Available and not total nitrogen in leaves explains key chemical differences between the eucalypt subgenera

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ABSTRACT

Many ecologists regard nitrogen as a key element in the life-histories of herbivore populations. Consequently, those studying interactions between plants and animals often attempt to link feeding to the concentration of nitrogen (N) in plants. This approach disregards the possibility that in many plants, especially those rich in tannins, animals cannot digest much of the N. The ubiquity of tannins in plants led us to hypothesise that the concentrations of available nitrogen may be more informative for ecologists than are measures of total N. Eucalypts provide a good model for examining this hypothesis because subgeneric differences in foliar chemistry cause dietary niche separation in marsupials.

We used an *in vitro* assay that integrates fibre, digestibility, tannins and N into a single measure of "available nitrogen" (AvailN) to compare the concentrations of total and available nitrogen in the leaves of 138 eucalypt species. There were distinct differences between the subgenera. Most notably, even though differences in total N were minor, species within *Eucalyptus* contain half the AvailN of those within *Symphyomyrtus* (0.27% vs. 0.59% dry matter). Among all species ($N = 138$), there was a relationship between AvailN and total N ($r^2 = 0.23$; $P < 0.001$). The relationship, however, was much stronger ($r^2 = 0.84$; $P < 0.001$) and the concentrations of AvailN much higher after inactivating tannins with polyethylene glycol (PEG).

By integrating several measures, we showed that tannins defend eucalypts against herbivory but this is pronounced in species within *Eucalyptus*. This likely explains the different feeding niches of arboreal folivores and likely affects many other processes in eucalypt forests and woodlands. Furthermore, it suggests that ecologists should be far more careful in their measure of N; AvailN rather than total N is important, especially when diets contain tannins.

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1. Introduction

Although plants provide the nutrients for the expansion and replacement of all animal populations, proving this point in wild herbivorous mammals is remarkably difficult. Part of the difficulty lies in the way that ecologists look at plant composition. While measures of total nutrients, such as leaf nitrogen (N), may tell us much about the life-histories of plants (Wright et al., 2005), they may be of limited value when considering the nutrients available to herbivores or to organisms that degrade litter. For example, the digestibility of the cell walls of grasses is often the factor limiting nutrient extraction by grazing animals (Van Soest, 1994) while non-grazing animals must also cope with a much wider range of plant secondary metabolites, especially phenolic compounds that bind nutrients, tannins being a prime example.

In this paper, our first hypothesis is that the concentration of available nitrogen (AvailN) in leaves rather than total N provides ecologists with a precise measure of the N that is important to animals. Likewise, it is the appropriate measure in studies of biological stoichiometry (e.g., Klaassen and Nolet, 2008). To explain this further, we identified three factors that hamper progress in understanding how the nutrients in plants influence the life-histories of animal populations. First, although ecologists have long known that it is the availability of nutrients – the proportion the animal can extract, that is important (e.g., McKey et al., 1978), they often ignore this fact. For example, they recognise that protein (measured as N) is important for animals but often try to relate herbivory to the total concentration of foliar N (White, 1993), rather than to AvailN. They do this even though they know that some compounds, particularly tannins, bind protein and render most N unavailable (e.g., McKey et al., 1978). Second, there are untold methods for analysing tannins in plant tissues, but no suitable method for isolating, identifying and measuring the effect of tannins on N availability and relating this

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to animal performance. Finally, there is uncertainty about how to combine traits, like N, fibre and tannins, statistically to explain herbivory, although some researchers have tried using soluble protein (e.g., Wait et al., 1998).

Eucalypts provide a good model for examining this first hypothesis because there are notable subgeneric differences in foliar chemistry that cause dietary niche separation in marsupials. In particular, although the two most populous eucalypt subgenera, *Symphyomyrtus* and *Eucalyptus*, commonly coexist, brushtail possums rarely eat *Eucalyptus* and thus avoid the high cost that tannins impose on their nitrogen (N) metabolism. In contrast, ringtail possums, which are adept at conserving N, largely through caecotrophy (Chilcott and Hume, 1985; McArthur and Sanson, 1993; Marsh et al., 2003b), prefer eating the leaves from species of *Eucalyptus*.

With over 900 species, most ecosystems in Australia contain eucalypts, but most eucalypts have a narrow climatic distribution covering less than 0.5% of the continent's area or about 30,000 km^2. In this paper, we use a broad range of taxonomically and geographically diverse species (that encompass wide-ranging rates of growth) to test our second hypothesis that there is no functional variation in the N chemistry of eucalypt leaves from the major subgenera. In particular, we use the *in vitro* assay of DeGabriel et al. (2008) to determine the AvailN content of leaves and thus give a perspective relevant to herbivores. By incorporating the tannin binding agent, polyethylene glycol (PEG) into the incubation, we also measured the effects of tannins on AvailN.

We sampled trees growing in a common garden – Currency Creek. We assumed that by sampling such diverse species, at the same time and place, that any differences between the subgenera would be authentic. In other words, there would not be any systematic bias on the results. This approach had another advantage: Schulze et al. (2006) found close correlations between specific leaf area (SLA) and the concentration of foliar N – two measures that interested us, in 45 species of eucalypt growing at Currency Creek or naturally along an aridity gradient. Given the ubiquity of tannins, if broad taxonomic differences occur in the concentration of AvailN in eucalypts – a group that contains a wide range of secondary chemicals, then they indicate the importance of measuring AvailN rather than total N in any taxa that contain tannins. This thesis assumes that variation in available N greatly exceeds variation in total N.

2. Materials and methods

2.1. Site

We collected all samples at the Currency Creek Arboretum 80 km south of Adelaide in South Australia over two days in April 2006, where major plantings have occurred since 1993 (Nicolle, 2003). There are now more than 900 species at the site, all grown from seed collected from natural stands. Temperatures drop to 2 °C in winter and rise to about 40 °C in summer while the average annual rainfall is 400–450 mm. Three eucalypts, all symphyomyrtles, grow naturally at the site – *E. fasciculosa*, *E. leucoxylon* and *E. odorata*, while several others (*E. calycogona*, *E. camaldulensis*, *E. cosmophylla*, *E. incrassata*, *E. leptophylla*, *E. ovata*, *E. phenax*, *E. socialis* and *E. viminalis*) grow within 10 km. No monocalypts occur naturally on the Currency Creek Arboretum site, but several occur naturally within 10 km, including *E. diversifolia*, *E. baxteri* and *E. obliqua*. The arboretum has an easterly aspect and the sandy loam on clay has neutral pH. Gravity-fed drip irrigation supports the planted tubestock for the first 2–4 years.

2.2. Selection of eucalypt species

Our interest was in collecting a diverse range of eucalypt species to maximise the chemical variation in the sample set and in doing so, we followed Brooker's (2000) classification. That said we were most interested in comparing the subgenera, *Symphyomyrtus* and *Eucalyptus*, to explore possible chemical explanations for niche separation among marsupials. Thus, we sampled 138 species (Table S1) from 11 of 13 subgenera, the two omissions being the monotypic *Cuboidea* and *Primitiva*. We sampled 83 species from *Symphyomyrtus* (11 of 15 sections that did not include species in the monotypic *Racemus*, *Similares* and *Pumilio* and any of the six species in *Platysperma*); 31 from *Eucalyptus* (10 of 11 sections that excluded the monotypic *Nebulosa*); 11 *Corymbia*, four *Eudesmia* (2 of 4 sections that excluded the monotypic *Complanatae* and *Ebbanoensis*), two *Angophora*, two *Blakella*, one *Minutifructus* (1 of 2 sections that excluded *Equatoria*) and the sole representatives from each of the remaining monotypic subgenera: *Acerosae*, *Cruciformes*, *Alveolata* and *Idiogenes*. Fig. 1 shows the sites of the original seed collections.

2.3. Leaf sampling and processing

We cut a small branch from the mid-canopy height of each shrub or tree and stripped about 60 g of mature leaf, which we sealed in a paper bag and placed in a portable freezer. Upon returning to Canberra, we immediately freeze-dried most of the leaves (chamber temperature −35 °C). Opinions differ about how to dry leaves for chemical analysis (e.g., Julkunen-Tiito and Sorsa, 2001), but given the distance from the field to the laboratory (1200 km), the time needed to sample and the amount of sample collected, freeze-drying was the best option. After grinding the leaves to pass a 1 mm sieve using a Cyclotec 1093 Mill (Tecator, Sweden), we stored the resulting powder at room temperature in the dark in clear plastic 50 mL specimen containers. We pressed the remaining leaves for later determination of leaf area and mass.

2.4. Analytical

2.4.1. Dry matter and ash

We used freeze-dried ground material for all of the analyses and corrected for residual moisture by drying 1.00 g of material to constant mass at 50 °C. The dry sample was then combusted at 600 °C for 12 h to measure the ash content.

2.4.2. Polyethylene glycol binding capacity

We measured PEG-binding largely by following the original method of Silanikove et al. (1996). Briefly, for each sample we weighed 0.8000–0.8100 g of dry material into two scintillation vials to which we added 7.5 mL of a solution containing 33.33 g L^{-1} of PEG 4000 (Sigma, analytical grade) in 0.05 M buffer Tris-BASE pH 7.1 (Sigma) spiked with 1.85 MBq [^{14}C]-labeled PEG 4000 (Amersham). We mixed the tubes on a vortex mixer before placing them in an oven at 37 °C for 24 h with occasional shaking. After incubation, we decanted about 1.5 mL of the supernatant into a 2 mL microtube (Sarstedt 72.694.100) and centrifuged it for 5 min. We then weighed (±0.0001 g) about 40 μL of supernatant into glass scintillation vials containing 10 mL of scintillant (Packard Emulsifier-Safe) and counted them for 10 min or to a precision of 1.5% in a Beckman LS 6500 scintillation counter. In a similar way, we prepared and counted triplicate 40 μL samples of the working solution and of 0.05 M Tris-BASE buffer to determine, respectively, the amount of radiation added to each tube and the background radiation. The loss of PEG from solution during the incubation indicates binding by tannins.

Fig. 1. The locations of the original seed collections for species of *Symphyomyrtus* N = 83 (●), *Eucalyptus* N = 31 (□), *Corymbia* N = 11 (★) and *Eudesmia* N = 4 (▲).

2.4.3. Nitrogen

The N content of foliage was determined on duplicate samples (250 ± 10 mg) using a semi-micro-Kjeldahl technique with a Tecator 2012 digester, selenium catalyst and a Gerhardt Vapodest-5 distillation and titration apparatus calibrated with ammonium sulphate. We repeated analyses in duplicate if the coefficient of variation exceeded 2%.

2.4.4. Available nitrogen and dry matter digestibility (DMD)

We determined *in vitro* AvailN and DMD in duplicate using the method of DeGabriel et al. (2008) that mimics digestion in a model hindgut-fermenting herbivore. The purpose of the assay was to rank trees with regard to AvailN and DMD, in the presence and absence of tannins, rather than trying to give precise *in vivo* values for these constituents. The assay involved digesting the samples in porous bags (Ankom F57, Ankom Technology, Macedon, New York), first with pepsin (24 h) and then with cellulase (48 h). Knowing the mass of DM in the bag at the end of the assay enabled us to calculate DMD, while analysing this residue for N gave us values for digestible or AvailN. We were interested in measuring the effects of tannins on AvailN and DMD so at the start of the digestion we incubated two bags per sample for 24 h either with the tannin binding agent, polyethylene glycol MW 4000 (33.3 g L^{-1} in 0.05 M Tris-BASE buffer), or with buffer alone (both 25 mL/sample). We then thoroughly washed the bags before drying them to constant mass at 50 °C and weighing them. We call the loss of mass, in this initial step, "solubles". We repeated samples in duplicate if the coefficient of variation for AvailN and DMD exceeded 5%.

2.4.5. Condensed tannins

We extracted condensed tannins by sonicating 50 mg of dry leaf powder in 5 mL 50% aqueous acetone at 4 °C, centrifuging for 5 min at $250 \times g$ and 4 °C before repeating the process twice with fresh solvent (Cork and Krockenberger, 1991). We used the acid butanol method (Mole and Waterman, 1987) to develop colour and calculated the concentration of condensed tannin against quebracho and an internal standard prepared according to Giner-Chavez et al. (1997). Both gave similar results so we express our results as quebracho equivalents.

2.4.6. Leaf mass and area

After removing leaves from the freeze drier, we scanned three leaves of each species, calculated their areas using ImageJ (Rasband, 2006), placed them in an oven at 45 °C for 72 h to remove residual moisture and then weighed them. We divided leaf area by leaf mass to give specific leaf area (SLA).

2.4.7. Statistical

We analysed the data using GenStat Release 10.2, Lawes Agricultural Trust (Rothamsted Experimental Station).

3. Results

3.1. Descriptive statistics of SLA and chemical traits for the major taxa (Table 1)

The specific leaf area (SLA) ($P = 0.84$) and its components (log-transformed) – leaf mass ($P = 0.628$) and leaf area ($P = 0.429$) were the only variables that did not differ (unbalanced ANOVA after log transformation) between the four subgenera for which we had sufficient samples. In contrast, apart from N ($P < 0.005$) and tannins ($P = 0.019$), all other variates showed highly significant differences ($P < 0.001$) between the subgenera. In the comparison that interested us most – that between *Symphyomyrtus* and *Eucalyptus*,

Table 1
Descriptive statistics for specific leaf area (SLA) and various measures of leaf chemistry expressed per unit dry matter.

Taxa	SLA (cm² g⁻¹)	PEGbind[a] (%)	Ash (%)	N[b] (%)	Tannin[c] (QE g⁻¹)	Available N in leaf(%)		Solubles (%)		Dry matter digestibility (%)	
							+PEG[d]		+PEG		+PEG
Corymbia											
Mean N = 11	4.94	10.46a	3.46a	0.93a	315ac	0.29a	0.73a	0.27a	0.24a	38.2a	57.8a
SD	1.20	2.82	0.93	0.15	104	0.087	0.14	0.06	0.05	5.8	4.74
CV%	24.3	27.0	26.6	15.8	33	30.0	17.9	22.2	19.28	15.1	8.2
Min	2.59	5.6	2.30	0.765	105	0.072	0.557	0.169	0.17	31.1	47.4
Max	6.62	14.6	5.15	1.177	467	0.364	0.965	0.350	0.31	47.1	62.8
Eudesmia											
Mean N = 4	4.38	17.04b	3.57a	1.19bc	617b	0.26a	0.86ab	0.33ab	0.17a	50.1ab	63.4ab
SD	0.70	4.77	0.34	0.15	412	0.56	0.26	0.08	0.10	18.3	13.7
CV%	15.91	28.0	9.6	12.9	67	217.8	30.9	25.3	61.5	36.5	21.6
Min	3.71	10.8	3.15	1.001	0	−0.188	0.617	0.258	0.094	35.1	51.3
Max	5.14	21.7	3.91	1.361	853	1.077	1.233	0.443	0.323	75.8	83.0
Eucalyptus											
Mean N = 31	4.76	13.15c	3.60a	1.00ab	400ab	0.27a	0.77a	0.30a	0.24a	46.2a	67.6b
SD	1.58	3.46	1.087	0.17	152	0.22	0.17	0.07	0.09	9.2	9.0
CV%	33.2	26.3	30.2	17.3	38	79.4	21.6	22.3	38.5	19.9	13.3
Min	2.44	4.8	2.15	0.600	84	−0.086	0.410	0.162	0.075	30.4	45.3
Max	9.69	20.5	8.13	1.517	723	0.834	1.208	0.500	0.508	77.1	83.4
Symphyomyrtus											
Mean N = 83	4.60	8.81a	4.70b	1.12c	315c	0.59b	0.90b	0.37b	0.35b	57.4b	70.6b
SD	1.54	3.54	1.264	0.23	231	0.28	0.20	0.09	0.3	11.8	8.9
CV%	33.52	40.2	26.9	20.6	73	46.9	21.5	25.7	36.0	20.6	12.6
Min	1.61	0.02	2.63	0.574	0	−0.091	0.449	0.184	0.117	30.9	53.2
Max	10.64	17.11	8.56	1.719	871	1.177	1.440	0.574	0.643	78.5	88.3
P	0.853	<0.001	<0.001	0.005	0.019	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lsd C vs. Eud	1.748	4.053	1.369	0.244	247	0.304	0.218	0.0999	0.131	12.80	10.184
Lsd C vs. Euc	1.051	2.436	0.823	0.146	148	0.183	0.131	0.0600	0.0786	7.695	6.121
Lsd C vs. S	0.961	2.227	0.752	0.134	136	0.167	0.120	0.0549	0.0718	7.036	5.596
Lsd Eud vs. Euc	1.591	3.688	1.246	0.222	225	0.276	0.198	0.0909	0.119	11.649	9.266
Lsd Eud vs. S	1.533	3.553	1.200	0.214	216	0.266	0.191	0.0876	0.115	11.225	8.928
Lsd Euc vs. S	0.630	1.461	0.494	0.088	85	0.109	0.078	0.0360	0.0471	4.615	3.671

Different letters next to mean values indicate significant differences between taxa ($P < 0.05$), separated by least significant difference (Lsd) tests.

[a] PEGbind is the mass (g) of polyethylene glycol (PEG) bound by 100 g of ground leaf.
[b] N is total nitrogen.
[c] Tannin is "condensed tannin" expressed as quebracho equivalents per g DM.
[d] +PEG refers to samples incubated with PEG.

leaves from *Symphyomyrtus* species contained less tannin, bound less PEG and contained more N and more AvailN in the presence and absence of PEG (unbalanced ANOVA). Furthermore, *Symphyomyrtus* leaves had higher concentrations of ash and solubles, while their dry matter was more digestible. Species of *Corymbia* and *Eudesmia* had traits common to both major subgenera. For instance, their mean ash contents resembled that of *Eucalyptus*. In contrast, leaves of the *Eudesmia* had relatively high N content while those of *Corymbia* contained a relative paucity, similar to leaves from *Eucalyptus*. The *Eudesmia* had by far the highest concentrations of tannins, which reflected them binding more PEG than did leaves of species from the other subgenera. Not surprisingly, three of the four species of *Eudesmia* had less than 0.1% DM of AvailN and two of these were negative values. In contrast, DMD in the *Eudesmia* resembled that in the *Symphyomyrtus*. The *Corymbia*, however, had low PEG-binding – similar to that in the *Symphyomyrtus*, but lower DMD in the presence and absence of PEG than found in the other subgenera.

Further examination of the N complex of data – N, AvailN, PEG-binding, etc., over all species shows a significant relationship between the foliar concentrations of AvailN and total N ($r^2 = 0.23$; $P < 0.001$; Fig. 2), but not nearly as strong as the negative relationship between AvailN and PEG-binding ($r^2 = 0.52$; $P < 0.001$; Fig. 3). Thus, when digestion occurs in the presence of PEG there is a remarkably strong relationship between AvailN and total N ($r^2 = 0.84$; Fig. 4). The marked increase in the explained variation suggests that tannins are key determinants of N digestibility in most species we examined. The strong relationship between AvailN

and total N and the comparison between species of *Eucalyptus* and *Symphyomyrtus* deserves special mention. Although species of *Eucalyptus* contain slightly but significantly less N than do *Symphyomyrtus* (1.00 vs. 1.12% DM), they contain less than half the AvailN (0.27 vs. 0.59% DM).
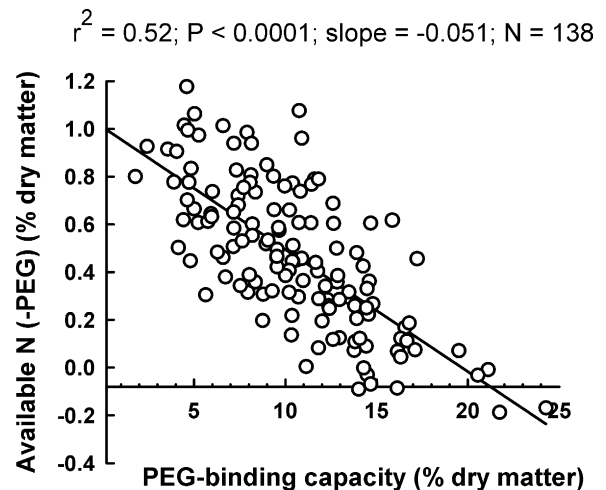

Fig. 2. The relationship between the concentrations of available nitrogen measured in the absence of PEG and PEG-binding capacity of leaves for 138 species of eucalypt.
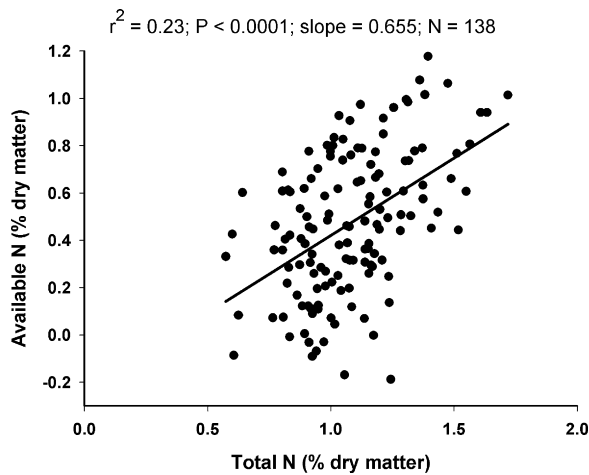
$r^2 = 0.23$; $P < 0.0001$; slope = 0.655; N = 138

Fig. 3. The relationship between the concentrations of available nitrogen measured in the absence of PEG and total nitrogen in 138 species of eucalypt.

3.2. Relationships between variables (Table 2)

Many of the variates were highly correlated, with roughly half of the correlations being highly significant ($P < 0.001$). We describe some below. In most cases, the relationships between variates for all 138 eucalypts were similar to those between the same variates within a major taxon. *Corymbia* provided the exceptions, which we will describe later. There were positive relationships between SLA and N ($r = 0.47$; $P < 0.001$) and between SLA and AvailN(+PEG) ($r = 0.35$; $P < 0.001$). Interestingly, there was no relationship between SLA and AvailN ($P = 0.90$). The degree of PEG-binding correlated negatively to the concentrations of N ($r = -0.31$; $P < 0.001$), AvailN ($r = -0.72$; $P < 0.001$), AvailN(+PEG) ($r = -0.58$; $P < 0.001$), DMD ($r = -0.42$; $P < 0.001$), DMD(+PEG) ($r = -0.46$; $P < 0.001$) and ash ($r = -0.34$; $P < 0.001$). There was a positive correlation between the N concentration of leaves and AvailN in the presence and absence of PEG ($r = 0.92$ and $r = 0.48$; $P < 0.001$, respectively). Finally, there were positive correlations between DMD in the presence and absence of PEG and AvailN ($r = 0.67, 0.69$; $P < 0.001$) and the ash content of leaves ($r = 0.37, 0.42$; $P < 0.001$).

The few *Corymbia* in the data make it difficult to interpret the results but two differences stand out. First, there is a pos-
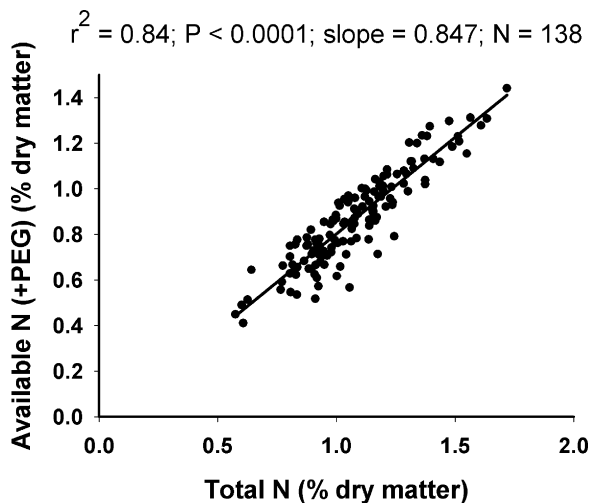

$r^2 = 0.84$; $P < 0.0001$; slope = 0.847; N = 138

Fig. 4. The relationship between the concentrations of available nitrogen measured in the presence of PEG and total nitrogen in 138 species of eucalypt.
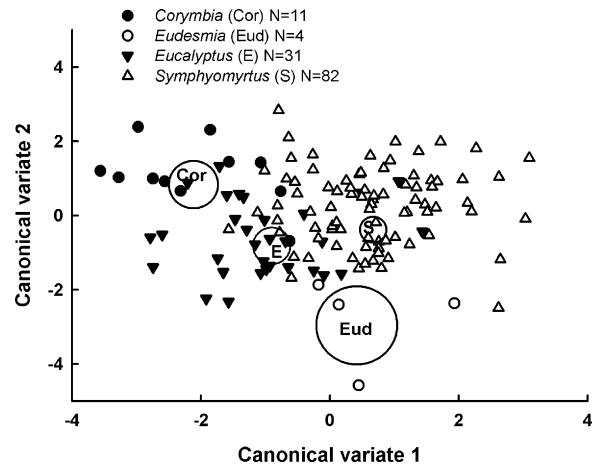

Fig. 5. A canonical variates discriminant plot showing individuals of *Corymbia* (Cor), *Eudesmia* (Eud), *Eucalyptus* (E) and *Symphyomyrtus* (S). The circles are the 95% confidence intervals around the subgenus means.

itive relationship between PEG-binding and solubles ($r = 0.60$; $P < 0.05$), which contrasts with the weak negative relationships overall ($r = -0.22$) and in subgenera *Eucalyptus* and *Symphyomyrtus* ($r = -0.1$). Also, the relationship between AvailN and DMD is not significant in *Corymbia* ($r = 0.29$; $P = 0.39$) whereas it was a highly significant positive relationship in *Eucalyptus* ($r = 0.808$; $P < 0.001$) and *Symphyomyrtus* ($r = 0.566$; $P < 0.001$).

3.3. Differences between subgenera (Fig. 5 and Table 1)

The first two components from a canonical variates analysis accounted for 64.0% and 33.8% of the variation among *Corymbia*, *Eudesmia*, *Eucalyptus* and *Symphyomyrtus*. The loadings (Table 3) indicate that the first canonical variate is largely an average of N and the soluble components in the leaf in the absence of PEG contrasting with the AvailN and solubles, both in the presence of PEG. The second is mainly a contrast between the AvailN content of leaves in the presence and absence of PEG. These do not change when we exclude the *Eudesmia* and *Corymbia*. Although our measures of chemistry separate the means and their 95% confidence intervals for the four taxa, there is clear overlap of the species. One *Eudesmia* (*E. erythrocorys*) and one *Corymbia* (*C. citriodora*) are distant from their respective means, while three *Eucalyptus* (*E. lacrimans*, *E. laevopinea* and *E. dives*) are distant from the *Eucalyptus* mean and much closer to the *Symphyomyrtus* mean than to the *Eucalyptus* mean. Thus, the *Eucalyptus* mean is close to an apparent boundary between representatives of *Eucalyptus* and *Symphyomyrtus*. The individual *Symphyomyrtus* are widespread also with one species (*E. hallii*) nested among the *Eucalyptus*, but with many species with high values for the first canonical variate.

4. Discussion

There is wide belief that the concentration of N (often expressed as crude protein, e.g., N*6.25) determines how herbivores respond to plant matter – whether they eat it and its nutritional value. For example, Ganzhorn et al. (2009) argued that the varying concentration of protein in fruit may explain the evolution of frugivory in primates. By analysing ripe fruits it was unlikely that tannins influenced the availability of N. In another example, Osier and Jennings (2007) showed that foliar N content varied widely between three species of deciduous sapling, was highest in those growing in the sun and that concentrations determined the growth of larvae eating the leaves. In these cases there may be no reason to look

Table 2
Correlations among the various leaf constituents.

	SLA	PEGbind	Tannin	N	AvailN	(+PEG)	Soluble	(+PEG)	DMD	(+PEG)	Ash
(a) All samples N = 138; bold = P < 0.0001; italic = P < 0.001											
SLA	1										
PEGbind	0.104	1									
Tannin	−0.028	**0.475**	1								
N	**0.470**	*−0.307*	−0.156	1							
AvailN	0.018	**−0.721**	**−0.613**	**0.482**	1						
AvailN(+PEG)	**0.354**	**−0.584**	**−0.419**	**0.915**	**0.737**	1					
Soluble	−0.265	−0.222	−0.244	*−0.271*	**0.432**	−0.056	1				
Soluble(+PEG)	−0.225	**−0.578**	**−0.543**	0.107	**0.619**	0.196	**0.768**	1			
DMD	−0.268	**−0.423**	**−0.334**	−0.045	**0.685**	0.208	**0.796**	**0.778**	1		
DMD(+PEG)	−0.229	**−0.456**	**−0.475**	−0.016	**0.674**	0.258	**0.798**	**0.827**	**0.892**	1	
Ash	−0.125	**−0.335**	−0.180	0.012	*0.273*	0.155	0.179	**0.436**	**0.369**	**0.416**	1
(b) Corymbia N = 11; bold = P < 0.01; italic = P < 0.05											
SLA	1										
PEGbind	0.408	1									
Tannin	0.453	*0.594*	1								
N	0.332	−0.341	0.266	1							
AvailN	−0.179	−0.503	−0.150	0.396	**1**						
AvailN(+PEG)	0.191	−0.478	0.126	**0.974**	0.468	1					
Soluble	0.553	0.596	0.392	0.005	0.180	−0.059	1				
Soluble(+PEG)	−0.097	**−0.696**	0.193	**0.697**	0.317	−0.064	1				
DMD	0.275	0.486	0.280	−0.284	0.288	−0.346	**0.690**	0.201	1		
DMD(+PEG)	*0.632*	0.380	*0.541*	0.245	0.380	0.182	**0.755**	0.251	**0.720**	1	
Ash	0.121	−0.434	0.296	*0.671*	0.315	*0.667*	−0.250	0.365	−0.064	0.164	1
(c) Eucalyptus N = 31; bold = P < 0.0001; italic = P < 0.001											
SLA											
PEGbind	0.053	1									
Tannin	0.011	0.300	1								
N	*0.466*	−0.446	0.093	1							
AvailN	−0.017	**−0.616**	−0.517	0.330	1						
AvailN(+PEG)	0.390	**−0.671**	−0.160	**0.918**	*0.556*	1					
Soluble	−0.259	−0.094	−0.409	−0.414	*0.486*	−0.180	1				
Soluble(+PEG)	−0.052	−0.599	**−0.642**	0.104	**0.818**	0.411	**0.687**	1			
DMD	−0.062	−0.320	−0.441	0.024	**0.808**	0.255	**0.685**	**0.773**	1		
DMD(+PEG)	−0.173	−0.439	−0.594	0.052	**0.761**	0.337	**0.612**	**0.865**	**0.777**	1	
Ash	0.014	−0.190	0.035	0.178	0.367	0.229	0.076	0.371	0.307	0.283	1
(d) Symphyomyrtus N = 83; bold = P < 0.0001; italic = P < 0.001											
SLA	1										
PEGbind	0.032	1									
Tannin	−0.069	**0.461**	1								
N	**0.570**	−0.255	−0.232	1							
AvailN	0.094	**−0.644**	**−0.642**	**0.459**	1						
AvailN(+PEG)	**0.481**	**−0.506**	**−0.477**	**0.921**	**0.713**	1					
Soluble	−0.315	−0.118	−0.179	**−0.429**	0.300	−0.239	1				
Soluble(+PEG)	−0.268	**−0.445**	**−0.481**	−0.311	**0.467**	−0.036	**0.784**	1			
DMD	*−0.349*	*−0.340*	−0.286	−0.295	**0.566**	−0.041	**0.773**	**0.752**	1		
DMD(+PEG)	−0.275	−0.326	**−0.518**	−0.251	**0.540**	0.014	**0.791**	**0.798**	**0.882**	1	
Ash	−0.114	−0.150	−0.238	−0.304	0.020	−0.171	0.124	*0.359*	0.268	*0.367*	1

further than the total N concentration in the leaves. Our results, however, show that eucalypt leaves are much different: a strong interaction between N and tannins means that the concentration of total N in leaves is not a reliable indicator of the concentration of AvailN. Moreover, the concentration of AvailN rather than total N in eucalypt leaves predicts life-history traits in *Trichosurus vulpecula* (DeGabriel et al., 2009). The relatively low concentration of AvailN and the link with life-history traits suggests that N may be limiting in herbivores that depend on eucalypts. If so, this diverges from the broad thesis on the ecology of Australia proposed by Orians and Milewski (2007) who cited evidence from Peeters (2002) and Cunningham et al. (1999) in arguing that N is not the controlling nutrient for herbivores in Australia and that rates of herbivory instead correlate with leaf fibre content or the ratio of N to phenols. Given the ubiquity of tannins in plant communities, our results suggest that differences of this scale between total N and AvailN may occur in other plant taxa with marked effects on herbivores and on ecosystem processes.

The second purpose of this study was to examine the N chemistry of a broad spectrum of eucalypts from the perspective of a marsupial folivore to test the null hypothesis that the taxa do not differ. Many researchers have tackled the subject because, relative to other angiosperms, eucalypt leaves contain low concentrations of N (Mattson, 1980) but high concentrations of tannins. Perhaps the best indication of the importance of tannins in the foliar chemistry of eucalypts comes from the study of Marsh et al. (2003a), who showed that brushtail possums would eat far more foliage from species within *Eucalyptus* when it was treated with PEG. This suggested that eucalypts use tannins to defend their N.

Using the *in vitro* assay of DeGabriel et al. (2008), we showed that the foliage from the main eucalypt subgenera differs in ways that are important to folivores. In particular, the mean AvailN concentration in foliage from 83 species of *Symphyomyrtus* (0.59% DM) was more than double the concentration in 31 species of *Eucalyptus* (0.27), 12 species of *Corymbia* (0.29) or four *Eudesmia* (0.26). Put differently, the digestibility of N was 53%, 27%, 31%, and 22%

Table 3
The contributions or loadings of the attributes of leaves to the canonical variates listed in order of explanatory power for the first canonical variate.

Variates	Loadings	
	Canonical variate 1	Canonical variate 2
Solubles (% DM)	8.319	−0.111
Nitrogen (% DM)	7.163	1.555
Available N content of leaf (+PEG) (% DM)	−3.318	−9.064
Solubles (+PEG) (% DM)	−3.293	0.955
Available N content of leaf (% DM)	−0.919	4.457
Ash (% DM)	0.472	0.103
Specific leaf area (cm^2 g^{-1})	−0.177	0.280
PEG-binding (% DM)	−0.089	−0.295
Dry matter digestibility (%)	0.055	−0.098
Dry matter digestibility (+PEG) (%)	−0.004	0.037
Tannin (quebracho equiv g^{-1} DM)	0.001	0.000

of total N in *Symphyomyrtus*, *Eucalyptus*, *Corymbia* and *Eudesmia*, respectively. The addition of PEG to the assay increased these values to 80%, 77%, 78% and 72%, indicating that tannins are largely responsible for the relatively low availability of N in three of the taxa and reduce AvailN appreciably in *Symphyomyrtus*. Presumably, the remaining 20–30% of N is indigestible for other reasons, such as bound to cell-wall constituents that cellulase did not hydrolyse, or to tannins that do not complex with PEG.

At Currency Creek, the leaves of 83 species of *Symphyomyrtus* contained more N than did the leaves of 31 species of *Eucalyptus* but the difference is small (1.12% vs. 1.00%) compared with the difference in Avail N. In contrast, Judd et al. (1996) and Noble (1989) wrote that species from the two major subgenera, typically sampled in natural stands, produce foliage with similar concentrations of N (1.19, SE = 0.07% and 1.21%, SE = 0.04 for monocalypts and symphyomyrtles). This difference prompted us to compare only the species at Currency Creek in common with those sampled by Judd et al. (1996). In this case, the mean N concentrations of 1.10 (SE = 0.06) for 10 monocalypts and 1.15 (SE = 0.05) for 34 symphyomrtyles were not significantly different. These concentrations suggest that the environment at Currency Creek has little influence on the N concentrations in leaves. This concurs with Schulze et al. (2006) who found no difference in SLA or N for 45 species of eucalypt growing at Currency Creek or in their natural habitats along an aridity gradient from south-western Western Australia to central Australia. They concluded that SLA and N are inherent characteristics of eucalypt species.

The finding of taxonomical differences in AvailN has eluded many biologists, who have sought to link the abundance of folivores to the chemistry of leaves by measuring traits such as total N, total phenolics and various types of fibre. In many instances, they have then correlated animal indices to ratios of chemicals, such as total N to total phenolics (e.g., Cork and Catling, 1996) or total N to fibre (e.g., Peeters, 2002). What we have done differs, simply because we examined plant tissue from an animal's perspective. Thus, AvailN is a combination of total N, digestibility and the N bound by tannins. The N in eucalypt foliage is of low digestibility (20–50%) and because eucalypt foliage contains low concentrations of N, it contains very little AvailN. The converse of this argument is that small differences in its components lead to large differences in AvailN. For example, *Symphyomyrtus* foliage contains more N, has higher DMD and lower PEG-binding than do leaves from species within the subgenus *Eucalyptus* resulting in a doubling of AvailN. Why might tannins be less important in symphyomyrtles? The likely answer is that these species also produce formylated phloroglucinol compounds (FPCs), the only compounds known to deter herbivory by eucalypt folivores (Moore et al., 2004). These taxonomic differences in AvailN and FPCs partly explain what we know about the separation of feeding along taxonomic lines by

marsupial folivores. Both the koala and the common brushtail possum tolerate limited amounts of FPCs and feed predominantly from *Symphyomyrtus*, while the common ringtail possum specialises on species of *Eucalyptus*. As predicted by Mattson (1980), the ringtail is well equipped to counter the low concentrations of AvailN by producing proline-rich salivary proteins that bind tannins and by practising caecotrophy to recover microbial protein (Chilcott and Hume, 1985; McArthur and Sanson, 1991).

The taxonomic differences in AvailN concentrations of leaves have implications that reach far beyond animal ecology. The polymeric phenol structure of proanthocyanidins causes hydrogen bonding with proteins in the gut but also in soil where it binds plant protein and microbial proteins (Hagerman, 1989; Kraus et al., 2003). Furthermore, the influence of tannins extends beyond the tree that synthesised them; they also have a cascading effect on processes in forests (reviewed by Kraus et al., 2003), through their influence on nutrient cycling. Because eucalypt forests and woodlands typically comprise species from the different subgenera (Pryor, 1959), we might expect the chemical patchiness of the forest floor to mimic that of the canopy. This, however, is unexplored.

The vast array of structures and the often poor correlation between tannin concentrations and herbivory, suggests that these compounds did not evolve solely for defence (Gershenzon, 1994). Our results allude to this effect and doubt relationships such as total N to tannins, to assess the nutritional value of foliage. For example, while highly significant (P < 0.001), the concentration of tannins explained only 22% of the variation in PEG-binding by leaves. But, incubating leaf samples with PEG invariably led to considerable increases in AvailN (median increases of 153%, 644%, 208% and 54% for *Corymbia*, *Eudesmia*, *Eucalyptus* and *Symphyomyrtus*, respectively). Our explanation is that PEG precludes tannins that would otherwise bind protein. As pointed out by Makkar et al. (1996), tannins interfere also with the detergent methods for analysing plant cell-wall constituents making the methods unsuitable for evaluating most leaves. This raises further doubt over using N to fibre ratios to predict the nutritional quality of foods rich in tannins and relationships to animal populations (Oates et al., 1990; Ganzhorn, 1992; Chapman et al., 2002, 2004, 2005; Wasserman and Chapman, 2003). Although these researchers all found significant relationships between N to fibre ratios and animal abundance, our results suggest that the reasoning may be wrong. Thus, rather than fibre diminishing the nutritional value it may be the influence of tannins on protein. Whatever the case, the AvailN assay integrates all of these factors while avoiding any statistical problems with ratios (Packard and Boardman, 1987). Furthermore, it is a functional trait and a common currency that enables ecologists to compare herbivory in different systems.

5. Conclusions

In conclusion, ecologists need to be far more careful with their measures of N. Animals have requirements for AvailN rather than total N so failing to detect a relationship between herbivory and the total concentration of N or of tannins in plant foods does not exclude the possibility that N is limiting in the animal's life-history. Conversely, while there are many examples of herbivores responding to total N in plants (e.g., Stiling and Moon, 2005; Huberty and Denno, 2006; Osier and Jennings, 2007), there may be a much better relationship between the trait of interest and AvailN, especially in herbivores ingesting foods rich in protein binding tannins.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foreco.2010.05.040.

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