining the variability in leaf structure and its effect on leaf physiology consider diverse species from different genera differing in LMA (Poorter and Evans, 1998; Wright et al., 2004; Flexas et al., 2008; Harrison et al., 2009; Poorter et al., 2009). In the present study, phyllogenetic variation was minimized by focusing on one genus (Banksia) with a great leaf structural diversity that allowed quantitative relationships between LMA and its components to be established with photosynthetic characteristics at the high end of the LMA spectrum.

**LMA and its anatomical correlates**

Among the 49 Banksia species examined, LMA (134–507 g m$^{-2}$), $T_{leaf}$ (193–700 µm), and $D_{leaf}$ (0.41–1.17 mg mm$^{-2}$)
varied 4-, 4-, and 3-fold, respectively, which is indicative of the broad range of leaf structure that is represented in this genus. Niinemets et al. (2009) found a 4.7-fold variation in LMA (66–313 g m\(^{-2}\)) and a 2.5-fold variation in T\(_{leaf}\) (274–594 \(\mu\)m) and D\(_{leaf}\) (0.29–0.56 mg mm\(^{-3}\)) across 35 Australian sclerophyllous species from 20 genera. Poorter et al. (2009) reported a 4-fold variation in leaf volume per area (equivalent to T\(_{leaf}\)) (100–700 \(\mu\)m) and a 7-fold variation in D\(_{leaf}\) (0.1–0.6 mg mm\(^{-3}\)) in a data set containing woody and herbaceous species from three functional groups. In their data set, most of the variation in LMA within functional groups is attributed to variation in D\(_{leaf}\), while differences in LMA between sclerophylls and mesophytes are usually due to variation in T\(_{leaf}\) (Poorter et al., 2009). Log–log scaling slope analysis in species from three functional groups showed that 80% and 20% of the variability in LMA was due to variability in D\(_{leaf}\) and T\(_{leaf}\), respectively (Poorter et al., 2009). The larger role of D\(_{leaf}\) in the data set of Poorter et al. (2009) is due to the fact that the range in D\(_{leaf}\) was much greater in their data set than that in the 49 Banksia species examined in this study (7-fold and 3-fold, respectively), whereas the ranges in T\(_{leaf}\) were very similar (~4-fold in both data sets). Moreover, the relationship between D\(_{leaf}\) and LMA is fairly similar for different functional groups, whereas the relationship between T\(_{leaf}\) and LMA differs between functional groups, such that T\(_{leaf}\) becomes a poorer predictor of LMA in the combined data set. It is also noteworthy that values of D\(_{leaf}\), T\(_{leaf}\), and LMA of some of the Banksia species extend far beyond the range found in the data set of Poorter et al. (2009).

The considerable variability in both D\(_{leaf}\) and T\(_{leaf}\) in the present data set indicates that even within the same genus there are various ways of achieving high LMA, with potential ecological significance. Niinemets et al. (2009) found that density tended to increase with decreasing water availability, and thickness increased with decreasing soil fertility in a comparison of Australian species from sites that differed in water and nutrient availability. A number of previous studies have also reported increases in leaf thickness with decreasing soil fertility as well as with other factors, such as decreasing rainfall and humidity and increasing irradiance (Beadle, 1966; Nobel et al., 1975; Chabot and Chabot, 1977; Givnish, 1978; Sobrado and Medina, 1980). High irradiance can result in increased T\(_{leaf}\) through the development of thicker epidermal tissues that confer photoprotection (Witkowski and Lamont, 1991;
High irradiance can also lead to high \( D_{\text{leaf}} \) (Chabot and Chabot, 1977) through addition of dense, sclerified tissues that increase the uniformity of illumination within thick leaves (Poulson and Vogelmann, 1990; Karabourniotis, 1998), although these tissues may also play other roles, such as providing support and enhancing the rigidity of long-lived high-LMA leaves.

\( D_{\text{leaf}}^* \) was an important predictor of LMA in Banksia leaves. Increases in \( D_{\text{leaf}}^* \) can result from increases in the proportion of non-photosynthetic supporting tissue, especially sclerified cells, and/or a general tendency for cells to have more structural mass. The latter can be due to thicker cell walls, but also to larger surface to volume ratios of smaller cells. In Banksia, mesophyll cells of high-LMA species had thicker cell walls compared with low-LMA species: a 4-fold range in LMA was accompanied by a 2-fold range in \( T_w \). This demonstrates that LMA does not simply scale proportionally with \( T_w \). Previous studies have reported a range of 0.15–0.4 \( \mu \text{m} \) for \( T_w \) (Hanba et al., 1999, 2001, 2002), with the Banksia species examined here being at the high end of this range, but with much higher LMA than the tree leaf LMA values from the above studies. Interestingly, the fraction of leaf volume occupied by the mesophyll was independent of LMA, indicating that high-LMA leaves used greater mesophyll volumes to achieve similar photosynthetic rates per unit leaf area than those of low-LMA leaves. As with the mesophyll fraction, the crypt, epidermal, and vascular tissue fractions were all independent of LMA, demonstrating that the volume of these tissues scales with leaf volume across a wide range of LMA.

Leaf thickening can occur through (i) addition of mesophyll cell layers; (ii) elongation of mesophyll cells; and/or (iii) addition of non-photosynthetic supporting tissue in the epidermal and hypodermal layers. The present results indicate that, in Banksia, all tissues contribute somewhat to increases in \( T_{\text{leaf}} \), but \( T_{\text{mesophyll}} \) contributes the most and is a better predictor of \( T_{\text{leaf}} \) than the epidermal thicknesses. Microscopic observations suggest that elongation of adaxial palisade cells was a major contributor to mesophyll thickening (Fig. 3). Abaxial palisade-like cells were observed in Banksia leaves alongside the crypts, a pattern that appears to be more common in high-LMA leaves, but more research is needed to elucidate their contribution to leaf thickening.

Given that \( A_{\text{area}} \) and \( f_{\text{mesophyll}} \) did not correlate with LMA, a question arises as to whether this indicates that the increase in mesophyll volume per area with increasing LMA is associated with a roughly proportional decrease in photosynthesis per unit mesophyll. Are there limits to how much photosynthetic tissue per area a leaf can have before it becomes inefficient in some way?

Leaf structure, photosynthesis, and mesophyll conductance

LMA has often been the trait of interest when looking at relationships between leaf structure and photosynthesis, but
since it is a product of two anatomical traits that often vary independently (T_{leaf} and D_{leaf}) and that may influence photosynthesis differently, a great variability is found in the relationship between LMA and A_{area} (Niinemets and Sack, 2006), which was also observed among Banksia species in this study. In contrast, a clearer negative relationship exists between A_{mass} and LMA (Fig. 7A; Wright et al., 2004). This can be at least partly attributed to the fact that high-LMA species have more structural material per unit dry mass, as indicated through their higher dry matter content.

Few studies have examined how the two components of LMA, D_{leaf} and T_{leaf}, relate to photosynthetic rates. In the present study, neither D_{leaf} nor T_{leaf} strongly correlated with A_{area} or A_{mass}. In a meta-analysis in a large data set, Niinemets (1999) found no significant relationship between A_{area} and D_{leaf}, but A_{area} scaled with T_{leaf} and LMA, while A_{mass} scaled negatively with D_{leaf} and LMA, being independent of T_{leaf}. While D_{leaf} and T_{leaf} are appealing parameters because they are easy to measure, their poor explanatory power suggests that other leaf traits that are more difficult to obtain are required to explain variation in photosynthetic rates.

The lower A_{mass} in combination with the lower N_{mass} at high LMA can explain the weak relationship obtained between PNUE (PNUE = A_{mass}/N_{mass}) and LMA. This is in contrast to previous studies, which reported a strong decrease of PNUE with increasing LMA (Poorter and Evans, 1998; Hikosaka, 2004), attributing this relationship to the increase of non-photosynthetic (structural) relative to photosynthetic tissue. In contrast to previous studies, which reported a strong decrease of PNUE in high-LMA leaves, and is consistent with the general trend of species at the high-LMA end of the spectrum (Wright et al., 2004). It would be worthwhile to measure LMA concentrations of mesophyll tissue across the range of LMA. The present data do not enable an estimation to be made of the mesophyll N concentrations; however, Chl per mesophyll volume was estimated based on the assumption that all leaf chlorophyll is located in the mesophyll. Chl_{mes} did not significantly decrease with increasing LMA. A similar pattern or a slight decrease with LMA (given the reduction in A_{mes} with LMA) may be expected for N per mesophyll volume.

An increasing body of evidence shows that g_{m} is an important factor limiting photosynthesis in C_{3} plants (Flexas et al., 2008; Evans et al., 2009). In seven Banksia species, g_{m} decreased significantly with increasing LMA (Fig. 6C and Hassiotou et al., 2009a). The negative relationship between g_{m} and LMA was mainly associated with D_{leaf} and not with T_{leaf}, since the latter correlated poorly with g_{m}. A factor contributing to the increase in D_{leaf} and directly to g_{m} was the increase in mesophyll cell wall thickness (Fig. 5B).

A_{mes}, A_{Chl}, and Chl_{mes} were better predictors and contributed more to the variability of A_{area} than f_{mesophyll} and MVA (although none of the corresponding slope analyses were significant) and these trends indicate that the photosynthetic capacity of the tissue is more responsible for the variation in A_{area} than the amounts of photosynthetically active tissue. Interestingly, A_{area} in the examined species reached values that were comparable with many mesophytic species of lower LMA (Flexas et al., 2008). Denton et al. (2007) found similar photosynthetic rates in field-grown Banksia plants.

A_{mes} decreased as LMA increased, since MVA increased with LMA. The chloroplastic CO_{2} concentration (C_{c}) was remarkably stable across the LMA range examined (Hassiotou et al., 2009a), so this does not explain a lower A_{mes}. Evans et al. (2009) reported a positive relationship between mesophyll resistance per unit of exposed chloroplast surface area and mesophyll cell wall thickness (T_{w}), and a negative relationship between the rates of photosynthesis per unit of exposed chloroplast surface area, A_{c}, and T_{w}. To the extent that A_{mes} reflects A_{c}, the data for Banksia confirm this trend. Lower A_{mes} may offset the impact of the increase in T_{w} in high-LMA leaves to moderate the CO_{2} drawdown from the substomatal cavity to the sites of carboxylation. A similar relationship was found by Terashima et al. (2006). Evidence suggests that at the high-LMA end of the spectrum, investment in chlorophyll is not a key component of A_{mes}. Instead, the lower A_{mes} of high-LMA leaves may reflect lower Rubisco
concentrations, lower Rubisco specific activity, lower Rubisco activation state, or reduced nitrogen allocation. The decreasing trend between PNUE (Fig. 8B) or nitrogen allocation to Rubisco or thylakoids (Fig. 9) and LMA suggests that reduced allocation of nitrogen to photosynthetic proteins may be causing the decline in $A_{\text{mes}}$. In Banksia, greater investment in photosynthetic machinery may not be advantageous in the extremely nutrient-impoveryed and seasonally dry habitats of these species where economic use of nutrients is vital and partial stomatal closure is common in the dry season (Veneklaas and Poot, 2003).

In addition to lower investment in photosynthetic machinery, lower $A_{\text{mes}}$ in high-LMA leaves could be a consequence of structural changes that result in irregular distribution of CO$_2$ and light across the leaf or greater diffusive limitations. For example, mesophyll cell wall thickness, which was greater in high-LMA leaves, may compromise $g_m$. Mesophyll surface area exposed to the intercellular spaces is another component of $g_m$ which needs to be measured in order to understand the anatomical basis of $g_m$. Difficulty in embedding the Banksia leaves has so far prevented this important parameter from being obtained. A meta-analysis showed that leaf structure was a more important determinant of photosynthesis than nitrogen (Niinemets, 1999).

There are some anatomical and physiological mechanisms that may reduce the negative effects of the structure of thick and dense leaves on CO$_2$ diffusion and light transmission. Increased presence of bundle sheath extensions and other sclerenchymatous tissues in high-LMA leaves facilitates light transmission to deeper leaf layers (Poulson and Vogelmann, 1990; Smith et al., 1997; Karabourniotis, 1998; Nikolopoulos et al., 2002), improving the uniformity of illumination across thick leaves. Stomatal crypts, present in most Banksia species (Hassiotou et al., 2009b), facilitate CO$_2$ diffusion to adaxial palisade cells.

**Conclusions**

The detailed analyses of the specific leaf structural and physiological traits contributing to variation in $A_{\text{area}}$ in Banksia leaves have provided new insights into the relationship between $A_{\text{area}}$ and LMA at the high end of the LMA spectrum. These leaves have large amounts of dense tissues that are not photosynthetically active, and therefore it is not surprising that they have lower $A_{\text{mass}}$. The present analysis of the factors that contribute to variation in $A_{\text{area}}$, however, shows that high-LMA leaves actually have more mesophyll per unit leaf area, but that the photosynthetic capacity of this tissue is lower. The net result is that photosynthetic rates per unit leaf area are independent of LMA. The lower photosynthetic capacity of the mesophyll of high-LMA leaves in Banksia could be due to structural limitations and partly to lower nitrogen concentrations. The contribution from these two limitations to the reduction of $A_{\text{mes}}$ at high LMA may differ between species. Future research must focus on how structural components (e.g. mesophyll surface area exposed to the intercellular airspaces) and the investment in photosynthetic machinery (e.g. Rubisco and nitrogen allocation to mesophyll) change with LMA, as potential explanations of the lower $A_{\text{mes}}$ of high-LMA leaves.

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**Appendix**

List of the 49 Banksia species examined (for nomenclature see Western Australian Herbarium, 1998)

<table>
<thead>
<tr>
<th>Species</th>
<th>Analysis</th>
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</tr>
<tr>
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<td>B. attenuata</td>
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<td>B. Lindleyana</td>
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exchange measurements (CO2 assimilation rate and leaf conduc-
tance); 3: 17 species (Fig. 6a, c), nitrogen content and photosynthetic
nitrogen use efficiency; 4: 14 species (Fig. 3a), leaf volume and
porosity; 5: 12 species (Fig. 8b), nitrogen allocated to thylakoids; 6: 10
nitrogen use efficiency; 4: 14 species (Fig. 3a), leaf volume and
porosity; 5: 12 species (Fig. 8b), nitrogen allocated to thylakoids; 6: 10
species (Figs 2a, 3b, 7), thickness of the different leaf layers,
mesophyll volume per unit leaf volume, and net CO2 assimilation rate
per unit mesophyll; 7: 7 species (Fig. 5c), mesophyll conductance; 8: 6
species (Fig. 4), mesophyll cell wall thickness; 9: 5 species (Fig. 2b),
palisade cell length; 10: 6 species (Fig. 8a), nitrogen allocated to
Rubisco.

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