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ANTIHERBIVORE CHEMISTRY OF *Eucalyptus*—CUES AND DETERRENTS FOR MARSUPIAL FOLIVORES

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Abstract—Formylated phloroglucinol compounds (FPCs) are the single most important factor determining the amount of foliage that marsupial folivores eat from individual *Eucalyptus* trees. Folivores need to recognize which trees contain FPCs if they are to avoid them and forage efficiently, they are challenged by great diversity in the types and quantities of FPCs present, even within eucalypt species. We investigated the relationship between FPCs and terpenoids in species with both simple and complex FPC profiles and found strong positive correlations between terpenes generally, and several monoterpenes in particular, and FPCs. Terpene cues also indicated qualitative differences in trees' FPC profiles. We describe significant qualitative and quantitative variation in FPCs in several species that are important food sources for marsupial folivores. New discoveries include the fact that macrocarpals occur as two major, distinct groups and several new dimeric acylphloroglucinols from *Eucalyptus strzeleckii*. These patterns add to the chemical complexity of the foraging environment for folivores.

Key Words—Formylated phloroglucinol compounds, terpenoids, conditioned flavor aversion, Koala, *Phascolarctos cinereus*, jensenal, *Eucalyptus strzeleckii*, macrocarpals, sideroxylonals, 1,8-cineole.

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INTRODUCTION

Trees of the genus *Eucalyptus* L'Hérit. possess complex mixtures of plant secondary metabolites, including terpenoids, cyanogenic glycosides, hydrolyzable and condensed tannins, flavonoids, long chain ketones, and formylated phloroglucinol compounds (FPCs; Brophy and Southwell, 2002). Folivores of *Eucalyptus* need to recognize and limit their intake of these compounds, because their ability to tolerate or detoxify them sometimes falls short of their concentrations in foliage (Lawler et al., 1998b). This task becomes easier if sensory cues reliably indicate the presence and/or concentration of relevant compounds.

Among trees belonging to the *Eucalyptus* subgenus *Symphyomyrtus*, which accounts for more than half of all eucalypt species, the most important single variable determining feeding by marsupial folivores is the concentration of FPCs (Lawler et al., 2000; Wallis et al., 2002; B. D. Moore, unpublished data). In several species, such as *E. polyanthemos* Shau. (Lawler et al., 2000) and *E. microcorys* F. Muell. (Moore et al., in press), FPC concentrations are strongly correlated with concentrations of the monoterpene, 1,8-cineole. Because of this, Lawler et al. (1998a) suggested that marsupials develop conditioned flavor aversions to high concentrations of volatile terpenes because their flavor is consistently associated with the negative postingestive consequences of ingesting FPCs. Lawler et al. (1999b) showed that in experiments with isolated compounds, 1,8-cineole could be used by common brushtail possums as a cue to the concentrations of deterrent compounds. However, the situation in species examined to date may be misleadingly simple, because many eucalypts possess numerous monoterpenes and sesquiterpenes in addition to 1,8-cineole (Brophy and Southwell, 2002), as well as numerous FPCs in addition to, or in place of, sideroxylonal (Ghisalberti, 1996; Eschler et al., 2000; Eyles et al., 2003).

The simplest FPCs are fully substituted, formylated acylphloroglucinols, such as jensenone (Figure 1). These units form the basis of dimeric acylphloroglucinols, such as sideroxylonals, grandinal, and robustaol A, and form adducts, such as euglobals and macrocarpals, with mono- and sesquiterpenes. Although the terpene moiety suggests an obvious link between the concentrations of macrocarpals and euglobals and those of terpenoids, simple and dimeric FPCs do not share a biosynthetic precursor with terpenoids. Mono- and sesquiterpenes are predominately products of the deoxyxylulose phosphate, or mevalonate-independent pathway (Dewick, 2002), whereas the phenolic moiety of FPCs must arise from chalcone synthase-type enzymes as part of the shikimate pathway. One possible explanation for the covarying synthesis of FPCs and terpenes is that regulation of these processes is closely linked at the genetic level. The expression of terpenes in eucalypts is highly variable and has a strong genetic basis (Doran, 1992; Dungey et al., 2000; Dunlop et al., 2000), and variation in FPC concentrations in natural populations occurs at a scale too small to be explained by environmental

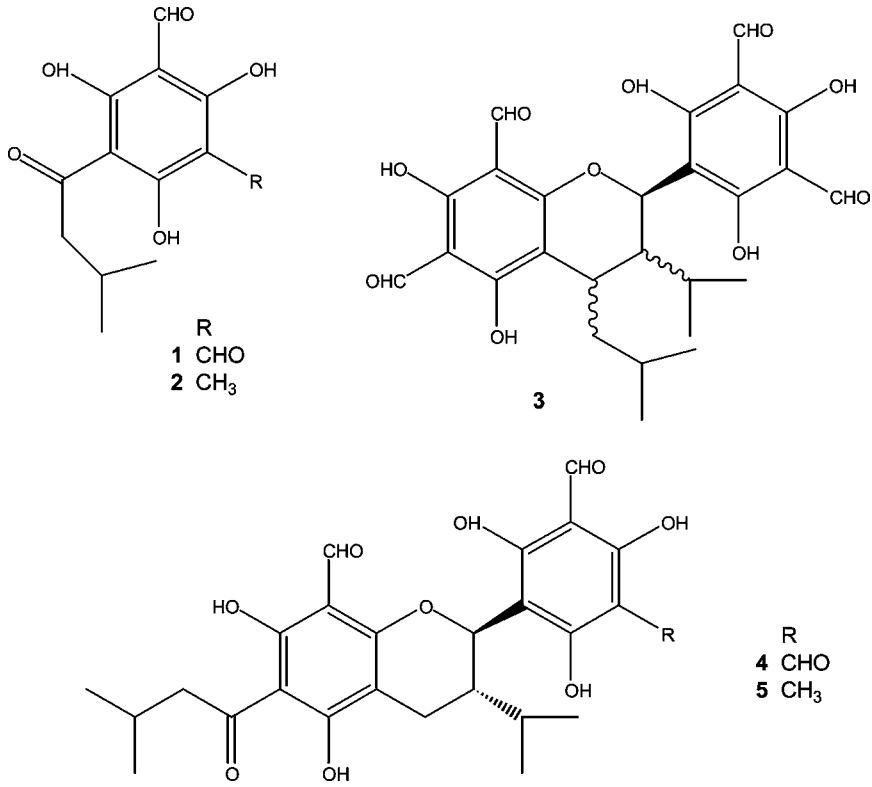

FIG. 1. Sideroxylonal (**3**), which occurs as 3 stereoisomers, is a dimer of jensenone (**1**), whereas grandinal (**4**) is a dimer of jensenone and grandinol (**2**). Structure **5** is a proposed grandinol dimer that would have a precise mass of 486.1890, consistent with ion peaks observed in *E. strzeleckii* extracts.

factors (R. Andrew, unpublished data). Given its interest from both ecological and biosynthetic perspectives, the link between FPCs and terpenes warrants further study. As a first step, this relationship must be better described, so we pursued this aim, using gas chromatography coupled to mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) to analyze and compare the terpene and FPC profiles from several *Eucalyptus* species. We considered two species that possess predominately sideroxylonal FPCs (*E. melliodora* A. Cunn. ex Schauer and *E. polyanthemos*) and two species possessing more complex FPC profiles (*E. globulus* Labill. and *E. viminalis* Labill.; Eschler et al., 2000).

Analytical limitations have restricted previous investigations of FPC content to eucalypt species that possess only sideroxylonal, so the qualitative variation in FPCs facing marsupial folivores remains largely unknown. Most FPCs have been

reported from only one or a few eucalypt species, and patterns of co-occurrence resulting from shared or overlapping biosynthetic pathways are poorly understood. Consequently, the other major aim of our study was to describe these patterns in detail for a large number of trees from several species. To achieve this, we used electrospray ionization, Fourier transform ion cyclotron mass spectrometry (ESI-FTMS), and HPLC to analyze foliage from *E. globulus*, *E. viminalis*, and *E. ovata* Labill. These species are widespread in south-eastern Australia and important food species for herbivorous marsupials. We also included a fourth species, *E. strzeleckii* K. Rule, for comparison with the closely related and sympatric *E. ovata*. *E. strzeleckii* is a recently described species (Rule, 1992) with a restricted distribution in the South Gippsland region of Victoria that, to our knowledge, has not been reported as a food species for vertebrate herbivores. All trees in this report were also used in an experiment, which will be reported elsewhere, to test the role of FPCs in determining the feeding preferences of koalas.

METHODS AND MATERIALS

To survey variation in FPCs, we collected foliage from 50 *E. globulus*, 29 *E. ovata*, 8 *E. strzeleckii*, and 51 *E. viminalis* trees in 1998 and 2002. Trees were selected randomly from Phillip Island, French Island, eastern Melbourne, and South Gippsland, in the state of Victoria. To study covariance in terpene and FPC concentrations, we collected foliage from a further 14 *E. globulus* and 15 *E. viminalis* from the same region, and 15 *E. melliodora* and 19 *E. polyanthemos* from near Canberra, Australian Capital Territory in 2002. We collected at least 100 g of mature foliage from the midcanopy of each tree and immediately froze samples at -20°C. Leaving aside 50 g subsamples from trees to be analyzed for terpenoids, we freeze-dried the samples and ground them to pass a 1-mm sieve in a Cyclotec 1093 cyclone mill (Tecator, Sweden). Further analyses were performed on solvent extracts made from this ground leaf material as follows.

We weighed 1.5 g of leaf material into a cellulose extraction thimble and refluxed this for at least 4 hr with 100 ml of 4:1 light petroleum spirit (40–60°C boiling point): acetone mixture in a Soxhlet extractor connected to a round-bottomed flask, heated to 85°C. We removed solvent from the flask by rotary evaporation at 50°C and transferred the crude extract into a glass vial using 4:1 dichloromethane:methanol. We determined the crude mass of extract after drying this solution under a stream of air for 24 hr and for a further 48 hr at room temperature.

Analysis of FPCs by ESI-FTMS. We randomly selected acetone–petrol extracts from 27 *E. globulus*, 26 *E. ovata*, 8 *E. strzeleckii*, and 30 *E. viminalis* samples collected in 1998 for analysis by ESI-FTMS. We dissolved $\sim$5 mg of dried crude extract in 1 ml MeOH and further diluted 10 μl in 1 ml MeOH. This solution

was continually infused at a flow rate of 1 μl min^{-1} into the external electrospray source (Analytica of Bradford, Bradford, CT) of a Bruker BioApex 47e Fourier transform ion cyclotron resonance mass spectrometer operating in negative ion mode with broadband [low resolution (6–10 k FWHM at m/z 500)] detection. Typically, the signal was averaged over 16 transients prior to Fourier transformation, requiring a data acquisition time of about 1 min, and the consumption of about 1 μg of the crude extract. The instrument was calibrated with sodium trifluoroacetic acid (TFA).

Analysis of FPCs by HPLC. We used HPLC to analyze all acetone–petrol extracts. We dissolved approximately 15 mg of extract in 5 ml of acetonitrile and analyzed between 12.5 and 25 μl of this solution with a Waters Alliance Model HPLC with photo diode array detector. The analytical column was a Wakosil 250 × 4 mm GL 3C18RS (SGE), and the column temperature was 37°C. Extracts were eluted under gradient conditions with 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B) as follows: 60% A/40% B for 5 min, linear gradient to 90% A/10% B at 60 min, hold for 10 min. Flow rate was 0.75 ml min^{-1}. We measured the peak response at 275 nm of 20 major peaks from the resulting chromatographs (Table 1).

TABLE 1. MAJOR HPLC CHROMATOGRAPH PEAKS FROM EUCALYPT FOLIAGE EXTRACTS

Retention time (min)	Formula	Identity	Standard curve?
11			
13	$C_{28}H_{42}O_7$	**Macrocarpal I**	Y
17	$C_{28}H_{42}O_7$	**Macrocarpal J**	
25	$C_{19}H_{18}O_5$	**Eucalyptin**	Y
27	$C_{28}H_{38}O_7$	Macrocarpal N?	
28	$C_{28}H_{38}O_7$	**Eucalyptone**	Y
32	$C_{28}H_{40}O_6$	**Macrocarpal A**	Y
34	$C_{28}H_{40}O_6$	Macrocarpal	Y
35	$C_{28}H_{40}O_6$	Macrocarpal	
37	$C_{28}H_{40}O_6$	**Macrocarpal B**	Y
39	$C_{28}H_{40}O_6$	Macrocarpal	
42	$C_{28}H_{40}O_6$	Macrocarpal	
43	$C_{26}H_{28}O_{10}$	**Grandianal**	Y
44	$C_{26}H_{28}O_{10}$	**Sideroxylonal A**	Y
45	$C_{26}H_{28}O_{10}$	**Sideroxylonal C**	Y
47	$C_{28}H_{40}O_6$	Macrocarpal	
53	$C_{28}H_{40}O_6$	**Grandinal**	Y
60	$C_{26}H_{28}O_{10}$	Jensenal	Y
		Monoterpene	
66	$C_{25}H_{30}O_9$	euglobal CHO	
70	$C_{23}H_{30}O_5$	**Macrocarpal G**	Y

We identified macrocarpals G, A, and B, the macrocarpal eucalyptone, sideroxylonals A and C, and grandinal on the basis of their coelution with authentic standards and by comparison of HPLC-UV data. We also collected fractions corresponding to most major peaks, which we analyzed with ESI-FTMS, as described above, to determine precise masses of the compounds. We collected between 2 and 6 mg each of compounds eluting at 13, 17, 25, 34, and 60 min from crude extract injected onto a Waters 300 × 7.8 mm Preparative NovaPak HRC18 column operated under similar gradient conditions to those described above, at a flow rate of 3.0 ml min^{-1}. ^1H NMR spectra of the 13, 17, and 25 min peaks confirmed identities as macrocarpals I and J and the flavonoid eucalyptin (Horn and Lamberton, 1963; Osawa et al., 1996). We determined extinction coefficients for a number of these compounds (Table 1). Several extracts containing compounds of interest were analyzed by MS and MS–MS, providing additional identification of these compounds as FPCs. We did not determine extinction coefficients for the compounds eluting as smaller peaks at 17, 27, 35, 39, 42, and 47 min, but used averaged extinction coefficients from other compounds with the same molecular formula to quantify them.

Extraction and Analysis of Terpenes. We used steam distillation to extract foliar terpenes from ∼50 g samples of frozen leaves. We estimated the dry matter content of this sample by drying a second portion of the leaves at 80°C for 48 hr. Our procedure was true steam distillation and not hydrodistillation, which is most commonly used to study *Eucalyptus* terpenes. Dunlop et al. (2000) showed that hydrodistillation alters the composition of distilled oils relative to those isolated by either vacuum distillation or steam distillation, probably because of rearrangements induced by pH changes in the water in which the leaves are distilled. We modified previously described methods (Foley et al., 1987) by suspending the leaves above boiling water on stainless steel mesh inside a 2 l flanged flask. Water returning from the collection burette was channelled via a funnel back to the base of the flask so that it did not drip though the leaf mass. The volume of oil recovered was measured, and the oil was dried over sodium sulphate and stored in vials with nitrogen at −20°C until it was analyzed.

Analytical gas chromatography (GC) (Shimadzu GC17A with FID) was used to quantify terpenes. Samples of distilled oil were injected on a column of DB-Wax (60 m × 0.5 mm × 1 μm) programmed to ramp from 50 to 220°C at 3°C min^{-1} with helium as a carrier gas. FID integrations were performed on a SMAD electronic integrator. GC–MS was used to identify oil components and performed on a VG Quattro mass spectrometer operating at 70 eV ionization energy. The GC column used was a DB-Wax (60 m × 0.32 mm × 0.25 μm) ramped from 35 to 220°C at 3°C min^{-1} with helium as carrier gas. Mono- and sesquiterpenes were identified by their identical GC retention times relative to known compounds and by comparison of their mass spectra with either known compounds or published

spectra (Stenhagen et al., 1974; Heller and Milne, 1978, 1980, 1983; Swigar and Silverstein, 1981; Adams, 1995; Joulain and König, 1998).

Statistical Analyses. To identify patterns of covariance among FPC constituents measured by HPLC, we constructed a correlation matrix describing pairwise correlations among the areas of all 20 peaks measured from chromatographs. Significance levels of correlation coefficients were adjusted by the serial Bonferroni procedure. For each species analyzed by GC–MS, we considered all possible pairwise correlations between the concentrations of independently occurring FPCs or groups of FPCs and all measured terpenes to identify the strongest correlations. Where we wished to further investigate the relationships between individual terpene and FPC groupings, we performed model II-type simple linear regressions, using major axis regression calculated by the computer program "Model II Regression" (Legendre, 2001).

RESULTS

Detection of Qualitative and Quantitative Variation in FPCs by ESI-FTMS. Most prominent ions detected from petrol–acetone extracts by ESI-FTMS were consistent with the molecular formulae of previously reported FPCs, free fatty acids or triterpene acids (Table 2). Several smaller peaks could be attributed to flavones and arenic and β-triketones. We detected masses consistent with most published FPCs, as well as several of the triterpene and sesterterpene FPCs and disubstituted monoterpene and sesquiterpene FPCs proposed by Eyles et al. (2003). The strongest ion peak from *E. strzeleckii* was at m/z 473.1798. Tandem MS analysis of this compound gave a strong product ion at m/z 237 and a weaker product ion at m/z 223, consistent with the structure of **6**, fragmenting at points indicated by (a) and (b) in (Figure 2). This compound, jensenal, has previously been isolated and characterized from *E. jensenii* Maiden (Midori Takasaki, personal communication). ^{1}H NMR and ^{13}C NMR spectra of the compound from *E. strzeleckii* were consistent with those from jensenal. *E. strzeleckii* extracts also produced strong peaks at m/z 488.1682 and 490.1859, which gave MS–MS spectra dominated by an m/z 237 product ion. The first mass is consistent with a compound recently reported from *E. saligna* (**8**, Figure 2; Mitaine-Offer et al., 2003), and the second with **7**. The smaller peak detected from *E. strzeleckii* at m/z 485.1811 may be **5** (Figure 1), a grandinol dimer.

ESI-FTMS revealed three basic FPC profiles (Figure 7). First, the spectra of all *E. globulus* and *E. viminalis* and of many *E. ovata* trees were dominated by macrocarpals, with other FPC peaks (including sideroxylonal) much less intense in comparison. Second, a number of *E. ovata* trees produced much stronger sideroxylonal peaks, although large macrocarpal peaks were still present. Third, all

TABLE 2. MAJOR ION PEAKS DETECTED BY ESI-FTMS FROM EUCALYPT FOLIAGE EXTRACTS

Calculated (m/z)	Measured (m/z)	Compound	Occurrence[a]				Mean r.i. (%)[b]	Reference[c]
			Eg	Es	Eo	Ev		
Formylated phloroglucinol compounds								
453.26	453.26	Macrocarpal G, sesquiterpene Euglobals	+++	+++	+++	+++	57.4	1,2
485.25	485.25	Eucalyptone, Macrocarpal N	+++	+++	+++	+++	20.7	3,4
471.27	471.27	Macrocarpals A,B,D,E,F,H,K,L,M,O	+++	+++	++	+++	68.2	1,5,6,3
385.20	385.20	Monoterpene euglobals	+++	+++	++	+++	8.2	2
675.46	675.46	Triterpene alcohol FPCs	++	−	++	+++	3.8	7
401.20	401.20	Oxidized monoterpene euglobals	++	+++	++	++	1.3	8
499.16	499.16	Sideroxylonal, grandinal	++	+++	++	+	11.2	9,10,11
657.45	657.45	Triterpene HCO FPCs	++	−	+	++	2.4	7
473.18	473.18	Jensenal (**6**)	+	+++	+	++	15.6	
589.39	589.38	Sesterterpene HCO FPCs	++	−	+	++	0.8	7
489.29	489.28	Macrocarpal I, J	++	+++	++	+	8.0	5
607.40	607.40	Sesterterpene alcohol FPCs	++	−	+	++	0.9	7
251.09	251.09	Grandional	+	+++	++	++	1.2	12
403.21	403.21	Monoterpene alcohol/ether FPCs	+	+++	++	++	1.5	7
703.35	703.35	Disubst, sesquiterpene HCO FPCs	+	++	++	+	4.4	7
485.18	485.18	Possible structure **5**	+	++	+	+	1.4	
635.29	635.28	Disubst. monoterpene HCO FPCs	−	+++	+	+	2.7	7
489.18	489.18	Possible strucutre **7**	+	+++	+	+	12.1	
487.16	487.16	Structure **8**	−	+++	+	−	7.2	
721.36	721.36	Disubst. sesquiterpene alcohol FPCs	−	+++	+	−	2.7	7

TABLE 2. CONTINUED

Calculated (m/z)	Measured (m/z)	Compound	Occurrence[a]				Mean r.i. (%)[b]	Reference[c]
			Eg	Es	Eo	Ev		
Flavones								
311.09	311.09	Sideroxylin	++	++	+++	+++	2.7	13,14
297.08	297.08	8-demethylsideroxylin	++	+	++	++	1.1	14
Ketones								
265.14	265.15	Apodophyllone, leptosermone	++	+++	++	+++	2.0	15
279.16	279.16	Torquatone, isotorquatone	+	+	+	+	0.7	15,16
251.13	251.13	Flavesone	+	−	−	+	1.0	17
235.10	235.10	Agglomerone	+	+	+	+	0.4	17

[a] +++ = ion present in 100% of trees; ++ = 50–100%; + = 0–50%; − = 0%; symbols are in bold type if mean r.i. > 10% for that species; Eg = *E. globulus*, Es = *E. strzeleckii*, Eo = *E. ovata*, Ev = *E. viminalis*.

[b] Mean relative intensity (r.i.) calculated from all non-zero values. r.i. does not accurately indicate relative concentrations of these compounds.

[c] Previous reports from *Eucalyptus*: 1 = Nishizawa et al., 1992; 2 = Amano et al., 1981; 3 = Shibuya et al., 2001; 4 = Osawa et al., 1995; 5 = Yamakoshi et al., 1992; 6 = Terada et al., 1999; 7 = Eyles et al., 2003; 8 = Eschler et al., 2000; 9 = Satoh et al., 1992; 10 = Eschler and Foley, 1999; 11 = Singh et al., 1997; 12 = Yoshida et al., 1988; 13 = Mitaine-Offer et al., 2003; 14 = Sarker et al., 2001; 15 = Menut et al., 1999; 16 = Brophy et al., 1996; 17 = Brophy and Southwell, 2002.

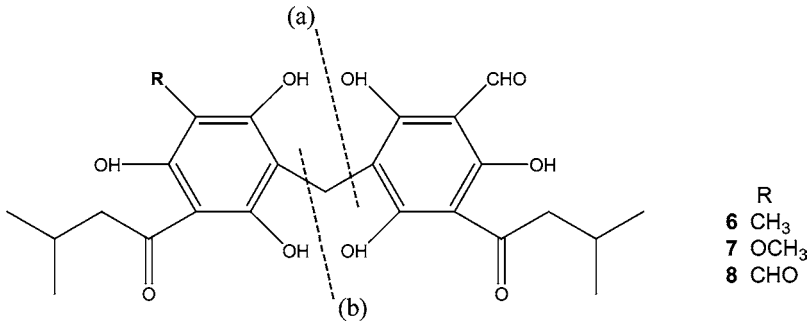

FIG. 2. Jensenal (**6**) and suggested structures for the compounds of precise mass 490.1859 (**7**) and 488.1682 (**8**) observed in *E. strzeleckii* extracts. The fragment to the right of the dashed line (a) has MW 237, which was observed in tandem MS analysis of these three compounds. The fragment of **6** to the left of (b) has MW 223, which was observed in tandem MS analysis of that compound.

E. strzeleckii trees produced small macrocarpal peaks, but large peaks attributable to jensenal and sideroxylonal and/or grandinal, as well as the peaks discussed above, which were absent from the other species.

Detection of Qualitative and Quantitative Variation in FPCS by HPLC. Example HPLC chromatographs are shown in Figure 3. Twelve of the 20 peaks quantified were attributable to macrocarpals, 2 to sideroxylonals, 2 to tautomers of grandinal, and 1 to jensenal. Only one of these major peaks was a euglobal, one was eucalyptin, and one was not identified.

Patterns of covariance among the 20 major peaks are shown in (Figure 4). Strong correlations indicate that certain peaks always co-occurred in constant proportions with other peaks. We refer to the group comprising the 70, 32, 37, 35, 39, 47, 28, and 27 min peaks (listed in order of decreasing peak size) as "group 1" macrocarpals, and the group consisting of the 34, 13, 17, and 42 min peaks as "group 2" macrocarpals. The isomers sideroxylonal A and C also occurred in a fixed ratio, and grandinal is a tautomer (Singh et al., 1997) that always produces two equal peaks. Although Figure 4 shows that grandinal and jensenal peak sizes are strongly correlated, the ratio describing their relative proportions is more variable than those for other groupings, and the significant correlation coefficient partly reflects these compounds' co-occurrence in all *E. strzeleckii* trees. The relative concentrations of compounds in each group are illustrated in Figure 5.

Total FPC concentration and the concentration of individual FPCs and groups of FPCs were highly variable in all species except *E. strzeleckii*, although this exception may reflect the smaller number of *E. strzeleckii* trees analyzed (Figure 6). HPLC confirmed and distinguished more clearly the existence of three basic FPC profiles, showing that macrocarpals were generally absent from the

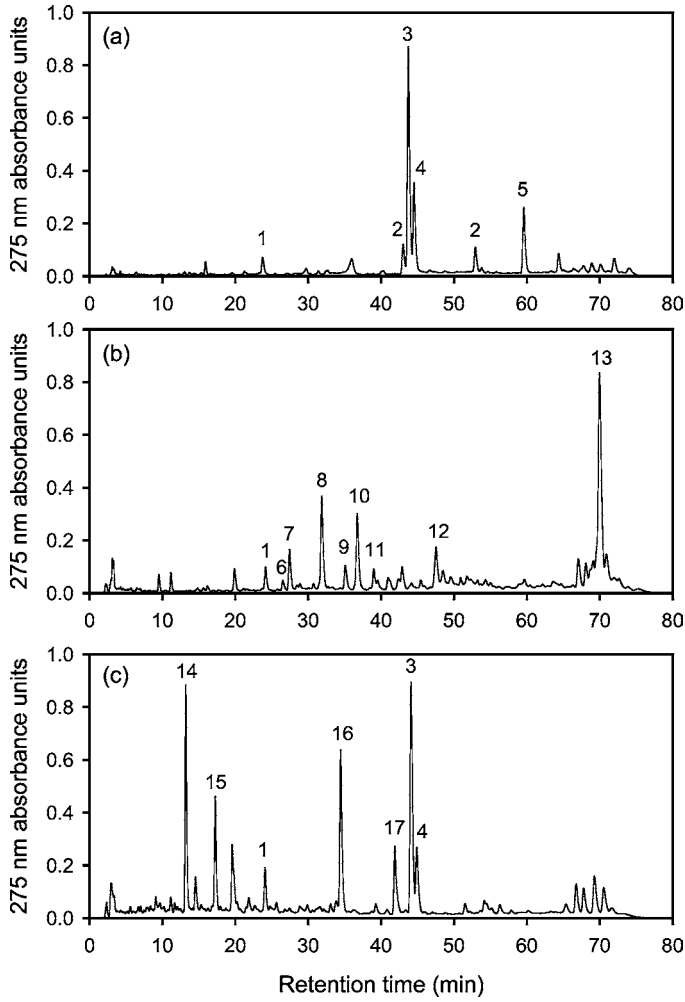

FIG. 3. HPLC chromatographs from foliage extracts of (a) *E. strzeleckii* (b) *E. viminalis* with group 1 macrocarpals and (c) *E. globulus* with group 2 macrocarpals and sideroxylonal. 1: eucalyptin, 2: grandinal, 3: sideroxylonal A, 4: sideroxylonal C, 5: jensenal, 6: macrocarpal ($R_t = 27$ min); 7: eucalyptone; 8: macrocarpal A; 9: macrocarpal ($R_t = 35$ min); 10: macrocarpal B; 11: macrocarpal ($R_t = 39$ min); 12: macrocarpal ($R_t = 47$ min); 13: macrocarpal G; 14: macrocarpal I; 15: macrocarpal J; 16: macrocarpal ($R_t = 34$ min); 17: macrocarpal J ($R_t = 42$ min).

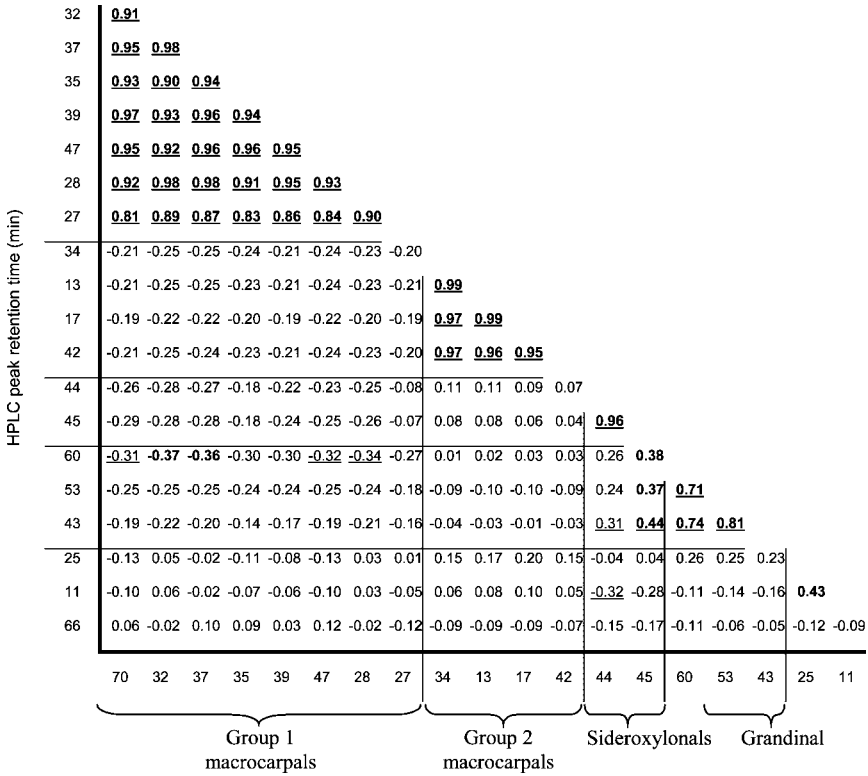

FIG. 4. Correlation matrix of HPLC peak responses from 130 eucalypt foliage extracts. (Correlation coefficients in bold and underlined are significant at $P < 0.001$; in bold at $P < 0.01$ and underlined, normal weight at $P < 0.05$ after sequential Bonferroni adjustment.)

sideroxylonal-rich *E. ovata* chemotype and *E. strzeleckii*. Macrocarpal-rich trees were generally dominated by "group 1" macrocarpals, however, in some trees, they were replaced by "group 2" macrocarpals or by a mixture of the two groups (Figure 6).

Comparison of HPLC and ESI-FTMS Results. Although ESI-FTMS detected macrocarpals in all trees, they were not detected by HPLC in sideroxylonal-dominated *E. ovata* or *E. strzeleckii*. Conversely, the intensity of ESI-FTMS sideroxylonal peaks was much less than its true concentration would suggest (Figure 7). We sought to quantify the difference between macrocarpal and sideroxylonal responses on the ESI-FTMS system by coinjecting authentic standards of sideroxylonal A and macrocarpal G in four different proportions. Relative to the quantity of compound injected, the macrocarpal G peaks were more than an

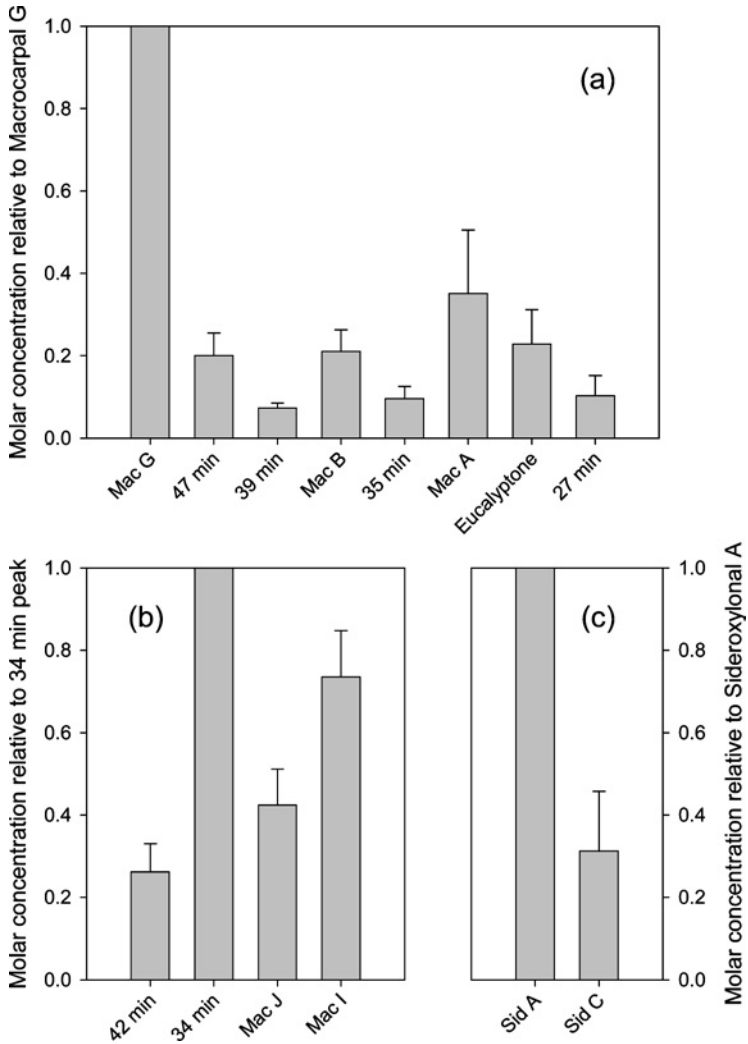

FIG. 5. Mean relative molar concentrations of (a) "group 1" macrocarpals ($N \geq 97$); (b) "group 2" macrocarpals ($N \geq 14$); (c) sideroxylonals ($N = 115$) measured by HPLC. Error bars represent 1 standard deviation.

order of magnitude greater than sideroxylonal A peaks. In the trees we analyzed, ESI-FTMS indicated the concentrations of differently-sized macrocarpals (e.g., macrocarpals with the formula $C_{28}H_{42}O_7$ and $C_{28}H_{38}O_5$; Figure 7) relative to one another, but not the relative concentrations of more structurally dissimilar groups, such as macrocarpals and sideroxylonals.

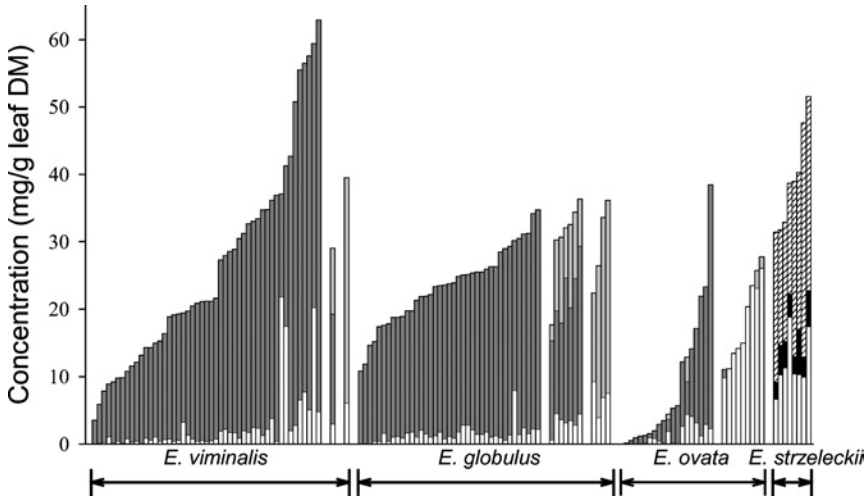

FIG. 6. Foliar concentrations of sideroxylonals (white bars), "group 1" macrocarpals (dark gray), "group 2" macrocarpals (light grey), grandinal (black), and jensenal (cross-hatched) in four eucalypt species, measured by HPLC. Bars representing different species and qualitatively dissimilar trees have been separated by gaps.

Terpene and FPC Relationships. We identified 76 terpenes in the four species analyzed (Table 3). All species were dominated by monoterpenes, particularly 1,8-cineole, with smaller amounts of α-pinene, limonene, *p*-cymene, and the sesquiterpene, globulol. Within each species, the oils obtained were mostly qualitatively similar, however, two *E. melliodora* trees had unusual terpene profiles—they produced moderate oil yields and were dominated by *p*-cymene and spathulenol, but contained little 1,8-cineole. One unusual *E. polyanthemos* tree possessed concentrations of most terpenes that were typical for that species, but also possessed a very high concentration of β-phellandrene. The FPC profiles of both *E. melliodora* and *E. polyanthemos* were dominated by sideroxylonals, but *E. melliodora* also contained small amounts of grandinal and some *E. polyanthemos* trees possessed small concentrations of "group 1" macrocarpals.

Sideroxylonal and total terpene concentrations were positively correlated in *E. melliodora* ($r^2 = 0.34$, $P = 0.02$), however, the two trees with unusual terpene profiles deviated from the general trend (Figure 8). We found stronger correlations between sideroxylonal and 1,8-cineole ($r^2 = 0.67$, $P < 0.001$) and sideroxylonal and limonene ($r^2 = 0.77$, $P < 0.001$; Figure 8). The correlation between sideroxylonal and total terpene concentration was stronger in *E. polyanthemos* ($r^2 = 0.62$, $P < 0.001$), although a tree with an unusual terpene profile formed an outlier in this species too (Figure 8). Sideroxylonal concentration in *E. polyanthemos* was

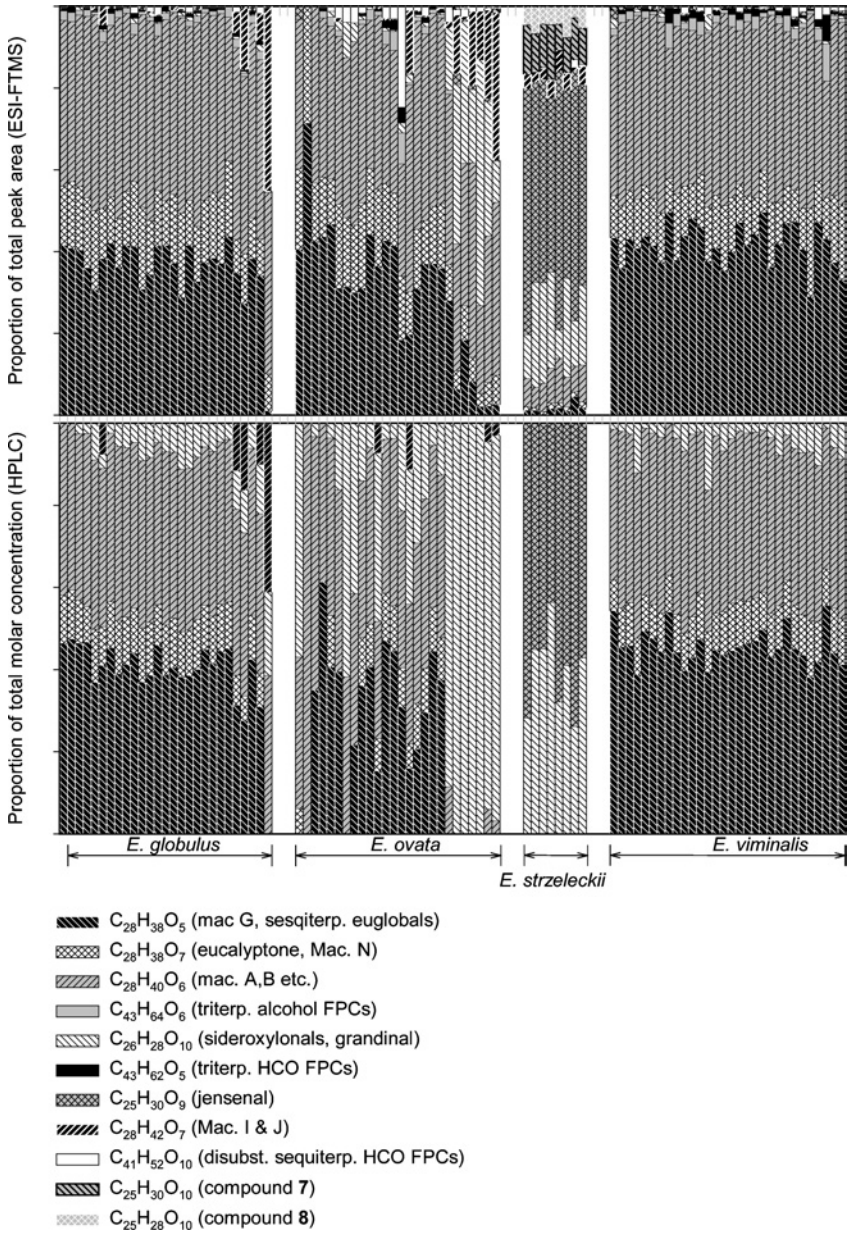

FIG. 7. Relative proportions of ESI-FTMS peak intensities (above) and relative molar concentrations determined by HPLC (below) of major FPC compounds in four eucalypt species.

TABLE 3. TERPENOIDS[a] IDENTIFIED BY GC-MS FROM STEAM-DISTILLED FOLIAGE EXTRACTS FROM *E. melliodora* (EM), *E. polyanthemos* (EP), *E. globulus* (EG), AND *E. viminalis* (EV)

	Em		Ep		Eg		Ev	
1,8-Cineole	**91.6**	(2.4–199.4)	**85.3**	(0–323.3)	**130.2**	(51.3–266.8)	**82.4**	(1.2–245.1)
α-Pinene	**15.0**	(1.6–29.5)	**5.9**	(0–24.3)	**40.7**	(16.8–79.1)	**33.8**	(0.1–137.5)
Limonene	**7.3**	(0.7–14.8)	**6.3**	(0–15.5)	**9.1**	(2.1–21.6)	**8.2**	(0.3–24.3)
p-Cymene	**6.1**	(0.5–31.9)	**4.6**	(0.4–18.2)	**4.5**	(0.3–14.8)	**9.3**	(0.6–27.1)
globulol	**1.7**	(0.1–8.7)	**1.2**	(0–3.3)	**8.0**	(0–15.2)	**12.4**	(6.0–21.3)
α-Terpineol	**3.4**	(0.4–7.4)	**5.4**	(0–23.9)	**2.4**	(0.5–7.7)	**2.3**	(0.1–16.2)
β-Eudesmol	—		—		**6.0**	(0–30.8)	—	
trans-Pinocarveol	**2.2**	(0–7.0)	**0.5**	(0–1.7)	**5.0**	(0.7–12.8)	**2.6**	(0–12.6)
Aromadendrene	**0.3**	(0.1–1.0)	**0.5**	(0–1.3)	**4.7**	(0.2–11.0)	**2.6**	(0.3–11.1)
γ-Terpinene	**0.8**	(0.0–6.9)	**1.1**	(0–6.0)	**0.6**	(0–3.8)	**4.9**	(0.1–24.6)
β-Phellandrene	**0.3**	(0–2.1)	**5.6**	(0–78.4)	—	(0–0.2)	**0.1**	(0–0.7)
Terpinyl acetate	**2.4**	(0–7.2)	**3.5**	(0–11.3)	—		—	
Spathulenol	**3.5**	(0.1–23.2)	**1.8**	(0.0–9.9)	**0.5**	(0–1.2)	**0.5**	(0.1–1.0)
α-Phellandrene	**1.7**	(0–11.9)	**2.5**	(0–18.7)	**0.4**	(0–1.5)	**0.8**	(0–6.5)
Terpinen-4-ol	—		—	(0–1.1)	**0.2**	(0–1.5)	**5.4**	(0–10.8)
Pinocarvone	**0.5**	(0.1–1.4)	**0.1**	(0–0.5)	**4.3**	(0.6–10.0)	**0.8**	(0–5.5)
Epiglobulol	—		—	(0–0.2)	**1.6**	(0–3.2)	**2.5**	(0–5.2)
β-Caryophyllene	**1.9**	(0.1–10.5)	**1.1**	(0–10.8)	**0.2**	(0–0.9)	**0.2**	(0–0.5)
Viridiflorene	**0.6**	(0–2.9)	**0.3**	(0–1.5)	**1.1**	(0–2.3)	**1.6**	(0.6–2.6)
$C_{15}H_{26}O_3$	—		—		**0.7**	(0–1.6)	**1.0**	(0–1.8)
α-Eudesmol	—		—		**1.8**	(0–13.3)	—	
Viridiflorol	**1.6**	(0–10.8)	**0.5**	(0–2.8)	**0.6**	(0–2.8)	**0.4**	(0–1.2)
Bicyclogermacrene	**1.8**	(0.1–15.0)	**1.1**	(0–6.7)		—	—	
γ-Eudesmol	—		—		**1.4**	(0–10.3)	—	
allo-Aromadendrene	**0.4**	(0–2.1)	**0.3**	(0–1.0)	**1.0**	(0.2–1.6)	**1.0**	(0–2.0)
trans-Menth-1(7),8-diene-2-ol	**0.5**	(0.1–1.4)	**0.4**	(0–1.4)	**1.1**	(0.2–2.4)	**0.4**	(0–1.4)

$C_{15}H_{26}O_2$	—		—		**0.6**	(0–1.5)	**0.5**	(0–0.8)
α-Bulnesene	**0.5**	(0.1–1.4)	**1.0**	(0–6.5)	**0.6**	(0.2–1.3)	—	
β-Pinene	**0.1**	(0–0.4)	**0.2**	(0–0.7)	**1.1**	(0.2–2.2)	**0.7**	(0–2.6)
Cubeban-11-ol	**0.8**	(0–5.9)	**0.3**	(0–1.0)	**0.4**	(0–0.7)	**0.5**	(0.2–1.0)
Isoamyl isovalerate	**0.6**	(0–1.8)	**0.4**	(0–3.2)	**0.1**	(0–0.6)	**0.5**	(0–1.8)
$C_{15}H_{26}O$	**0.6**	(0–4.5)	**0.2**	(0–1.7)	—		—	
cis-Menth-1(7),8-diene-2-ol	**0.4**	(0–1.2)	**0.2**	(0–0.8)	**0.7**	(0–1.5)	**0.3**	(0–1.0)
$C_{15}H_{26}O$	**0.5**	(0–4.6)	**0.3**	(0–2.8)	—		—	
Thymol	—		—		—		**0.6**	(0–2.8)
$C_{15}H_{26}O$	**0.4**	(0–3.3)	**0.1**	(0–0.5)	—		—	
Borneol	**0.4**	(0.1–1.1)	**0.3**	(0–3.2)	**0.2**	(0–0.6)	—	
Ledol	**0.2**	(0–1.1)	**0.1**	(0–0.8)	**0.2**	(0–0.4)	**0.4**	(0.1–0.6)
Neral	—		—		—		**0.4**	(0.1–0.9)
trans-Menth-1,8-dien-6-ol	**0.1**	(0–0.3)	**0.2**	(0–2.0)	**0.4**	(0–1.3)	**0.1**	(0–0.5)
Myrcene	—	(0–0.2)	**0.3**	(0–4.6)	**0.2**	(0–1.7)	**0.3**	(0–1.7)
Terpinolene	**0.1**	(0–0.4)	**0.2**	(0–1.0)	**0.2**	(0–0.6)	**0.2**	(0–1.3)
α-Gurjunene	**0.1**	(0–0.6)	—		**0.2**	(0–1.2)	**0.3**	(0–0.9)
Myrtenol	—		—		**0.1**	(0–0.3)	**0.2**	(0–0.5)
Ketone 142	—		—		**0.4**	(0–1.9)	—	
Palustrol	**0.2**	(0–1.3)	**0.2**	(0–1.6)	**0.1**	(0–0.2)	**0.1**	(0–0.2)
Carvone	—		—		**0.3**	(0–0.7)	—	
Caryophyllene oxide	—		**0.2**	(0–4.6)	—		—	
p-Cymene-8-ol	—	(0–0.2)	—	(0–0.4)	**0.2**	(0–0.8)	**0.1**	(0–0.5)
β-Gurjunene	—		—		**0.3**	(0–1.8)	—	
Z-β-Ocimene	—		—		—		**0.2**	(0–0.7)
Menth-1-en-7-al	—	(0–0)	**0.2**	(0–3.8)	—		—	
δ-Terpineol	**0.1**	(0–0.6)	**0.1**	(0–0.8)	**-**		**0.2**	(0–0.4)
Carvacrol	—		—		—		**0.2**	(0–0.4)
$C_{10}H_{14}$	—		—		**0.2**	(0–0.5)	—	
Fenchol	**0.2**	(0–0.5)	**0.1**	(0–1.1)		—	—	
E-β-Ocimene	**0.1**	(0–0.2)	**0.1**	(0–0.4)	—		**0.1**	(0–0.3)

TABLE 3.CONTINUED

	Em		Ep		Eg		Ev	
Piperitol	—	—			**0.1**	(0–0.4)	—	
cis-Menth-1,8-dien-6-ol	—	(0–0.2)	—	(0–0.2)	**tr**		—	(0–0.1)
Cryptone	—		**0.1**	(0–2.9)	—		—	
α-Terpinene	—	(0–0.1)	**0.1**	(0–1.4)	**tr**		**0.1**	(0–0.3)
α-Campholenic aldehyde	—		—		**0.1**	(0–0.9)	—	
Isovaleraldehyde	—		—	(0–1.3)	**0.1**	(0–0.6)	**0.1**	(0–1.0)
Humulene	**0.1**	(0–0.5)	**0.1**	(0–0.5)	—		—	
Camphene	**0.1**	(0–0.2)	**tr**		**0.1**	(0–0.2)	—	(0–0.3)
Phenylethyl isovalerate	—		—		**0.1**	(0–0.4)	—	
α,*p*-Dimethyl styrene	—		—		**tr**		**0.1**	(0–0.4)
α-Fenchene		(0–0.1)	**tr**	**tr**			—	
$C_{10}H_{14}$	—		—		**0.1**	(0–0.2)	—	
hex-3-en-1-ol	—		—		**0.1**	(0–0.2)	—	
Sabinene	—		**0.1**	(0–2.2)	—		—	
Ethyl-isovalerate	—		—		—		**tr**	
Isoamyl acetate	—			(0–0.2)	**tr**		—	
Isobutyl Isobutyrate	—		—		—		**0.1**	(0–0.1)
α-Thujene	—			(0–0.6)	—		—	
Cuminal	—			(0–0.3)	—		—	

[a] All values are concentrations (mg/g DM), mean concentrations are in bold type, and minima and maxima are provided in brackets.

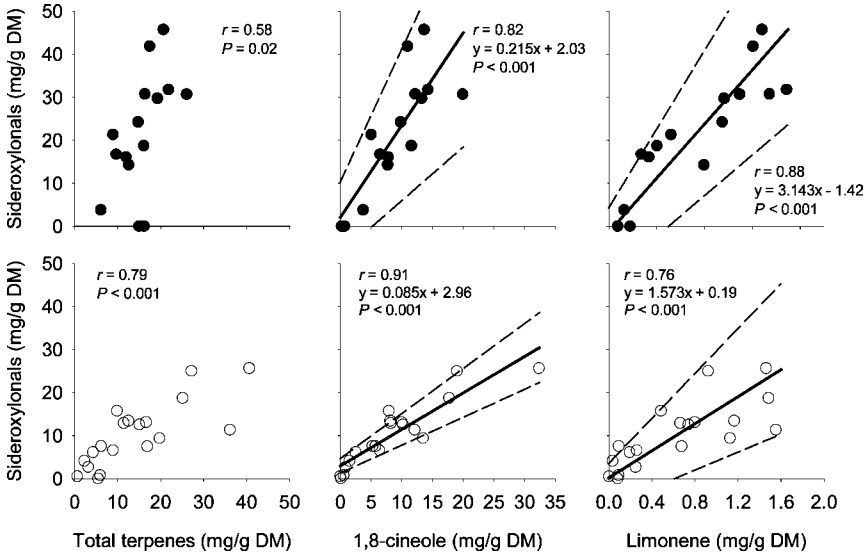

FIG. 8. Plotted FPC and terpene concentrations in 15 *E. melliodora* trees (closed circles) and in 19 *E. polyanthemos* trees (open circles). Listed on each figure are Pearson's correlation coefficient (r) and the associated significance level (P). Solid lines indicate major axis regressions and dashed lines 95% confidence intervals.

most strongly correlated with 1,8-cineole ($r^2 = 0.83$, $P < 0.001$) and limonene ($r^2 = 0.58$, $P < 0.001$; Figure 8). The major axis regression coefficient (x = cineole, y = sideroxylonal) was significantly greater for *E. melliodora* (95% confidence interval: 0.125–0.308) than for *E. polyanthemos* (0.065–0.105), reflecting the higher ratio of sideroxylonal: cineole in the former species.

In *E. globulus* (Figure 9), total FPC concentration was positively correlated with total terpenes ($r^2 = 0.67$, $P < 0.001$), 1,8-cineole ($r^2 = 0.72$, $P < 0.001$), and limonene ($r^2 = 0.66$, $P < 0.001$). In this species, the positive correlation between total terpenes and total macrocarpals was weaker ($r^2 = 0.53$, $P = 0.03$), but that between total terpenes and sideroxylonals was stronger than for FPCs overall ($r^2 = 0.79$, $P < 0.001$). In *E. viminalis* (Figure 10), total terpene concentration was a better predictor of total FPCs ($r^2 = 0.79$, $P < 0.001$) than 1,8-cineole ($r^2 = 0.59$, $P < 0.001$). Total terpene concentration was also correlated with "group 1" macrocarpals ($r^2 = 0.67$, $P < 0.001$) and sideroxylonals ($r^2 = 0.45$, $P = 0.006$), but not with "group 2" macrocarpals, which occurred in less than half of the trees ($r^2 = 0.13$, $P = 0.19$). The major axis regression coefficient (x = total terpenes, y = total FPCs) for *E. globulus* was significantly less (95% confidence interval: 0.244–0.643) than that for *E. viminalis* (0.811–1.524). The y-intercept of the *E. globulus* regression was greater than zero.

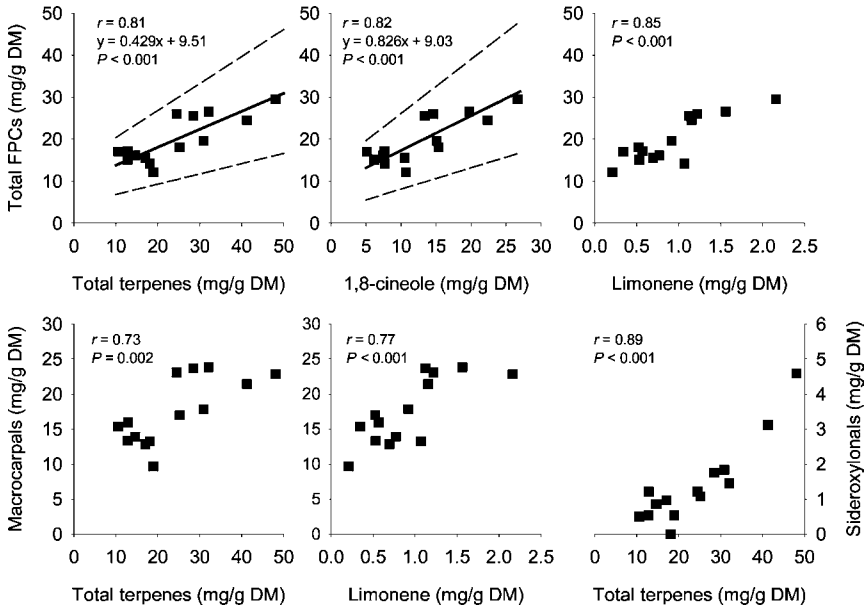

FIG. 9. Plotted FPC and terpene concentrations in 14 *E. globulus* trees. Listed on each figure are Pearson's correlation coefficient (r) and the associated significance level (P). Solid lines indicate major axis regressions and dashed lines 95% confidence intervals.

Of the 14 *E. globulus* trees, two possessed "group 2" macrocarpals but no "group 1" macrocarpals. For eight, the reverse was true, and the other four contained both groups. Among the small number of trees considered, higher concentrations of total terpenes and of 1,8-cineole corresponded to higher "group 2" macrocarpal concentrations when they were present (Figure 11). "Group 2" macrocarpal concentration was also strongly correlated with the covarying concentrations of β-, α-, and γ-eudesmol. We only detected eudesmols in *E. globulus* possessing "group 2" macrocarpals. Few strong correlations occurred between "group 1" macrocarpals and individual terpene concentrations in *E. globulus*, and the strongest of these, between "group 1" macrocarpals and globulol, only occurred amongst trees possessing "group 2" macrocarpals (Figure 11).

DISCUSSION

The great inter- and intraspecific variation in the types and concentrations of FPCs in the species that we considered has important implications for marsupial

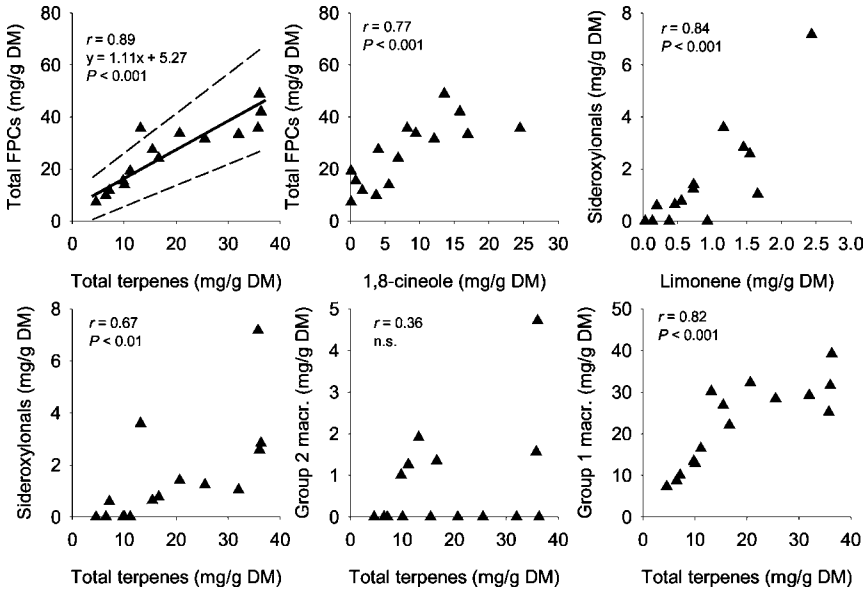

FIG. 10. Plotted FPC and terpene concentrations in 15 *E. viminalis* trees. Listed on each figure are the Pearson's correlation coefficient (r) and the associated significance level (P). The solid line indicates a major axis regression and the dashed lines indicate the 95% confidence interval.

folivores. In addition, the strong relationships between major terpene and FPC constituents may facilitate the development of conditioned flavor aversions that allow these animals to feed selectively. This study identified several previously unreported FPCs from *E. strzeleckii*, and for the first time described patterns of covariance amongst FPCs. This is a critical first step towards understanding the biosynthesis.

ESI-FTMS detected ion masses consistent with most known and several novel FPCs in our extracts, although jensenone (m/z 265.241) only occurred in one tree. One possible explanation of jensenone's rarity is that it is produced in these species, but only as a precursor of larger-molecular weight FPCs (Ghisalberti, 1996). The paucity of euglobals in our extracts is also surprising, given the large number of euglobal isomers previously reported from these species. ESI-FTMS indicated that flavones and cyclic polyketones, which differ from simple FPCs primarily in their functional groups, were generally only present in low concentrations. However, pentacyclic triterpenes such as ursolic acid produced strong ion peaks. The implications (if any) of these biologically active compounds for marsupial herbivores remain unknown.

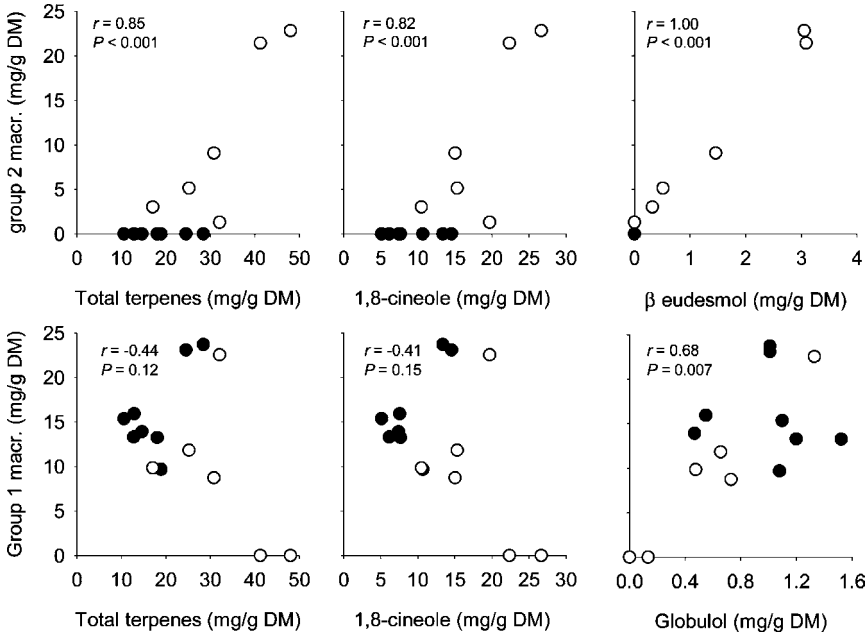

FIG. 11. Plotted FPC and terpene concentrations in 14 *E. globulus*, including trees possessing "group 2" macrocarpals (open circles) and trees without "group 2" macrocarpals (closed circles). Listed on each figure are Pearson's correlation coefficient (r) and the associated significance level (P), calculated across all 14 trees.

Like *E. melliodora* and *E. polyanthemos*, *E. strzeleckii* and the sideroxylonal-rich chemotype of *E. ovata* did not possess significant concentrations of most macrocarpal-type acylphloroglucinol-terpene adducts, including the sesterterpene and triterpene adducts proposed by Eyles et al. (2003). This suggests the possibility that a single enzyme may be responsible for combining acylphloroglucinol and terpenoid residues to produce a range of macrocarpals.

Several compounds present in *E. strzeleckii*, including jensenal, **7**, and **8**, differ from the more common dimeric acylphloroglucinols, such as sideroxylonals and grandinal, in that the 5-carbon acyl functions are not incorporated in the bonds forming the dimer. Consequently, these acyl functions may influence the effectiveness of these compounds as antifeedants. Hydrogen bonding of equivalent carbonyl groups with phenolic hydroxyl groups is essential to the action of grandinol as an inhibitor of both germination (Bolte et al., 1985) and activation of the Epstein-Barr virus (Takasaki et al., 1990). Jensenal, **5**, and **7** also differ from most FPC molecules in possessing only one formyl group compared to macrocarpals with two, and sideroxylonals with four. Although the formyl groups are thought

to be prerequisite to the antifeedant activity of FPCs (Lawler et al., 1999a), it is unclear as to whether their number is important.

In isolation, our mass spectrometry results imply that sideroxylonals were present only in trace amounts in *E. globulus* and *E. viminalis* and in lower concentrations than macrocarpals in most *E. ovata*. The only previous survey of FPC distributions used ESI-FTMS to assess the presence and the absence of known FPCs in 41 species of *Eucalyptus* (Eschler et al., 2000). In that study, sideroxylonals were the most frequently identified group of compounds overall, but were not detected in *E. globulus*. Our HPLC results indicated that sideroxylonals were present in substantial concentrations in many of our trees, including *E. globulus* and *E. ovata*. We showed that macrocarpal G produces a peak more than an order of magnitude greater than that from an equivalent molar concentration of sideroxylonal A. The most likely explanation of this difference is that macrocarpals are more readily ionized than sideroxylonal.

In most cases, the strongest terpene–FPC relationships involved specific monoterpenes, usually either 1,8-cineole or limonene. This pattern is well illustrated by *E. melliodora* and *E. polyanthemos*, in which sideroxylonal concentrations fell on the regression line predicted by 1,8-cineole and were not influenced by the high concentrations of other terpenes. There were strong correlations between terpene concentrations and each of the classes of FPCs when considered individually, but unsurprisingly these relationships were weaker for less dominant FPC classes. Correlations between FPC and monoterpene concentrations may result if the regulation of their synthetic enzymes is linked.

The relationship between "group 2" macrocarpals and α-, β-, and γ-eudesmol in *E. globulus* may be more direct because these sesquiterpenes and the terpene moiety attached to the epimeric macrocarpals I and J share a eudesmane-type skeleton (Osawa et al., 1996). Hence, the synthesis of these compounds may be limited by a common sesquiterpene precursor. In *E. globulus* trees that contain "group 2" macrocarpals, the concentrations of "group 1" macrocarpals are positively correlated with globulol and structurally similar terpenes, as well as with aromadendrene and its similar terpenes. Globulol is structurally identical to the terpene moiety of macrocarpals A and B, and aromadendrene matches the terpene moiety of macrocarpal G. These terpene moieties probably share a common origin in bicyclogermacrene (Ghisalberti, 1996). The fact that these correlations did not exist in trees without "group 2" macrocarpals suggests that the synthesis of "group 2" macrocarpals and related sesquiterpenes competes with the synthesis of "group 1" macrocarpals and related terpenes. Although the availability of specific sesquiterpene precursors may determine the type of macrocarpals produced, the positive correlations between terpene and total macrocarpal concentrations suggest that allocation to macrocarpal acylphloroglucinol precursors may be determined upstream from the formation of the final terpene adducts.

Our results confirm the possibility of dominant volatile monoterpenes acting as cues to folivores feeding on eucalypt species containing complex mixtures of FPCs. However, the relationship between terpene and FPC concentrations is species-specific. Major axis regression coefficients indicated that sideroxylonal concentrations in *E. melliodora* trees were typically more than twice those in *E. polyanthemos* trees with the same 1,8-cineole concentrations. Similarly, FPC concentrations in *E. globulus* were almost twice those seen in *E. viminalis* with similar terpene profiles. As terpene-rich species are not necessarily FPC-rich, folivores' feeding decisions must be informed by both the concentration of terpenes in the foliage and the tree species.

The headspace concentration of terpene over the leaf surface may vary according to weather conditions, light intensity, time of day, and leaf age (Zini et al., 2002), affecting the reliability with which folivores can gauge actual foliar concentrations by olfaction. However, qualitative differences in terpene composition should be consistently distinct. Hence, the distinctive terpene profiles of the two *E. melliodora* trees that possessed negligible amounts of cineole and sideroxylonal, but substantial amounts of *p*-cymene and spathulenol, could potentially provide a cue for a conditioned flavor preference. Folivores may find it easier to identify trees that smell different than to distinguish between similar "weak" and "strong" smells, and may be able to detect the presence of particular terpenes more reliably than the absence of others. Similarly, the correlation between eudesmol terpene concentrations and "group 2" macrocarpals in *E. globulus* potentially allows herbivores to discriminate among trees on the basis of the types of FPCs that are present. Although "group 1" and "group 2" macrocarpals do not appear to differ in their effectiveness as antifeedants for koalas (B. D. Moore, unpublished data), compositional cues may be important where the biological activity of compounds does differ. Even if the composition of hydrodistilled oils does not correspond to headspace terpene composition, similar between-tree qualitative differences can be expected. Several studies have found that eucalypt headspace volatiles are richer in sesquiterpenes, including compounds known to play infochemical roles in other plant genera, than are hydrodistilled oils (Betts, 2000; Zini et al., 2003).

Our results show striking variation in the absolute concentrations of FPCs, particularly in *E. viminalis* and *E. ovata*, including trees with almost no FPCs and trees in which FPCs accounted for more than 6% of the dry mass of foliage. Although high FPC concentrations deter folivore feeding (Lawler et al., 2000), this variation means that some less defended trees will usually be available to animals. In this context, the lesser degree of variation in *E. globulus* and *E. strzeleckii* is noteworthy. In both cases, even the least defended trees contained considerable concentrations of FPCs.

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