Ecotype adaptation and acclimation of leaf traits to rainfall in 29 species of 16-year-old *Eucalyptus* at two common gardens

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Summary

1. Relationships of leaf traits with rainfall at the place of origin of seed (*R*<sub>PO</sub>) are a function of acclimation and adaptation. To disentangle these effects we studied 29 species of 16-year-old *Eucalyptus* at a productive and an unproductive common garden (mean annual increments of above-ground stem volume = 21 ± 11 and 8 ± 5 m<sup>3</sup> ha<sup>−1</sup> years<sup>−1</sup>, respectively). We tested three hypotheses: (i) leaf traits vary between sites, but relationships among them do not; (ii) relationships of leaf traits with *R*<sub>PO</sub> do not vary between sites; and (iii) ecotypes originating from low-rainfall areas allocate a small fraction of nitrogen to thylakoid proteins and Rubisco, and have small SLA, small and narrow leaves, and large water-use efficiency (WUE).

2. Eleven leaf traits (leaf area, leaf thickness, leaf width/length, specific leaf area, fresh weight/dry weight, N, chlorophyll *a*,/b*, carotenoids/chlorophyll, thylakoid N%, Rubisco N%, WUE derived from <sup>13</sup>C content) were measured in 1-year-old sun leaves.

3. Site had a large effect on not only the absolute values of leaf traits, but also relationships between pairs of traits. There were 20 significant correlations between pairs of traits. Three of the correlations had different slopes between sites, while a further nine had different intercepts. Hence the majority of significant correlations were not independent of site.

4. Leaf area and leaf width/length were the only traits related to *R*<sub>PO</sub>. There was no evidence that N allocation to Rubisco or thylakoid proteins was related to *R*<sub>PO</sub>, or that WUE was greater in ecotypes from dry areas.

5. For *Eucalyptus*, and perhaps other genera, physiological leaf traits may play a minor role in adaptation to water availability. There is large phenotypic plasticity in many leaf traits affecting not only the absolute values of traits, but also relationships among them.

Key-words: leaf morphology, Rubisco, nitrogen, specific leaf area, water-use efficiency

Introduction

It is axiomatic that plant growth and survival in unproductive habitats (xeric and/or oligotrophic) is associated with a suite of interrelated leaf ‘stress-tolerance’ traits (Fonseca *et al*. 2000; Wright *et al*. 2001). One of the most commonly observed trends in leaf morphology is the reduction in leaf size and specific leaf area (SLA, leaf area per unit dry mass) with decreasing supplies of water or nutrients (Givnish 1984; Cunningham *et al*. 1999; Fonseca *et al*. 2000).

Survival in unproductive habitats may be associated with a smaller proportion of leaf nitrogen allocated to photosynthetic functions (e.g. a smaller percentage of N as Rubisco or thylakoid proteins). The reasoning behind this is that species from oligotrophic and/or xeric habitats require greater investment of N in chemical
and physical defences (Field & Mooney 1986). Directly measuring the allocation of N to compounds with photosynthetic (or other) functions is a logical first step towards testing this hypothesis, but there are few data for mature trees and most studies have measured only total N.

Species from unproductive habitats (xeric and/or oligotrophic) are sometimes characterized by greater water-use efficiency (WUE) than species from productive habitats (Field et al. 1983), although this is far from clear for the genus Eucalyptus. In many studies with Eucalyptus, WUE has been estimated indirectly via its correlation with the stable carbon isotope composition (δ13C, Farquhar et al. 1982) of leaves (Macfarlane et al. 2004). No consistent relationship between WUE and habitat aridity has emerged in studies of multiple Eucalyptus species sampled across aridity gradients (Miller et al. 2001; Wright et al. 2001; Schulze et al. 2006), but this may partly reflect the large phenotypic plasticity in WUE of Eucalyptus (Pate & Arthur 1998; Cernusak et al. 2003; Macfarlane et al. 2004). Nevertheless, results have been contradictory even in cases where plants were grown under identical conditions in common gardens or glasshouses. The common-garden experiment of Anderson et al. (1996) and the glasshouse study of Sefton et al. (2002) demonstrated that variation in WUE among Eucalyptus species was related to water availability at the place of origin of the seed. However, another glasshouse study comparing seedlings of nine species (Warren & Adams 2004a) found no such relationship.

Many traits exhibit considerable phenotypic plasticity (Sultan & Bazzaz 1993; Cordell et al. 1998; Hovenden & Vander Schoor 2004), thus confounding of environmental with genetic influences is a common issue in studies of leaf traits. For example, trait correlations may shift in size and sign between contrasting environments (Antonovics 1976; Schlichting & Levin 1984). This has not been considered explicitly in many ecological studies of relationships among leaf traits (Cunningham et al. 1999; Wright et al. 2005). In such studies phenotypic plasticity almost certainly contributes (an unknown proportion) to correlations among traits and to correlations of traits with environmental variables. One aim of the present study was to explore phenotypic vs ecotypic influences on relationships among leaf traits.

We used two common-garden experiments with 35 ecotypes of 16-year-old Eucalyptus trees. The 35 Eucalyptus ecotypes (representing 29 species) were grown at a productive common garden (mean annual increment in above-ground stem volume = 21 ± 11 m3 ha−1 years−1; mean ± SD, n = 35 ecotypes) and an unproductive common garden (8 ± 5 m3 ha−1 years−1). These differences in productivity were related to greater rainfall at the productive site (1220 mm year−1) than the unproductive site (600 mm year−1) and differences in nutrient availability (Table 1). All 35 ecotypes are canopy dominants in their natural habitat, and this study thus at least partially avoids problems that arise from comparing ecologically different species as all belong to the same 'functional group': canopy trees.

Adaptation to water availability is a major factor in the evolution of the genus Eucalyptus (Parsons 1969a, 1969b; Noble 1989; Adams 1996). The 35 ecotypes used here were collected as seed from locations in southeast Australia with mean annual rainfall from 545 to 1938 mm year−1 (rainfall at the place of origin of seed, Rm). This is a large enough range to test hypotheses
Variation in leaf traits among Eucalyptus

regarding adaptation of Eucalyptus species to water availability. Using two common-garden field sites varying in productivity allowed us to examine the effect of environment (phenotypic plasticity) on traits and their correlations. By using trees at an advanced stage of growth (16 years old), we hoped also to avoid the many well known ‘juvenile’ characteristics that often do not reflect those of more mature trees. We tested the following hypotheses.

(i) The mean value of leaf traits varies between sites due to phenotypic plasticity, but relationships among leaf traits do not vary between sites because they reflect adaptation.

(ii) Relationships of leaf traits with rainfall at the place of origin reflect adaptation (to rainfall), and thus do not vary between sites.

(iii) Low-rainfall ecotypes are characterized by distinct biochemical, morphological and physiological traits: allocation of a small fraction of N to thylakoid proteins (as indicated by chlorophyll) and Rubisco, low SLA, leaves that are small and narrow (relative to their length), and a high long-term WUE (as indicated by C isotope composition, Farquhar et al. 1982).

Materials and methods

COMMON-GARDEN SITES

Full details of the common-garden sites may be found in Duncan et al. (2000); Duncan (2001). The two common-garden sites were in south-eastern Australia, a region where water deficits are common in summer, but the frequency and severity of such deficits varies throughout the region. Site PPFD is unknown but is unlikely to vary given that the two sites are at very similar latitudes, and do not differ in aspect (both are approximately flat) or the number of cloudy days. Rainfall at the productive site, Mt Worth, was more than twice as great as at the unproductive site, Glencoe (Table 1). Availability of N and P at Mt Worth was also greater than at Glencoe (see also differences in $N_{max}$ Table S1 in Supplementary material). Additional data on N availability are given by Warren (2006).

Both common gardens were established in 1987 with the planting of 88 seed lots (‘ecotypes’) representing 33 Eucalyptus species. Ecotypes were planted in a complete block design as 12-tree plots (three rows spaced at 3.6 m, four trees spaced at 2.8 m) with five replicate plots. Our primary interest was variation among species, thus our sampling was restricted to 35 ecotypes representing 29 species (Table S1). Multiple ecotypes of E. obliqua, E. elata, E. viminalis and E. saligna were included in the sampling and analysis as they originated from geographically isolated places with disparate rainfall. Multiple ecotypes (of the same species) were considered independent because they were separated by hundreds of kilometres. The names of ecotypes and their assignment to subgenera follows Brooker’s recent classification of Eucalyptus (Brooker 2000).

RAINFALL AT PLACE OF ORIGIN

For each place where seed was collected, we estimated mean annual rainfall ($R_{ma}$), rainfall of the driest quarter and potential evapotranspiration (areal point potential, Morton 1983) based on long-term records of the Australian Bureau of Meteorology (http://www.bom.gov.au/climate/averages).

COLLECTION OF LEAVES

We collected 1-year-old sun leaves from the upper one-third of the canopy from between three and five trees of each ecotype. The light environment in the lower canopy probably differed among ecotypes because of ecotype differences in size and leaf-area index. Hence we used sun leaves from the upper one-third of the canopy to ensure that leaves of all ecotypes developed in the same (full-sun) light environment. Trees were used as our experimental units for all leaf traits. Sun leaves from the upper one-third of the canopy were collected by shotgun in early November 2003, when trees were 16 years old. At the time of sampling the new season’s growth was commencing (the ‘spring flush’), but we restricted our sampling to the previous year’s growth (1-year-old leaves that were fully expanded and not yet senescing). Five leaves from each tree were stored in sealed plastic bags for measurements of leaf morphology, N and $\delta^{13}$C. Another five leaves from each tree were sampled for later analysis of Rubisco and chlorophylls. These leaves were sampled with a cork borer (4 x 0.56 cm$^2$ discs per leaf) and the 20 leaf discs per tree were placed in a 2-ml Eppendorf microfuge tube (Safe-Lock tube 2.0 ml, Eppendorf AG, Hamburg, Germany), frozen in liquid N and stored at $-80 \degree$C until analysis.

LEAF MORPHOLOGY

For each of the five leaves per tree we made a series of morphology measurements, these were subsequently averaged giving a mean for each tree. Fresh weight of each leaf was measured immediately after removal of leaves from plastic bags so as to minimize changes in mass due to transpiration. Leaf thickness was measured in two to four places between major veins with a Vernier calliper (Mitutoyo Corp, Kawasaki, Kanagawa, Japan). Total length and maximum width were measured to the nearest 1 mm with a steel ruler. The frequency and severity of such deficits varies throughout the region. Site PPFD is unknown but is unlikely to vary given that the two sites are at very similar latitudes, and do not differ in aspect (both are approximately flat) or the number of cloudy days. Rainfall at the productive site, Mt Worth, was more than twice as great as at the unproductive site, Glencoe (Table 1). Availability of N and P at Mt Worth was also greater than at Glencoe (see also differences in $N_{max}$ Table S1 in Supplementary material). Additional data on N availability are given by Warren (2006).

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Table 2. Primary variables used in analyses and their replication

<table>
<thead>
<tr>
<th>Units</th>
<th>Description</th>
<th>Replicates per ecotype (no. trees)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainfall (mm a⁻¹)</td>
<td>Rainfall at place of origin</td>
<td>–</td>
<td>Estimated from long-term climatic records</td>
</tr>
<tr>
<td>Leaf area (cm²)</td>
<td>Projected area of one leaf</td>
<td>3–5 (mean of 5 leaves per tree)</td>
<td>Directly measured by IRMS</td>
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<tr>
<td>Leaf thickness (mm)</td>
<td>Leaf thickness</td>
<td>3–5 (mean of 5 leaves per tree)</td>
<td>Determined on dried leaves by IRMS</td>
</tr>
<tr>
<td>Width/length</td>
<td>Leaf width/leaf length = leaf shape</td>
<td>3–5 (mean of 5 leaves per tree)</td>
<td>Extracted with DMSO and measured colorimetrically</td>
</tr>
<tr>
<td>FW/DW</td>
<td>Fresh mass/dry mass</td>
<td>3–5 (mean of 5 leaves per tree)</td>
<td>Extracted with DMSO and measured colorimetrically</td>
</tr>
<tr>
<td>SLA (m² kg⁻¹)</td>
<td>Specific leaf area</td>
<td>3–5 (mean of 5 leaves per tree)</td>
<td>Total amount of Rubisco protein was measured by capillary electrophoresis; Rubisco N% calculated assuming Rubisco is 16-7% N by mass</td>
</tr>
<tr>
<td>N₉₅ (mg g⁻¹)</td>
<td>Nitrogen content per unit mass</td>
<td>3–5 (5 pooled leaves per tree)</td>
<td>Thyakoid N% was calculated assuming 50 mol thyakoid N per mol of chlorophyll</td>
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<tr>
<td>δ¹³C (%)</td>
<td>Discrimination against ¹³C relative to Peedee belemnite (PDB) standard</td>
<td>3–5 (5 pooled leaves per tree)</td>
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<tr>
<td>Chl alb (mol mol⁻¹)</td>
<td>Molar ratio of chlorophyll alb</td>
<td>3–5 (5 pooled leaves per tree)</td>
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</tr>
<tr>
<td>Carot/chl (mmol mol⁻¹)</td>
<td>Ratio carotenoids : total chlorophylls</td>
<td>3–5 (5 pooled leaves per tree)</td>
<td></td>
</tr>
<tr>
<td>Rubisco N%</td>
<td>Percentage of N accounted for by Rubisco</td>
<td>3–5 (5 pooled leaves per tree)</td>
<td></td>
</tr>
<tr>
<td>Thylakoid N%</td>
<td>Percentage of N accounted for by thylakoid proteins</td>
<td>3–5 (5 pooled leaves per tree)</td>
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</table>

All measured variables determined at 16 years. All leaf traits were measured on 1-year-old sun leaves.

NITROGEN AND STABLE CARBON ISOTOPE COMPOSITION

Pooled samples, comprising five dried leaves per tree, were ground to a fine powder in a matrix mill (MM 301, Retsch, Haan, Germany). Samples were analysed for N and δ¹³C by isotope ratio mass spectrometry (IRMS) at the University of California, Davis Stable Isotope Facility.

CHLOROPHYLLS, CAROTENOIDS AND RUBISCO CONTENT

Chlorophylls and carotenoids were extracted with dimethyl sulfoxide (DMSO) and determined colorimetrically using the extinction coefficients of Wellburn (1994). Thylakoid N was calculated from chlorophyll concentrations using an empirical relationship (Evans 1989): 50 mol thylakoid N mol⁻¹ chlorophyll. This relationship is widely used and varies little among species or as a function of rainfall (Evans 1989).

Rubisco was quantified by capillary electrophoresis using a method modified from Warren et al. (2000a). Capillary electrophoresis was performed with a Beckman P/ACE MDQ system (Beckman-Coulter, Fullerton, CA, USA) fitted with a photodiode array detector and controlled by a computer running SYSTEM GOLD software (Beckman-Coulter). The separation of proteins was performed in sodium dodecyl sulfate (SDS) 14-200 gel buffer (Beckman-Coulter) in an SDS-coated fused-silica capillary (100 μm internal diameter × 31.2 cm long, eCap SDS 14-200 capillary, Beckman-Coulter).

Standard curves for purified Rubisco were highly linear (r² = 0.99) over the range of 0.05–1 g l⁻¹. Standard curves were also highly linear (r² = 0.98 or better) when constructed by serial dilution of leaf extracts from E. globulus, E. obliqua or E. rubida. In addition, there was 95% recovery of Rubisco in spike and recovery tests with leaf extracts from E. globulus, E. obliqua and E. rubida. These findings indicate that quantification was unaffected by the complex matrix of Eucalyptus leaves (Warren et al. 2000b). The fraction of N present as Rubisco was calculated assuming that Rubisco is 16-7% N by weight.

STATISTICS

All statistics were performed with MINITAB 14 for WINDOWS (Minitab Inc., Tulsa, OK, USA). Univariate analyses of relationships between pairs of traits were based on ecotype means. Input variables were chosen on the basis that they were not logically or mathematically autocorrelated and would provide the most ‘information’, and thus only a subset of variables was used (Table 2). Where there was a choice between area- and mass-based parameters, we used mass-based parameters as our primary measure of leaf structure (SLA) is mass-based. Using a mixture of mass- and area-based parameters leads to problems with interpretation because cross-correlations then include information on SLA in addition to the variables of interest. Normality of leaf traits was determined by the Ryan–Joiner test (similar to the Shapiro–Wilks test) and four traits were log-10 transformed to make their distribution normal (Underwood 1997).
## Table 3. ANCOVA and Pearson correlations among 11 leaf traits

<table>
<thead>
<tr>
<th>Leaf area*</th>
<th>Thickness*</th>
<th>Width/length</th>
<th>FW/DW</th>
<th>SLA*</th>
<th>N\textsubscript{mass}</th>
<th>(\delta\text{^13C})</th>
<th>Chl (a/b)</th>
<th>Carot/chl</th>
<th>Rubisco N%</th>
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<td>Width/length</td>
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<td>0.52</td>
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<td>SLA*</td>
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<td></td>
<td></td>
<td>Int***, -0.88</td>
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<tr>
<td>N\textsubscript{mass}</td>
<td></td>
<td>Int***, -0.87</td>
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<tr>
<td>(\delta\text{^13C})</td>
<td></td>
<td>0.24</td>
<td>0.51</td>
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<tr>
<td>Chl (a/b)</td>
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<tr>
<td>Carot/chl</td>
<td></td>
<td>Int***, -0.85</td>
<td></td>
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<tr>
<td>Rubisco N%</td>
<td></td>
<td>-0.32</td>
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<td></td>
<td></td>
<td>0.38</td>
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<tr>
<td>Thylakoid N%</td>
<td></td>
<td>Int***, -0.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Slope*</td>
<td>-0.75</td>
<td>Int***, 0.68</td>
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</tbody>
</table>

ANOVA with site as a fixed factor was used to test first for the significance of relationships between traits and homogeneity of slopes (model terms = site, covariate, site \times covariate). No further analyses were done if traits were not significantly related \((P > 0.05)\) or slopes were significantly heterogeneous between common gardens. For significant and homogeneous relationships the effect of site on intercepts was determined by another covariate, site (model term = site). Data are for 35 ecotypes (29 species) of Eucalyptus growing at two common gardens. Traits marked * were log-10 transformed. Where no numbers are shown, relationships were not significant. A Pearson correlation coefficient is the sole number where relationships were significant \((P < 0.05)\) and did not differ between sites. Slope = heterogeneous slope between sites. Int = homogeneous slope, but with a significant effect of site on intercept. Numbers following ‘Int’ are Pearson correlation coefficient for the ‘common’ relationship. Significance of differences in slope or intercept: *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).

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Analysis of covariance (ANCOVA), with a leaf trait as the covariate, was used to determine whether relationships among traits varied between common gardens. With ANCOVA we first tested whether relationships were significant and, if so, whether slopes were homogeneous (between sites). No further analyses were performed if relationships were not significant or slopes were heterogeneous. For significant and homogeneous relationships we then tested whether site affected the intercept of relationships.

Multivariate statistics were performed essentially as described by (Tausz et al. 1998). Principal components analysis (PCA) was based on 11 leaf traits (see Table 4) measured in individual trees. Normality of leaf traits was determined by the Ryan–Joiner test, and six traits measured in individual trees. Normality of leaf traits was determined by log-10 transformation to make their distribution normal. An acceptable principal component solution was determined based on visual examination of the Scree plot and the Kaiser criterion (all eigenvalues >1).

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### Results

#### Water Availability at Place of Origin

The 35 ecotypes originated from a wide range of annual rainfalls and potential evapotranspirations (Table S1). Mean annual rainfall at the place of origin of seed \((R_{\text{org}})\) varied from a minimum of 545 to a maximum of 1938 mm year\(^{-1}\), while rainfall of the driest quarter varied from 66 to 285 mm. Potential evapotranspiration varied from around 1000 to 1700 mm year\(^{-1}\). Among ecotypes there was a very wide range in \(\Delta W\) from –916 to +838 mm year\(^{-1}\) \((\Delta W = \text{annual rainfall} – \text{potential evapotranspiration})\).

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### Relationships among Leaf Traits: Do They Reflect Adaptation, Acclimation, or Both?

There were 20 significant correlations among pairs of traits, but the majority of these (12/20) varied between sites (Table 3), indicating that site affects not only the mean value of traits, but also relationships among traits.

The SLA is a case in point. It was significantly correlated with seven other leaf traits, but only two of these relationships \((\delta^{13}C\) and Rubisco N\%) did not vary between sites (Table 3; Fig. 2). Intercepts were significantly different in the correlations of SLA with four traits (thickness, width/length, FW/DW, thylakoid N\%) (Figs 1 and 2), while the slope of the SLA–N\textsubscript{mass} relationship differed between sites (Fig. 2). In fact, fitting a correlation to combined SLA and N\textsubscript{mass} data for the two sites would have led to a more than twofold overestimation of the actual slope (Fig. 2).

Multivariate relationships among traits were examined by PCA (Fig. 3; Table 4). PCA with four axes explained 68–71% of variation in the data set. Communalities (total variance explained by the four axes) were >50% for all variables except Rubisco N\%. Certain groups of traits were consistently loaded with one or other PC axis, indicating that the major directions of variation were relatively conservative. The first PC axis was highly loaded (>0.7) with thickness and SLA, irrespective of whether data for the two sites were combined or analysed separately (Table 4). The PCA using data for the two sites contained a unique second PC axis that did not exist in PCAs for either site on its own. This second axis of variation was highly loaded with N\textsubscript{mass} and FW/DW (Table 4) and separated the two sites (Fig. 3). PC2 (one site) or PC3 (both sites combined) was a ‘chloroplast pigment axis’ that was highly loaded...
with chl alb, carotenoids/chlorophyll (carot/chl) and thylakoid N%. A ‘leaf size and shape axis’ was represented by PC3 (one site) or PC4 (both sites combined), which were highly loaded with leaf area and leaf width/length. In comparing PCAs for the two sites, we note that the first three PC axes were similar and it was only PC4 that differed between sites (Table 4).

**RELATIONSHIP OF LEAF TRAITS WITH RAINFALL AT PLACE OF SEED ORIGIN**

Leaf area and leaf width/length were the only leaf traits correlated with rainfall at the place of origin. Relationships of width/length ($r = 0.31$) and leaf area ($r = 0.40$) with rainfall at the place of origin were unaffected by site (ANCOVA). In simple terms, species from dry habitats tended to have leaves that were narrow and small.

Somewhat surprisingly, there was no correlation between $\delta^{13}C$ (our indicator of WUE) and rainfall at $R_{p}$, despite the fact that $\delta^{13}C$ varied between (ecotype means) $-27.7\%$ and $-31.9\%$. Similarly, there was no evidence that N allocation to Rubisco or thylakoid proteins varied among ecotypes as a function of $R_{p}$, or that ecotype differences in SLA or thickness were related to $R_{p}$.

**DO LEAF TRAITS DIFFER BETWEEN SITES?**

The mean values of most leaf traits differed significantly between sites (Table S1), indicating significant...
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Results of this study show that phenotypic plasticity affects not only the absolute value of many leaf traits (Cordell et al. 1998; Hovenden & Vander Schoor 2004), but also relationships among traits (Table 3; Figs 1 and 2, Antonovics 1976; Schlichting & Levin 1984; Nicotra et al. 1997). More than half (12 out of 20) of the correlations between pairs of traits were affected by site. Consequently, correlations fitted to combined data for the two sites were spurious in many cases. For example, a correlation between N\textsubscript{mass} and SLA for the two sites would have led to a more than twofold overestimation of the actual slope (Fig. 2; cf FW/DW, Fig. 1). We cannot offer a mechanistic explanation as to why there are differences in relationships between a productive and an unproductive site. It could be due to differences in growth rate or, more likely, the determinants of growth rate: water availability and/or nutrient availability. Irrespective of the ultimate cause, these findings indicate that traits are strongly affected by site conditions, and that relationships among traits are far from universal.

Multivariate analysis indicated consistent relationships among traits, with some of these affected by phenotypic plasticity (Table 4; Fig. 3). PCA was affected by phenotypic plasticity insofar as the PCA using data for the two sites contained a unique second PC axis (Table 4; Fig. 3) that separated the two sites and was strongly loaded with FW/DW and N\textsubscript{mass}, traits that are almost certainly strongly affected by the large difference between sites in water and nutrient availability. Three of the four major multivariate axes were loaded with similar trait groupings, indicating that the directionality of (three out of four) trait groupings is rather conservative and thus relates to patterns of genetic variation. For example, the primary multivariate axis did not vary between sites and was in all cases strongly loaded with SLA and/or thickness (Table 4), confirming previous studies (Wright et al. 2004). Traits grouped together on PC axes were functionally related, lending support to the notion that functionally related traits respond similarly (Berg 1960; Schlichting 1986; Nicotra et al. 1997). For example, trait loadings showed that PC2 (one site) or PC3 (both sites combined) was a ‘chloroplast pigment axis’ highly loaded with chl \textit{ab} and carot/chl and thylakoid N%. A ‘leaf size and shape axis’ was represented by PC3 (one site) or PC4 (both sites combined) that were highly loaded with leaf area and leaf width/length.

The problem of confounding of genetic variation by phenotypic plasticity is a function of the relative sizes of phenotypic vs genotypic variation. Our finding that the majority of relationships were affected by site perhaps reflects modest genetic variation in traits among Eucalyptus ecotypes (Table S1). A much larger genotypic range in traits, such as that available across multiple life forms, could reduce the influence of phenotypic plasticity on these relationships. However, we know of no common-garden experiments that include varying multiple life forms with adequate replication.

Fig. 3. Scores on PC1 and PC2 of 35 ecotypes of Eucalyptus growing at two common gardens. Data are mean of five individual trees from each of 35 ecotypes growing at two common-garden sites (unproductive, ●; productive, ○). Principal components analysis was based on 11 variables measured in individual trees (Table S1). The x- and y-axes are normalized with zero corresponding to the mean PC score.

Discussion

RELATIONSHIPS AMONG LEAF TRAITS: DO THEY REFLECT ADAPTATION, ACCLIMATION, OR BOTH?

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functional relationships. Studies across climatic gradients have found negative correlations of SLA with WUE or surrogates such as δ13C (Wright et al. 2001; Lamont et al. 2002). Our results from common gardens suggest that this trend is not solely phenotypic (Fig. 2). Differences in δ13C are unlikely to be related to the biochemical capacity for photosynthesis, given that correlations of WUE with Nmass, Rubisco N% and thylakoid N% were either not significant or negative (Table 3). Stomatal conductance, and its relationship with photosynthetic capacity, seems the most likely explanation for relationships of δ13C with SLA.

In general, species with a larger SLA allocated a greater fraction of N to Rubisco and thylakoid proteins (Fig. 2), as has also been found in the few studies to measure directly the amount of Rubisco (Poorter &

Table 4. PCA Principal components (PC) solution of 11 leaf traits measured in three to five trees from each of 35 ecotypes (29 species) of Eucalyptus

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>Communality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area*</td>
<td>0.77</td>
<td>-0.77</td>
<td>67.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness*</td>
<td>-0.94</td>
<td>-0.94</td>
<td>89.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Width/length*</td>
<td>-0.86</td>
<td>0.86</td>
<td>76.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW/DW</td>
<td>-0.77</td>
<td>0.77</td>
<td>75.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLA*</td>
<td>0.92</td>
<td>-0.92</td>
<td>85.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nmass</td>
<td>-0.69</td>
<td>0.69</td>
<td>66.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ13C</td>
<td>0.60</td>
<td>-0.60</td>
<td>57.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl ab</td>
<td>0.68</td>
<td>-0.68</td>
<td>50.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carot/chl*</td>
<td>0.87</td>
<td>0.87</td>
<td>77.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubisco N%*</td>
<td>-0.63</td>
<td>-0.63</td>
<td>36.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoid N%</td>
<td>0.63</td>
<td>0.63</td>
<td>70.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Explained variance (%) 24.3 16.4 15.6 11.4 67.7

b) Unproductive

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC1</th>
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<th>PC4</th>
<th>Communality (%)</th>
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</thead>
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<td>0.63</td>
<td>70.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Explained variance (%) 22.6 17.2 16.0 14.6 70.4

c) Productive

PCA was based on individual trees of (a) unproductive and productive common gardens combined; (b) unproductive and (c) productive. Data are PC loadings and communalities determined after Varimax axis rotation. PC loadings <0.6 are not shown. Traits marked * were log-10 transformed.
Evans 1998; Onoda et al. 2004; Takashima et al. 2004). Nevertheless, these results contrast with our earlier study of seedlings of nine Eucalyptus species, where we found no such relationship (Warren & Adams 2004b). Of greater consequence is the lack of a definitive mechanistic explanation of the relationship between SLA and N allocation to Rubisco and thylakoid proteins. The common presumption is that allocation patterns reflect differential N allocation to cell walls and defensive compounds (Gleadow et al. 1998; Takashima et al. 2004), but this remains poorly tested.

**RELATIONSHIP OF LEAF TRAITS WITH RAINFALL AT PLACE OF SEED ORIGIN**

In general, few leaf traits were correlated with rainfall at the place of origin. The only consistent (genotypic) trend was for Eucalyptus ecotypes originating from drier habitats to have narrower and smaller leaves than ecotypes from wetter habitats. It is noteworthy that leaf width/length and leaf area were the only two traits that were related to $R_{\text{PO}}$, and were two of the three traits that did not vary between sites. This suggests they are under strong genetic control, and confirms other studies showing that ecotypes from drier habitats have narrower and smaller leaves (Gates et al. 1968; Smith 1978; Werger & Ellenbroek 1978; Fonseca et al. 2000). There was fourfold variation in width/length among ecotypes, thus differences in leaf boundary-layer conductance and temperature offer a plausible explanation for genotypic trends (Gates et al. 1968; Smith 1978).

High WUE is frequently proposed as a trait of species from low-rainfall habitats (Sun et al. 1996; Cregg & Zhang 2001; Lamont et al. 2002; Sefton et al. 2002; Wright et al. 2003), yet there is substantial experimental evidence to the contrary (Lauteri et al. 1997; Zhang et al. 1997; Warren et al. 2001), and this is especially the case for Eucalyptus. Ours is the largest common-garden study of $\delta^{13}$C in the genus Eucalyptus and we did not observe a correlation of $\delta^{13}$C with $R_{\text{PO}}$. Given that $\delta^{13}$C is related to WUE (Farquhar et al. 1982), our data cast doubt on a general relationship of WUE with $R_{\text{PO}}$ in Eucalyptus. Nevertheless, relationships have been observed in a common-garden study with 11 Eucalyptus species (Anderson et al. 1996); a glasshouse study with seedlings of three Eucalyptus species (Sefton et al. 2002); some studies across aridity gradients (Miller et al. 2001; Wright et al. 2001; Schulze et al. 2006), but with notable exceptions (Schulze et al. 2006). It is becoming increasingly clear for Eucalyptus spp., and indeed for other genera, that there is no simple relationship between $R_{\text{PO}}$ and WUE.

The SLA commonly decreases (and thickness increases) with decreasing rainfall (Givnish 1984; Cunningham et al. 1999; Fonseca et al. 2000; Schulze et al. 2006) – a trend we also observed between sites (a phenotypic trend). However, among 35 ecotypes of Eucalyptus there was no relationship of SLA with $R_{\text{PO}}$ (Table S1), which casts doubt on the existence of a genotypic relationship. The absence of a (genotypic) relationship of SLA with $R_{\text{PO}}$ was also found in a common-garden planting of 15 ecotypes of Eucalyptus sideroxylon ssp. tricarpa (Warren et al. 2005) and a glasshouse study of seedlings of nine Eucalyptus species (Warren & Adams 2005). One explanation for the absence of correlations between SLA and $R_{\text{PO}}$ among Eucalyptus ecotypes might be that the range in SLA is too small (sensu Reich 1993).

Almost 20 years ago it was argued that species from unproductive (xeric and/or oligotrophic) habitats allocate a smaller fraction of N to compounds with photosynthetic functions (Field & Mooney 1986). To date, however, there have been too few direct measurements of N allocation in multiple species to test this hypothesis adequately. In the case of our 35 Eucalyptus ecotypes, there was no evidence that N allocation to Rubisco or thylakoid proteins was related to $R_{\text{PO}}$. A glasshouse study with seedlings of nine Eucalyptus species also failed to find evidence that N allocation to Rubisco and thylakoid proteins is related to $R_{\text{PO}}$ (Warren & Adams 2004b). While we may be reasonably confident of the lack of a general relationship of N allocation with $R_{\text{PO}}$ for eucalypts, we cannot speculate as to whether this applies to other genera, owing to a lack of multispecies common-garden or glasshouse experiments where the amounts of Rubisco have been directly measured.

**WHY ARE SO FEW LEAF TRAITS CORRELATED WITH RAINFALL AT PLACE OF SEED ORIGIN?**

There are many reasons why so few leaf traits are correlated with rainfall at the place of seed origin. First, we acknowledge that the leaf-level traits reported here are difficult to interpret in isolation because they are affected by variables such as leaf area per plant, leaf phenology and hydraulic architecture (Warren & Adams 2000). Hence individual leaf traits may be unrelated to water availability because they are selectively neutral or even offset by other traits that were not measured (Gutschick 1988; Warren & Adams 2000; Gutschick & BassiriRad 2003). It may well be the case that other drought adaptations – particularly those related to seed germination, seedling establishment, root development and above- vs below-ground allocation – may be more important for survival. Other reasons that leaf traits may be unrelated to rainfall at the place of seed origin include: (i) the climatic distribution of ecotypes may not be determined solely by physiological traits; (ii) rainfall may not be the sole climatic determinant of species’ distributions and leaf traits; and (iii) mean annual rainfall and other long-term rainfall measures (e.g. rainfall of the driest quarter) may be poor indicators of water availability.

**ABSOLUTE VALUES OF MOST LEAF TRAITS DIFFER BETWEEN SITES**

The mean values of most leaf traits differed significantly between sites (Table S1), generally in the fashion...
one would expect for sites differing in productivity, water and nutrient availability. For example, SLA was ≈20% greater at the productive site than the unproductive site, consistent with observations that SLA is positively related to supplies of water and/or nutrients (Givnish 1984; Cunningham et al. 1999; Fonseca et al. 2000). Nitrogen concentrations were also greater at the productive site than the unproductive site, not surprising given the large differences in nutrient availability (Table 1).

Leaf size (leaf area) and leaf shape (width/length) did not vary between sites (Table S1), despite varying among ecotypes as a function of $R_{\text{g}}$. Hence the genotypic trend for Eucalyptus from low-rainfall habitats to have smaller and narrower leaves than congerenics from high-rainfall habitats is not matched by a corresponding phenotypic trend. For Eucalyptus, at least, we may be reasonably confident that the general observation that leaves get smaller and narrower with decreasing water and nutrient availability (Givnish 1984; Cunningham et al. 1999; Fonseca et al. 2000) is largely genotypic.

The absence of a difference in $\delta^{13}$C between sites in this study suggests that photosynthetic capacity and stomatal conductance were co-ordinated so as to maintain a similar WUE. This may be due to covariation in water and nutrient availability between sites. In other words, the reduced N concentrations and thus photosynthetic capacity at the unproductive site may have offset the larger stomatal limitation due to lesser water availability. Alternatively, it might reflect co-ordination of photosynthesis with stomatal conductance (Cowan 1977).

Conclusions

Few leaf traits of Eucalyptus are correlated with rainfall at place of origin. For example, there is no evidence that WUE or N allocation to Rubisco varies between Eucalyptus species of xeric and mesic habitats. The only consistent trends were for ecotypes from low-rainfall habitats to have smaller and narrower leaves than ecotypes from wetter habitats. These data suggest that for Eucalyptus, and perhaps other genera, physiological leaf traits play a minor role in adaptation to water availability.

It is well known that the mean value of leaf traits varies between sites due to phenotypic plasticity, but results of this study indicate that plasticity also affects relationships among traits. Relationships among traits are often seen as being indicative of adaptation and functional relationships, but the existence of considerable phenotypic plasticity argues against the existence of universal trait relationships as an expression of universal functional constraints. It seems more likely that relationships are partially coincidental and partly functional, and there is considerable scatter in such relationships.

Acknowledgements

This work was supported by funding from the Australian Research Council (discovery grants and a linkage international award). C.W. was supported by an APD fellowship and then a QEII fellowship from the Australian Research Council. None of this would have been possible without the support of Hancock Plantations Victoria, its employees, and staff of the Department of Sustainability and Environment. The helpful comments of anonymous reviewers are gratefully acknowledged.

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subsp. tricarpa) grown in a common garden? Tree Physiology 25, 1369–1378.

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Supplementary material
The following supplementary material is available as part of the online article (full text) from http://www.blackwell-synergy.com

Table S1. Taxonomic background, rainfall at place of seed origin and leaf variables measured in 35 ecotypes growing in two common-garden field sites. Leaf variables were measured on five trees per ecotype (see Table 2 and Materials and methods for further details). (a) Means for each ecotype; (b) means for subgenera and common gardens.