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A Metabolomic Approach to Identifying Chemical Mediators of Mammal–Plant Interactions

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Abstract Different folivorous marsupials select their food from different subgenera of *Eucalyptus*, but the choices cannot be explained by known antifeedants, such as formylated phloroglucinol compounds or tannins, or by nutritional quality. Eucalypts contain a wide variety of plant secondary metabolites so it is difficult to use traditional methods to identify the chemicals that determine food selection. Therefore, we used a metabolomic approach in which we employed ^{1}H nuclear magnetic resonance spectroscopy to compare chemical structures of representatives from the two subgenera and to identify chemicals that consistently differ between them. We found that dichloromethane extracts of leaves from most species in the subgenus *Eucalyptus* differ from those in *Symphyomyrtus* by the presence of free flavanones, having no substitution in Ring B. Although flavanoids are known to deter feeding by certain insects, their effects on marsupials have not been established and must be tested with controlled feeding studies.

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Introduction

Plants contain a wide array of chemicals and nutrients that influence how animals respond to them. Although ecologists have long understood the value or liability of general classes of compounds, such as "tannins" or "terpenes" (Swihart et al. 2009), they are just beginning to recognize how specific compounds or particular molecular structures influence how animals choose their diets. Without this knowledge of structure and function, it is impossible to evaluate ideas about dietary constraints on an herbivore's life history, habitat selection, fitness, competition, and coevolution. Although this applies to all herbivores, it is particularly acute in studies of larger mammalian herbivores. It is widely believed that differential responses to secondary compounds allow mammalian herbivores to partition the available resource to reduce interspecific competition (Marsh et al. 2003a). Without detailed chemical knowledge, these ideas cannot be assessed and incorporated into conservation and monitoring programs.

Several factors make it difficult to understand interactions between animals and plants. The first is the complexity of the chemical profile in plants. Related to this is the need to consider intraspecific as well as temporal and spatial variation in chemical makeup. This variation makes it hard to design specific assays for compounds that might be active. Bioassay guided fractionation has been used successfully to identify specific antifeedant compounds in arctic plants (Reichardt et al. 1985; Bryant et al. 1989) and in Australian eucalypts (Pass et al. 1998). In

this procedure, compounds are isolated progressively from a plant and fed to animals to identify the compound of interest. Among other problems, this approach requires great effort to fractionate large volumes of extracts to use in long-term feeding tests with mammals.

McIlwee et al. (2001) suggested that spectra of plants obtained with near infrared reflectance spectroscopy (NIRS) could be related to how much animals eat of those plants. In this way, NIRS could be used to predict feeding in vertebrate browsers independent of a detailed understanding of the underlying chemistry. While that approach has been useful in eucalypts (Foley et al. 1998; Wallis and Foley 2003), NIRS is not suitable for deciphering the underlying chemistry that contributes to differences in the NIR spectra of plants. Nonetheless, spectroscopic approaches are attractive because they give a more comprehensive view of plant chemistry than do assays of broad classes of compounds. In contrast to NIRS, ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) is a spectroscopic approach that can provide structural information about the underlying chemical differences between groups of plants. In this paper, we evaluate the use of ^{1}H NMR spectroscopy combined with principal components analysis (PCA) of the spectra to identify significant chemical differences between subgenera of *Eucalyptus* that support known differences in feeding behavior.

Within *Eucalyptus* there are distinct differences among the subgenera in the extent to which marsupial (and insect) herbivores use the foliage as food. In particular, the two largest subgenera, *Symphyomyrtus* (common name symphyomyrtles) and *Eucalyptus* (common name monocalypts) are used differentially by marsupial browsers. Koalas (*Phascolarctos cinereus*) and common brushtail possums (*Trichosurus vulpecula*) feed predominately on foliage from species in the *Symphyomyrtus*, whereas common ringtail possums (*Pseudocheirus peregrinus*) and greater gliders (*Petauroides volans*) eat leaves mostly from the subgenus *Eucalyptus* (Moore et al. 2004a).

The aversion of ringtail possums to symphyomyrtles is due to their sensitivity to the nauseating effects of formylated phloroglucinol compounds (FPCs) such as sideroxylonal-A (Fig. 1, structure **1**) (Moore et al. 2004b). These compounds are absent from the monocalypts but variably present in the symphyomyrtles, which explains diet selection by koalas and brushtail possums in both the laboratory and the field (Wallis et al. 2002; Scrivener et al. 2004; Moore and Foley 2005) as follows. Both species feed mainly from symphyomyrtles but select leaves to limit their ingestion of FPCs. In contrast, captive brushtail possums eat very little monocalypt foliage but they eat much more when the foliage is coated with the tannin-blocking agent, polyethylene glycol (PEG), while PEG

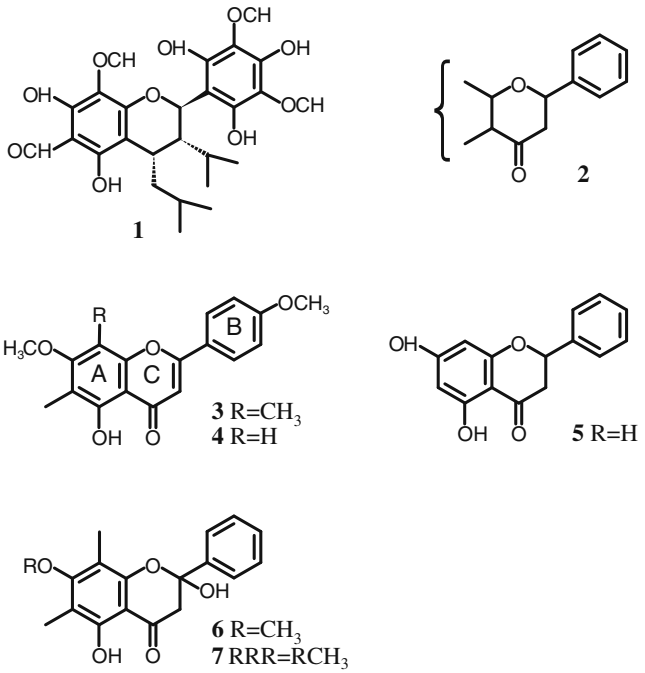

Fig. 1 The structures and partial structures of compounds referred to in the text. **1** sideroxylonal-A; **2** proposed partial structure of a compound in the extract of *E. agglomerata*; **3** eucalyptin; **4** demethyleucalyptin; **5** pinocembrin; **6** and **7** novel 2-hydroxyflavanone structures from *E. rossii*

has no effect on feeding by the common ringtail possum (Marsh et al. 2003b). In mixed stands of eucalypt trees, representatives from both major subgenera tend to co-dominate (Pryor 1959). Thus, by specializing on the PSMs produced by a particular eucalypt subgenus, different folivores could inhabit the same tracts of forest without competing for food (Marsh et al. 2003a). Along with hyrax (Hoeck 1975) and bamboo lemurs (Tan 1999; Yamashita et al. 2009), this is one of the few clear examples of dietary partitioning by vertebrates. If we could identify the factors in monocalypts that deter feeding by the koala and common brushtail possum or the mechanism by which the common ringtail possum processes the monocalypt compounds, then we would have a better understanding of niche partitioning by sympatric vertebrate herbivores.

In this research, we compared extracts from the leaves of a wide variety of monocalypt and symphyomyrtle species. We hypothesized that, unlike the symphyomyrtles, the monocalypts contain specific compounds that are unpalatable to koalas and brushtail possums, and that ^{1}H NMR spectra could reveal these compounds. We added additional control to the study by including several *E. melliodora* (*Symphyomyrtus*) with varying concentrations of sideroxylonals, expecting those with low concentrations of sideroxylonals to group closer to the monocalypts than do those with higher concentrations.

Methods and Materials

Preliminary Collection We did an initial study of a small number of species growing around Canberra, ACT, Australia, to see if ^{1}H NMR spectroscopy would detect consistent differences between the eucalypt subgenera and, if so, would identify the types of compounds that contribute to the differences. By beginning with a small number of species, we could also examine intraspecific variance. Leaf samples were collected from mid-canopy from five individuals of each of the following species: *E. agglomerata, E. dives, E. elata, E. macrorhynca,* and *E. rossii* (monocalypts); *E. globulus ssp bicostata, E. mannifera*, and *E. polyanthemos* (symphyomyrtles). We also collected leaves from 10 individuals, known to vary in FPC concentration, of another symphyomyrtle, *E. melliodora*. Leaves were frozen upon collection and later freeze dried but left unground.

Large-scale Collection The preliminary study gave promising results so we undertook a larger study of a diversity of taxa from *Eucalyptus* and *Symphyomyrtus*. In this way, we could determine whether there are consistent chemical differences between the subgenera. All leaf samples were collected during a single field trip to the Currency Creek Arboretum 80 km south of Adelaide in South Australia in April 2006. The collection included 83 symphyomyrtles (11 of 15 sections that did not include species in the monotypic *Racemus*, *Similares,* or *Pumilio,* or any of the six species in *Platysperma*) and 31 monocalypts (10 of 11 sections that excluded the monotypic *Nebulosa*). A small branch from mid-canopy height of each shrub or tree was cut, and from it about 60 g of mature leaf were stripped, sealed in a plastic bag, and placed in a portable freezer. Upon returning to Canberra, the leaves immediately were freeze dried and ground to pass a 1 mm sieve by using a Cyclotec 1093 Mill (Tecator, Sweden). The resulting powder was stored in the dark in clear plastic 50 ml specimen containers.

Extraction For the preliminary study, we crushed 2.00± 0.05 of leaves into a conical flask, added 40±1 ml of AR grade dichloromethane (DCM), stoppered the flask, and extracted the sample for 48 hr. The solvent was decanted into a round-bottomed flask through a sintered glass funnel fitted with a Whatman No. 41 filter paper and rotary-evaporated at 40°C. The resulting material was redissolved in a small amount of DCM, transferred to a glass vial, dried under a stream of air for 48 hr, and freeze–dried.

For the large-scale collection, we weighed 2.00±0.01 g of dry ground leaves into a conical flask, containing 40± 1 ml DCM. After 5 min of sonication, the extraction was continued for 23 hr with occasional stirring. The solvent was decanted into a round-bottomed flask through a sintered glass funnel fitted with a Whatman No. 41 filter paper, and the leaf residue was re-extracted for about 1 hr in 10 ml DCM. The extract was filtered, added to the first extract, and the solvent was removed by rotary evaporation at 40°C. The resulting crude material was transferred quantitatively with five aliquots of DCM (2 ml each) to a pre-weighed glass vial. We then dried the extracts under a gentle stream of N_2 for about 24 hr, freeze–dried them to remove any remaining solvent, and reweighed them to give the amount of extract per g of dry leaf.

NMR Spectroscopy ^{1}H NMR spectra were measured using a Bruker Avance 750 spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) with a 5 mm TXI XYZ ^{1}H -^{13}C -^{15}N probe, operating at 749.66 MHz (^{1}H). Crude leaf extracts were dissolved in $CDCl_3$ at a concentration of 10 mg/ 750 μl. Spectra were recorded at 298 K and referenced to the residual $CHCl_3$ signal (δ7.26), and chemical shifts are expressed in ppm downfield from TMS. The following parameters were used for ^{1}H spectra: (pulse program:zg) 9.014 KHz spectral width, 64 K data points, 32 scans, 90° pulse (8.0 μs), 1 sec recycle delay. No exponential line broadening was applied prior to Fourier transformation. The 2D-NMR spectra were measured using either a Bruker Avance 750 spectrometer, operating at 749.66 MHz (^{1}H) and 188.52 MHz (^{13}C) with conditions as described above and using the following parameters: COSY: (pulse program: cosydfph) 4 K × 760 data points, 8 scans; HSQC: (pulse program: hsqcetgpsi2) 2 K × 512 data points, 24 scans; HMBC: (pulse program: hmbcgplpndqf) 4 K × 512 data points, 24 scans, or a Bruker Avance 300 spectrometer with a 5 mm BBIz probe, operating at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C) using the following parameters: COSY: (pulse program: cosygpmftp) 1 K × 512 data points, 4 scans; HSQC: (pulse program: invieagssi) 1500 × 512 data points, 16 scans; HMBC: (pulse program: inv4gplplrnd) 1500 × 512 data points, 32 scans.

Statistical Analysis Spectra were binned and Principal Component Analysis (PCA) was done using the Bruker Amix software package (version 3.6.8). Spectra were binned from 10.6 to 0 ppm, excluding the regions from 7.3 to 7.2 and 1.3 to 1.2 ppm, with a bin width of 0.1 ppm or from 10.6 to 7.3 ppm with a bin width of 0.05 ppm. Bins were scaled relative to the largest bin. A number of principal components sufficient to explain at least 95% of the variance were calculated (typically 6–12 principal components).

Large-scale Extraction of E. rossii (monocalypt) and Fractionation of the Dichloromethane Extract Freeze–dried intact leaves (260 g) were extracted by soaking (twice) for 24 hr with petroleum spirit (bp 60–70°C, about

1.5 l per extraction) at room temperature, followed by two extractions with redistilled DCM under the same conditions. The two DCM extracts were combined to yield, after rotary evaporation, a green gum (4.6 g, *ca* 2% of dry leaf mass). On redissolving the extract in DCM, a small quantity of a white, DCM-insoluble residue remained in the flask. This material was identified as ursolic acid by 2D-NMR spectroscopy and confirmed by comparison of NMR and melting point data with literature values. The soluble portion of the extract was subjected to flash column chromatography on silica gel (Merck Keiselgel 60, 40–63 μm, 100 g). Elution with DCM yielded a fraction containing crude 2,5-dihydroxy-7-methoxy-6,8-dimethylflavanone (35 mg), while further elution with 10% ethyl acetate/DCM yielded a fraction from which yellow crystals of 2,5,7-trihydroxy-6,8-dimethylflavanone (10 mg) were obtained.

Results

Preliminary Collection A large broad singlet peak at δ1.29, from lipid and/or wax components dominated the ^{1}H NMR spectra of the DCM extracts of the leaves of all species. As this peak was common to both subgenera, it was considered unlikely to be associated with antifeedant properties and it was omitted from the spectra prior to PCA to avoid its effect masking the contribution from minor constituents. The scores plot from the PCA reasonably separates the subgenera along the Principal Component 2 (PC2) axis (Fig. 2a), the major loadings being 1.05 (i.e., 1.10–1.00 ppm in the negative direction, correlating to the symphyomyrtle species) and 1.35 (positive, monocalypts) (Fig. 2b). Both of these bins may contain lipid methylene (δ1.35) and methyl (δ1.05) resonances, particularly those of unsaturated lipids, and so they were considered to be poor potential indicators of chemical differences between the subgenera. Further analyses showed that optimum separation occurred only when the regions of the ^{1}H NMR spectra between δ 10.6 and 7.3 ppm were included in the analysis (Fig. 3a). Here, PC1 produces a tight grouping of monocalypt species, with the exception of *E. elata* and two individuals of *E. macrorhynca*. Although these individuals have a similar score in PC1, they are separated from the remaining monocalypts in PC2. The symphyomyrtles also show a tight grouping with the exception of four out of five of the individuals of *E. melliodora* known to have a low FPC content (Fig. 3a). The major loadings in the positive direction (*Symphyomyrtus*) of PC1 were 10.13 (10.15–10.10 ppm) and 10.23, due to the aldehyde proton resonances of FPCs (Fig. 3b), while the monocalypts were typified by loadings at 7.48 and 7.43 (Fig. 3b).

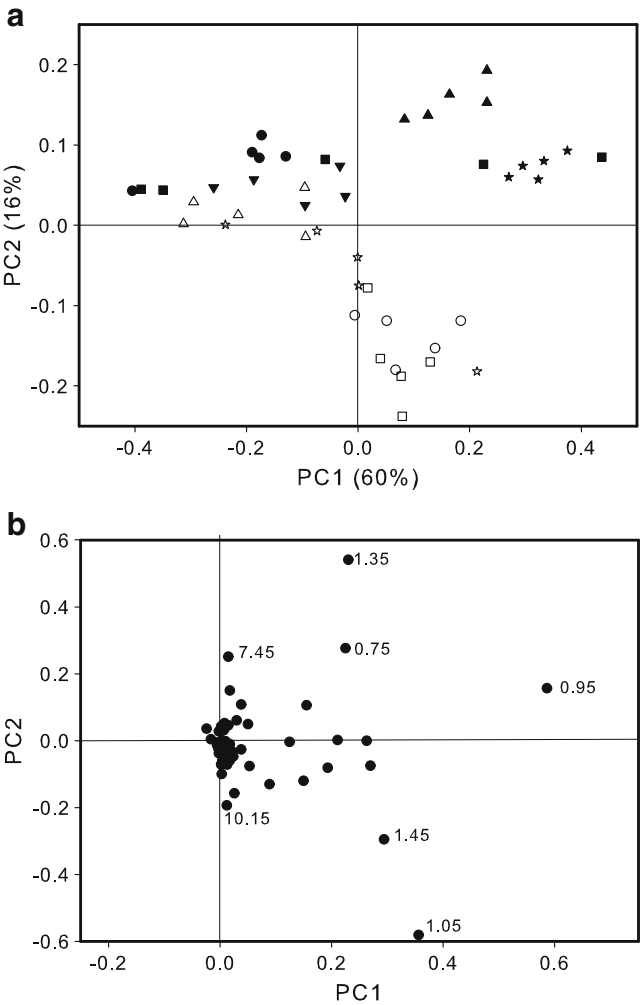

Fig. 2 Results of principal components analysis for ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves. Bins: 0.1 ppm wide, δ10.6–δ0.0 (δ7.3–7.2, δ1.3–1.2 excluded). **a** Scores plot. Filled symbols: *Eucalyptus* species. ▲ *E.agglomerata;* ▼ *E. rossii*; ● *E. dives*; ★ *E. macrorhynca*; ■ *E. elata*. Open symbols: *Symphyomyrtus* species. Δ *E. melliodora* (low FPC variety); ∇ *E. melliodora*; ○ *E. globulus*; ☆ *E. polyanthemos;* □ *E. mannifera*. **b** Loadings plot

The region between δ7.5 and δ7.3 of most of the monocalypt ^{1}H NMR spectra revealed a pattern of one doublet and two triplets, with integrals of 2, 2, and 1, respectively (Fig. 4), typical of a monosubstituted aromatic ring. There were two exceptions: the spectra from *E. rossii*, in which the doublet signal was further downfield (δ7.71), and those of *E. elata*, which had no such resonances (Fig. 4). These signals were absent from the spectra of the symphyomyrtle species, as typified by *E. globulus* (Fig. 4), which suggests that monocalypt species differ from symphyomyrtle species by the presence of a phenolic compound bearing a monosubstituted ring. However, the small differences between species in the chemical shifts of these resonances suggest that the monocalypts share a class

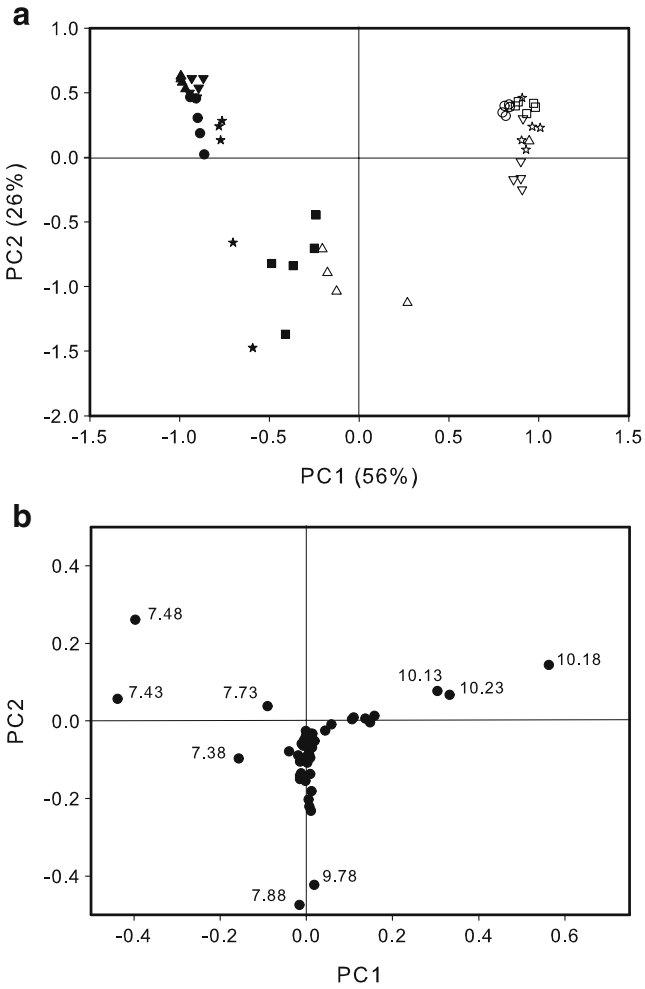

Fig. 3 Results of principal components analysis for ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves. Bins: 0.05 ppm wide, δ10.6–δ7.3. **a** Scores plot. Filled symbols: *Eucalyptus* species. ▲ *E. agglomerata;* ▼ *E. rossii*; ● *E. dives*; ★ *E. macrorhynca*; ■ *E. elata*. Open symbols: *Symphyomyrtus* species. Δ *E. melliodora* (low FPC variety); ∇ *E. melliodora*; ○ *E. globulus*; ☆ *E. polyanthemos;* □ *E. mannifera*. **b** Loadings plot

of compounds having similar structures, rather than a specific compound.

To gain more information about the structure of these phenolic compounds, 2D-NMR spectra were run on one sample of the DCM extract of *E. agglomerata*. The COSY, HSQC, and HMBC spectra confirmed that the three resonances, at δ7.47, 7.44, and 7.38, were due to a monosubstituted aromatic ring. However, apparent splitting of the peaks at δ7.44 and 7.38 suggested that these signals were due to at least two sets of overlapping resonances. The HMBC spectrum allowed the assignment of the quaternary carbon of the aromatic ring (at δ138.8) and revealed a connection from that resonance to another three proton coupling system (again overlapping pairs of almost equal intensity) consisting of resonances at δ5.42, a doublet of doublets (dd) (J 13.2 and 3.2 Hz, overlapped with a similar resonance at δ5.40), δ3.05 (dd, J 13.2 and 17.0 Hz, overlapped with δ3.02) and δ2.85 (dd, J 3.2 and 17.0 Hz, overlapped with δ2.845). Those resonances were coupled further to a carbon resonance at δ196.1. This evidence leads to the partial structure (Fig. 1, structure **2**) and suggests that the major phenolic constituents of the extract were a mixture of two flavanone compounds in approximately equal proportions, both having no substitution on Ring B and differing only in their substitution in Ring A. The complexity of the HMBC spectrum, due to overlap of some aromatic resonances and the presence of many similar minor constituents, prevented the full elucidation of the Ring A substitution patterns of these compounds. However, the ^{1}H NMR data were largely compatible with a mixture of two compounds having Ring A substitution patterns analogous to those of the commonly occurring flavones (ring-C unsaturated analogues of flavanones), eucalyptin (Fig. 1, structure **3**) and demethyleucalyptin (Fig. 1, structure **4**), although there were some discrepancies between the ^{1}H chemical shifts (and those of connected carbons found from the HMBC) for these compounds and those reported for the corresponding flavanone analogues (Diaz et al. 1987; Hsieh et al. 1998; Mayer 1990).

In summary, PCA analyses performed only on the ^{1}H NMR spectra identified the region between 7.5 and 7.3 ppm as being the major point of difference between species of the two subgenera. Inspection of 1H NMR spectra reveals

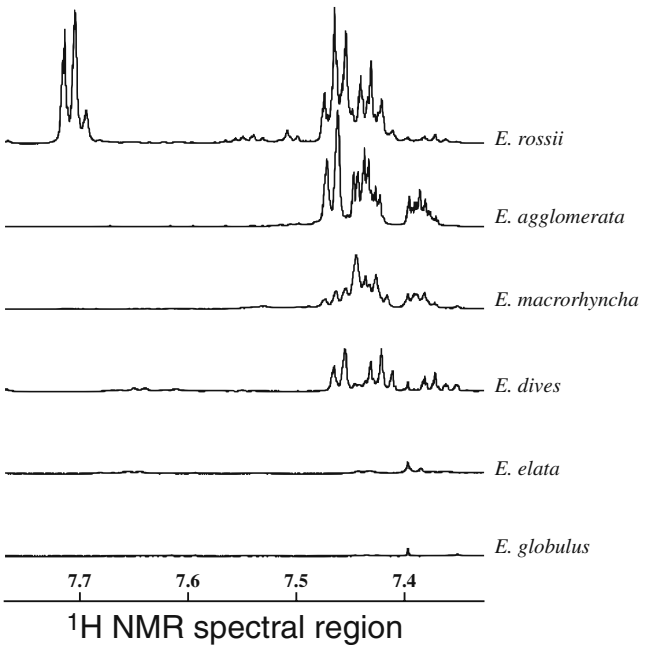

Fig. 4 The region of the ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves between δ 7.75 and δ 7.35. All are monocalypts except *E. globulus*, which gives a trace typical of *Symphyomyrtus.* One monocalypt, *E. elata*, tends to group away from other monocalypts

that the resonances present in this region can be attributed to a monosubstituted aromatic ring, while the 2D NMR data prove that the monosubstituted aromatic ring is the B ring of a flavanone. However, even within single species, a complex mixture of compounds with varying A-ring substitution patterns occurs. The exact nature of these substituents remains uncertain until the compounds are isolated from a larger collection of the plant material. That said, structures similar to that of the widely occurring unsubstituted B-ring flavanone, pinocembrin (Fig. 1, structure 5) seem likely.

Large-scale Collection A PCA of the ^{1}H NMR data of extracts from the large scale collection of 83 symphyomyrtle and 31 monocalypt species produced results (Fig. 5a) similar to those obtained for the preliminary collection. Again, the monocalypts largely group together with major loadings at 7.48 and 7.43 ppm (Fig. 5b). This suggests that there are consistent chemical differences between the leaves of species from these two major eucalypt subgenera.

A few symphyomyrtle species group close to the monocalypts in the PCA (Fig. 5). Inspection of their ^{1}H NMR spectra reveals that this is due to a combination of two features. First, they show very small or no resonances corresponding to FPCs (e.g., *E. nitens*), similar to our observations in the preliminary experiment for *E. melliodora* with low concentrations of the FPC, sideroxylonal (Fig. 3a). Second, the symphyomyrtles that grouped close to the monocalypts show one or more peaks in the region from δ 7.5–7.4. However, the chemical shift and multiplicity of these resonances suggest that they are not due to unsubstituted B-ring flavanones. In some cases, the resonance was a doublet, with a coupling partner at lower field (ca. δ 7.8–7.7) suggesting a 4′-substituted flavone, such as eucalyptin (Fig. 1, structure 3) or related compounds. In contrast, the spectra of three species, *E. dundasii*, *E. salubris,* and *E. pleurocorys*, had resonances with multiplicity typical of a monosubstituted aromatic system (i.e., a one proton triplet, a two proton triplet, and a two proton doublet) with chemical shifts of δ 7.55, 7.44, and 8.04, respectively, but the latter shift suggests a flavone with an unsubstituted B-ring rather than a flavanone derivative. The presence of a resonance at δ 6.6 in the spectra of these species, perhaps due to H-3, supports this suggestion. Another species of interest is *E. grandis*, which also has resonances in its spectrum due to a monosubstituted aromatic ring. However, in this case the shifts (δ7.30 (2H, t), 7.26 (2H, d) and 7.21 (1H, t) are not compatible with those of the B-ring of a flavone or a flavanone. Leaves of *E. grandis* contain the β-triketones, flavesone, leptospermone, and isoleptospermone (Boland et al. 1991), and it seems likely that these aromatic resonances may be due to the aromatic analogue, grandiflorone, which has not been

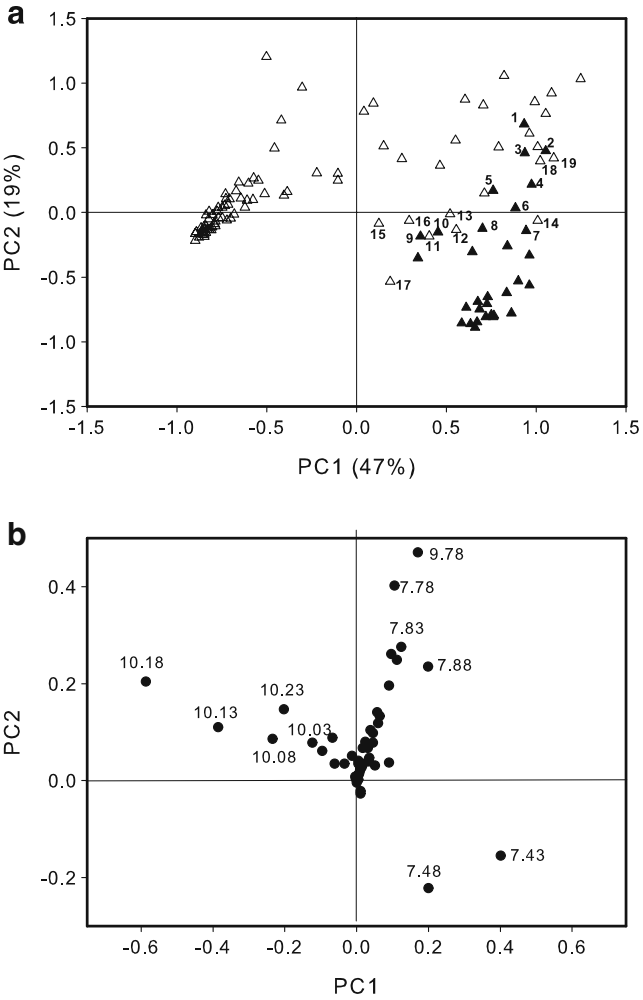

Fig. 5 Results of principal components analysis for ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves from the large scale collection. Bins: 0.05 ppm wide, δ 10.6–δ 7.3. **a** Scores plot. ▲ *Eucalyptus* species, *N*=31; Δ *Symphyomyrtus* species, *N*=83. Some species are labelled as follows: (*Eucalyptus*) 1—*E. dives*; 2—*E. brevistylis*; 3—*E. staeri*; 4—*E. buprestium*; 5—*E. marginata*; 6—*E. lacrimans;* 7—*E. moorei*; 8—*E. amydalina;* 9—*E. elata;* 10—*E. laevopinea;* (*Symphyomyrtus*) 11—*E. nitens*; 12—*E. dundasii*; 13—*E. salubris*; 14—*E. caleyi*; 15—*E. brunnea*; 16—*E. angustissima*; 17—*E. dawsonii;* 18—*E. pleurocorys;* 19—*E. grandis*. **b** Loadings plot

reported previously in eucalypts. The only ^{1}H NMR data reported for grandiflorone was at 60 MHz (Hellyer and Pinhey 1966), at which frequency the aromatic protons are unresolved, but the shift of δ 7.22 agrees reasonably well with those found at 750 MHz in this work.

Of the outlying monocalypts, two species, *E. amygdalina* and *E. moorei*, contained substantial quantities of unsubstituted B-ring flavanones, but a large score in PC2 separated them from most monocalypts. The loadings responsible for this score were at 7.83 and 7.87 ppm due to eucalyptin and 8-demethyleucalyptin (confirmed by comparison of the ^{1}H NMR spectra of these species with literature data) (Sarker et al. 2001). Horn and Lamberton

(1963) found these flavones in both monocalypt and symphyomyrtle species, while we found NMR evidence of many further occurrences in both sub-genera, thus suggesting that they have no chemotaxonomic significance. The other outlying monocalypt species (*E. brevistylis*, *E. buprestium*, *E. dives*, *E. elata*, *E. lacrimans, E. laevopinea, E. marginata,* and *E. staeri*) contained no unsubstituted B-ring flavanones.

The literature provides some support for our observation that flavanones with unsubstituted B-rings occur in monocalypts but not in symphyomyrtles. A wide variety of flavonoids have been isolated from symphyomyrtle species, including C-methylated flavones, C-methylated flavonols and flavonols (Wollenweber and Kohorst 1981; Conde et al. 1997). In contrast, the unsubstituted B-ring flavanones pinocembrin, alpinetin, and O,O-dimethylpinocembrin, have been reported only in the monocalypt *E. sieberi* (Bick et al. 1972). A reanalysis of negative ion ESIMS data published by Eschler et al. (2000) revealed that the spectra of all the monocalypt species contained an ion at m/z 255, which plausibly could be the M-H^+ ion of pinocembrin. The m/z 255 ion was present in substantially lower relative abundance in the two species *E. andrewsii* and *E. haemastoma*.

Large-scale Extraction of E. rossii and Fractionation of the DCM Extract We attempted to isolate and characterize the compounds responsible for the major aromatic resonances observed in the ^{1}H NMR spectrum of the leaf extract of *E. rossii* leaves. The appeal of the species was that its ^{1}H NMR spectrum suggested the presence of a flavonoid with an unsubstituted B-ring that differed in the aromatic region from that of most other monocalypt species. The DCM extract yielded two compounds tentatively assigned as novel 2-hydroxyflavanones (Fig. 1, structures **6** and **7**). The structural elucidation of these compounds will be reported elsewhere.

Discussion

We demonstrated that a broad metabolomic approach using ^{1}H NMR spectra of the DCM extracts of leaves can detect consistent chemical differences between the two major eucalypt subgenera. In particular, monocalypt leaf extracts contain flavanones having an unsubstituted B-ring, typified by resonances in the region from δ 7.4–7.3, while species of symphyomyrtle do not contain this class of compounds. In contrast, the symphyomytles have unique signals in the vicinity of 10 ppm that correspond to FPCs. These differences provide a correlative explanation for why brushtail possums refrain from eating monocalypt foliage (Marsh et al. 2003a). Furthermore, these differences help explain dietary niche partitioning in marsupial folivores and provide clues about the evolutionary relationships between eucalypts and the animals that eat them. As pointed out previously, most eucalypt forests contain mixed stands with representatives from both major subgenera tending to co-dominate (Pryor 1959). This, together with the high intraspecific chemical variation in eucalypts (Moore et al. 2004b) that is largely under genetic control (Andrew et al. 2007), creates a patchy landscape for folivores with likely effects on the ecosystem (Whitham et al. 2008).

While we have no direct evidence that unsubstituted B-ring flavanones are responsible for the feeding behavior of brushtail possums, many flavonoids have been reported to deter feeding by insects (Treutter 2006). The reported isolation of free flavonoids from eucalypt epicuticular wax may support an antifeedant role for these compounds (Wollenweber and Kohorst 1981). Furthermore, flavonoids with an unsubstituted B-ring differ, both in biological activity and chemical reactivity, from flavonoids with mono or di-substitution in Ring B (Pannala et al. 2001; Jia et al. 2003). Thus, such compounds may have antifeedant activity that differs from that of other flavonoids, which are more widely distributed across the eucalypt genera. These results suggest two worthwhile directions for feeding studies. It would be reasonable to compare feeding by brushtail possums offered monocalypt species that have unsubstituted B-ring flavonoids to those few species that do not have these compounds (*E. brevistylis*, *E. buprestium*, *E. dives*, *E. elata*, *E. lacrimans, E. laevopinea, E. marginata,* and *E. staeri*). In addition, we suggest a comparison of feeding by possums offered artificial diets containing isolated pinocembrin (an unsubstituted B-ring flavanone, Fig. 1, structure 5) to those offered diets containing flavonoids with B-ring substitution.

We acknowledge that monocalypts may contain compounds with feeding deterrent activity that were not extracted with DCM and were thus not detected in our study. Indeed, more polar compounds including flavonoid glycosides and proanthocyanidins (condensed tannins) are well known to have antifeedant activity (Marsh et al. 2003b) but would not be present in a DCM extract. On the other hand, the exclusive production of unsubstituted B-ring flavanones by monocalypts, revealed in this research, indicates that these species are capable of biosynthetic pathways unavailable to symphyomyrtles. Such processes may produce unique flavonoid glycosides and proanthocyanidins, as these compounds share common biosynthetic precursors with flavanones (Veitch and Grayer 2008). This prompts a similar study using methanol extracts to determine whether the more polar metabolites of these subgenera show similar differentiation to that seen in DCM extracts.

A key message of this paper is the value of spectral approaches and metabolomics for elucidating ecological problems. The literature contains numerous examples in which the study design was based on the assumption that a particular nutrient, especially nitrogen, was limiting. One advantage of spectral approaches is that they require no preconceived ideas about chemical or nutrient composition, but provide a holistic view of the material. Approaches such as NIRS have been used to discriminate groups, such as leaf samples from different species or with differing susceptibility to herbivory, but this method is of limited value for deciphering the underlying chemistry. The better method is broad metabolite profiling, in which one aims to identify as many metabolites as possible in a single extract to provide the biochemical phenotype of the organism (Allwood et al. 2008). One of the preferred methods, gas chromatography-mass spectrometry, can be used routinely to determine the concentrations of 300–500 polar and non-polar metabolites per sample (Lisec et al. 2006). Its disadvantages include the need for compounds to be in mass spectral libraries (i.e., known compounds), the need to derivatize non-volatile compounds, and the need for a calibration system to account for differences in detection sensitivity among compounds. Therefore, GC-MS is not suitable for poorly understood systems, such as *Eucalyptus*. In contrast, ^{1}H NMR spectroscopy offers several benefits (Nicholson et al. 1998): 1) samples require minimal treatment; 2) detection is universal, since virtually all organic compounds give ^{1}H NMR signals; 3) resonance intensity is directly proportional to the concentration of compound; 4) the bins responsible for the major loadings in the PCA provide structural information about compounds.

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