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Foliar quality of co-occurring mallee eucalypts: balance of primary and secondary metabolites reflects past growing conditions

Martin J. Steinbauer · Ian R. Wallis · Noel W. Davies · Simon J. Watson

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Abstract Foliar quality for herbivores is determined by the balance of primary and secondary metabolites which is dependent on leaf age which in turn is determined by the periodicity of flushing and the rate of abscission. We conducted a 10-month longitudinal study in northeastern Victoria, Australia, of the quality of fully expanded leaves of *Eucalyptus gracilis*, *E. socialis*, *E. dumosa* and *E. incrassata*. We measured N and available N (AvailN) as well as sideroxylonals and other formylated phloroglucinol compounds (FPCs) and related changes in concentration to tree phenology (flushing and flowering) and climatic conditions (temperature and rainfall). Concentrations of N, sideroxylonals and other FPCs differed significantly between species and with time as well as among trees within a species. AvailN also differed significantly between species and among trees within a species and with time for *E. gracilis*. Our analyses indicated that tree phenology affected N concentrations in *E. gracilis* only while climatic conditions affected N concentrations in *E. gracilis*, *E. socialis* and *E. dumosa*. Nitrogen concentrations in *E. incrassata* were unaffected by phenological or climatic factors. Tree phenology affected concentrations of sideroxylonals in *E. gracilis* and *E. dumosa* while climatic conditions affected concentrations of the same in *E. socialis*, *E. dumosa* and *E. incrassata*. The concentration of primary metabolites in expanded leaves of these eucalypts was relatively consistent compared to the concentration of quality reducing secondary metabolites which varied with the conditions experienced when leaves were expanding. Our results show that the foliar quality of leaves can be highly variable, with variations mediated by phenology and climate. These variations are likely to partially explain fluctuations in the abundance of chewing insect herbivores reliant on expanded leaves.

Keywords Sclerophylly · Allocation patterns · Antifeedants · Antibiosis · Terpenes · Epicuticular waxes

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M. J. Steinbauer (✉) · S. J. Watson
Department of Zoology, La Trobe University, Melbourne, VIC 3086, Australia
e-mail: M.Steinbauer@latrobe.edu.au

I. R. Wallis
Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia

N. W. Davies
Central Science Laboratory, University of Tasmania, Private Bag 74, Hobart, TAS 7001, Australia

Introduction

Foliar quality determines the suitability of different species of plant as food for herbivores and consequently the severity of defoliation they may sustain. The balance between primary (nutritional) and secondary (potentially defensive) metabolites in leaves defines their quality and is intimately linked to their ontogeny. Changes in temperature, moisture and light control vegetative growth but it is the magnitude of seasonal fluctuations in one or more of these exogenous factors which dictate the timing and magnitude of plant responses. For example, in aseasonal forests, such as lowland (equatorial) tropical rainforest, leaf phenology is largely under internal (endogenous) rather than exogenous control but in seasonal, temperate forests

comprising both deciduous and perennial species it often has a temperature-related periodicity (Reich 1995; Basler and Körner 2014). In tropical forests with wet (monsoon) and dry seasons, leaf phenology may be endogenously regulated via acclimative physiological processes (Reich 1995). Specifically, changes in internal water status, which reflect plant access to soil moisture and the rate of foliar evapotranspiration, dictate when flush is produced and leaves are shed in many deciduous and perennial species (Williams et al. 1997). Species of eucalypt endemic to sub-tropical Australia (summer-dominant rainfall) exhibit a bimodal periodicity of flushing with peaks in autumn and spring; flushing occurs when there is sufficient available water and solar radiation and ambient temperatures exceed 16–18 °C (Specht and Brouwer 1975). Understanding the causes and patterns of plant phenology and leaf ontogeny is fundamentally important for understanding ecosystem function because foliar quantity and quality are likely to regulate the dynamics of herbivore populations.

In both deciduous and perennial species of plant, a common pattern is for nitrogenous primary metabolites to decline in concentration as leaves age while plant secondary metabolites (PSMs) rise (Haukioja et al. 2002; Riipi et al. 2002; Brunt et al. 2006)—although some secondary metabolites provide exceptions to this pattern (Lindroth et al. 1987). Most species conforming to this pattern are deciduous, i.e., leaves with lifespans less than one year. In perennial species (leaves with lifespans greater than one year), it has been suggested there may be a trade-off between investment in leaf toughness (positively correlated with leaf age) and investment in PSMs. Specifically, because leaf toughness can by itself be an effective defence against chewing insects, some plants with sclerophyllous leaves may produce less PSMs (specifically phenolic compounds) as leaves become tougher with increasing age (Read et al. 2003; Brunt et al. 2006). However, a large number of sclerophyllous species, including eucalypts, do not conform to this trend (Read et al. 2009). Moreover, not all phenolic compounds have evolved to protect plants against chewing insects, but rather in response to abiotic stressors (Close and McArthur 2002), so their expression may not be linked to leaf ontogeny (Stark et al. 2008). Of the broad classes of PSMs, eucalypts are perhaps most renowned for their high concentrations of terpenoids (Keszei et al. 2008; Steinbauer 2010). Although empirical data are limited, quantities of oils in eucalypt leaves (of the same morphological type) increase as they age, plateauing when fully expanded; this is attributed to an increase in the volume of individual oil glands during leaf expansion (Boland et al. 1991; Goodger et al. 2013a, b) which was not accounted for in some earlier studies that reported higher concentrations of oils in younger leaves (e.g., Larsson and Ohmart 1988). In addition to being the sites of terpenoid synthesis and storage, oil glands synthesise other non-volatile PSMs (Goodger et al. 2009; Heskes et al. 2012).

Eucalypts exhibiting the mallee growth habit (multiple stems generally reaching <10 m in height arise from an underground lignotuber) produce flush foliage during the Austral summer and into autumn (December to April); growth begins when air temperature is greater than 18 °C (Specht 1981). This phenology is out of phase with rainfall in this Mediterranean climate where rainfall is winter-spring dominant. This rainfall asynchronous phenology has been suggested to be a relict physiological response evolved during the sub-tropical climate of the Tertiary (Specht 1981). While there are no vertebrate folivores in mallee ecosystems, they support a diverse community of insect herbivores. The herbivore community on three species of mallee in Victoria comprised 3.5 % chewing insects (represented by Coleoptera, Lepidoptera, Orthoptera, Phasmatodea and Collembola) and 5.9 % sucking insects (represented by Hemiptera and Thysanoptera); the leaf age preferences of the species of chewing insect are not known (Yen 1989). In discussing the insect fauna supported by mallee eucalypts, Yen (1989) wrote "that foliage invertebrate communities are complex, highly variable and inconsistent". Drought can greatly reduce, and even cause the local disappearance of, insect populations on eucalypts (Bell 1985).

Terpenoid (Boland et al. 1991; King et al. 2006; Goodger et al. 2013b), phenolic (Goodger et al. 2013b) and cyclitol (Merchant et al. 2007) PSMs of some mallee eucalypts have been quantified. Eucalypt terpene composition generally differs inter- and intra-specifically as well as with tree and leaf ontogeny (e.g., Li et al. 1994, 1996; Steinbauer et al. 2004; King et al. 2006; McArthur et al. 2010; Goodger and Woodrow 2012; Goodger et al. 2013a, b). Terpene composition has been found to change over time in one species of *Angophora* (genus closely allied to *Eucalyptus*; Leach and Whiffin 1989) and four species of eucalypt (Simmons and Parsons 1987; Brooker et al. 1988). In the leaves of *A. costata*, the percentage composition of some terpenes increased with temperature while others decreased but the authors did not consider that seasonal patterns adequately explained the correlations they obtained (Leach and Whiffin 1989). Simmons and Parsons (1987) reported that different chemotypes of *E. camphora* and *E. ovata* exhibited unique variations in terpene composition which were considered likely to be best explained by leaf ageing rather than seasonal effects. Brooker et al. (1988) reported that 1,8-cineole concentrations in the leaves of *E. kochii* ssp. *kochii* and *E. kochii* ssp. *plenissima* were slightly higher in January–February.

In Australia, mallee (eucalypts and associated vegetation) covers 250,420 km^2 or approximately 3.3 % of the landmass (Clarke et al. 2010). The lack of information

about mallee eucalypt leaves as potential food for insect herbivores limits our capacity to understand insect diversity and rates of defoliation across this geographically extensive ecosystem. We conducted a longitudinal study to compare the foliar quality of four widespread species to address two questions: (1) how does foliar quality respond to tree phenology (flushing and flowering)? and (2) how does foliar quality respond to climatic conditions (temperature and rainfall)? We focussed on expanded leaves because this is the most abundant and persistent leaf age class available to insect herbivores. Furthermore, in perennial plants, expanded leaves are sources of N for expanding leaves which is achieved by the mobilisation of the photosynthetic protein ribulose bisphosphate carboxylase/oxygenase (Rubisco; Wendler et al. 1995). The cost of the mobilisation of Rubisco on expanded leaves is dependent upon the plant's access to soil nutrients. We measured N and AvailN; AvailN is a functionally significant measure of foliar N because it accounts for the fraction of N that is complexed by cell walls and tannins (DeGabriel et al. 2008; Wallis et al. 2010)—notwithstanding current evidence that tannins have no effect on protein digestion by insect herbivores (Barbehenn and Constabel 2011). We also measured concentrations of a group of PSMs known as formylated phloroglucinol compounds (FPCs); these are non-tannin phenolic compounds apparently unique to the members of the informal subgenera *Symphyomyrtus* and *Alveolata*, the synthesis of which is probably linked to enzymes regulating monoterpene concentrations (Eschler et al. 2000; Wallis et al. 2003; Moore et al. 2004a). FPCs are known to have antifeedant activity against vertebrate herbivores of eucalypts and evidence is emerging that they can have antifeedant and antibiotic activity against chewing insects (Steinbauer and Matsuki 2004; Östrand et al. 2008; Matsuki et al. 2011). This combination of primary and secondary metabolites provides a comprehensive picture of the quality of expanded mallee eucalypt leaves to insect herbivores.

Materials and methods

Study area and trees selected

The mallee growth habit is adapted to persistence on shallow, skeletal soils in low rainfall, fire-prone habitats. "Mallee is an unmistakably Australian habitat found mainly in the southern part of the continent, on wind-blown calcareous soils. In Victoria it occurs in the northwestern corner, where there are hot dry summers associated with low and erratic rainfall, usually in winter" (J. H. Willis, circa 1980, Australia's Wildlife Heritage, p 2858). The type of mallee ecosystem in which the study was conducted is classified as "*E. socialis-E. dumosa* alliance" according to Specht (1981) which falls within "mallee with grassy/herbaceous/chenopodiaceous understory" or "shrubby mallee" according to (Haslem et al. 2010). The study was conducted using trees in Bronzewing Flora and Fauna Reserve which is approximately 15 km due south of Ouyen, Victoria (35°12′17.17″S, 142°21′13.13″E; elevation 57 m). The site chosen provided the opportunity to select 40 trees (ten trees of each species) that were within approximately 50 m of each other. Four species of mallee eucalypt belonging to the *Symphyomyrtus* coexist at the site, namely *E. gracilis* F.Muell. (Yorrell or White mallee), *E. socialis* F.Muell. ex Miq. (Red or Grey mallee), *E. dumosa* Cunn. ex Oxley (Dumosa or White mallee) and *E. incrassata* Labill. (Ridge-fruited or Yellow mallee; as *E. costata* F.Muell. and Behr ex F.Muell. in New South Wales). *Eucalyptus gracilis* forms a mallee or tree to 7 m tall; *E. socialis* subsp. *socialis* grows to 10 m tall; *E. dumosa* grows to 10 m tall; *E. incrassata* grows to 8 m tall (EUCLID 2006). Figure 1 shows the distributions of the four species. While recruitment of seedling mallees is linked to the incidence of fire, seedling survival post-fire can be highly variable (Davies and Myerscough 1991). Hence, it is difficult to comment on the relatedness of conspecific trees within the study area. We used climatic data for the Bureau of Meteorology's station at Ouyen Post Office (station number 076047) because it is the closest to the site (15.5 km due north).

Collection of leaves

Leaves from each tree were harvested on six occasions (30 July, 23 September, 26 November 2008, 29 January, 30 March and 27 May 2009) over 10 months. Expanded leaves (which are not flexible as are expanding leaves) between nodes 3 and 6 at numerous locations around the canopy were chosen for harvesting. Sufficient leaves to produce a minimum of 3 g of dried powder were harvested which, because each species' leaves differ in area and thickness, meant different numbers of leaves from each species had to be harvested. Adult leaves of *E. gracilis* [mean dry weight 123 mg (±40 SD), $n = 26$ leaves; min. 50 and max. 190 mg] are narrowly lanceolate to linear to slightly falcate; adult leaves of *E. socialis* [mean dry weight 305 mg (±92 SD), $n = 25$ leaves; min. 170 and max. 470 mg] and *E. incrassata* [mean dry weight 524 mg (±95 SD), $n = 24$ leaves; min. 320 and max. 700 mg] are lanceolate and adult leaves of *E. dumosa* [mean dry weight 568 mg (±255 SD), $n = 25$ leaves; min. 270 and max. 1,240 mg] are lanceolate to falcate (EUCLID 2006). Consequently, at each harvest the greatest number of leaves was collected from individual *E. gracilis* and the least from *E. incrassata*; roughly equal numbers were harvested from

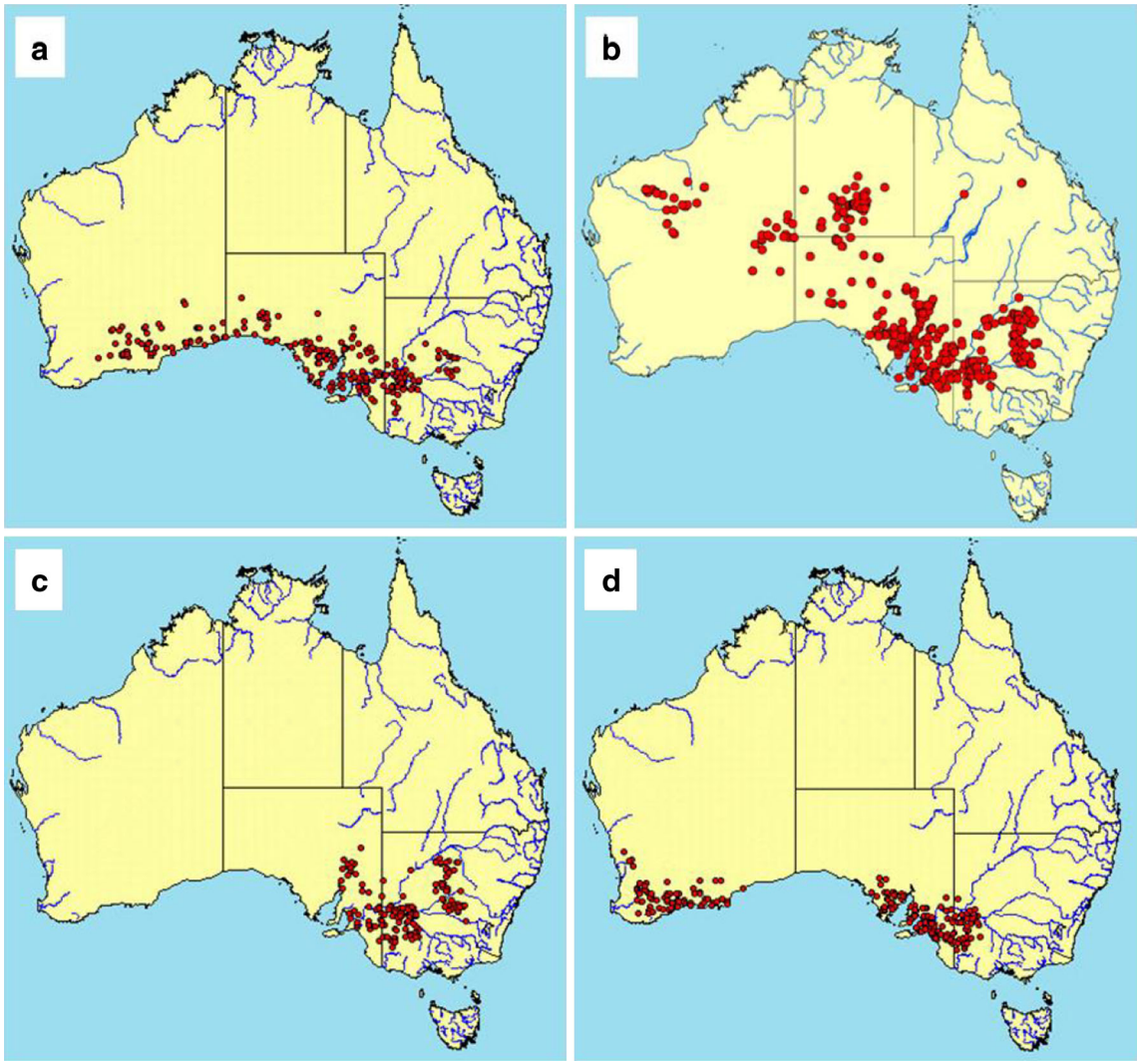

Fig. 1 Distributions of the mallee eucalypts studied. **a** *Eucalyptus gracilis*, **b** *E. socialis* subsp. *socialis*, **c** *E. dumosa* and **d** *E. incrassata*. Distributions taken from EUCLID (2006)

E. dumosa and *E. socialis*. The production of flush foliage and whether individual trees were flowering were noted at the time of harvesting. Leaves minus petioles were placed in labelled brown paper bags following collection and allowed to air dry for one month prior to grinding. Air drying preserves nitrogenous compounds and FPCs in eucalypt leaves (Ian Wallis unpublished). Air-dried leaves were ground through a 0.5 mm screen in a Retsch ZM200 centrifuge mill at 6,000 rpm and stored in 70 ml Sarstedt plastic sample vials and kept in the dark prior to analysis.

We measured concentrations (expressed on a dry matter basis determined by drying 1.00 g of air-dried material to constant mass at 50 °C) of metabolites likely to affect post-ingestion metabolism of chewing insects, including primary (N and AvailN) and secondary (total sideroxylonals and total other FPC) metabolites (Steinbauer and Matsuki 2004; Östrand et al. 2008; Matsuki et al. 2011). We also characterised terpenes and waxes linked to host location and selection behaviours of specialist and generalist insect herbivores of eucalypts (Steinbauer et al. 2004; Östrand et al. 2008; Matsuki et al. 2011). Since terpene and wax compositions were characterised for a single tree on only one occasion, we did not use these data in statistical analyses but instead calculated terpene and wax uniqueness and modified uniqueness indices to compare species (Steinbauer 2010).

Near infrared reflectance spectroscopy (N and AvailN)

We used near infrared reflectance spectroscopy (NIRS) to estimate concentrations of N and AvailN. NIRS links spectra for a subset of samples to values obtained via chemical analyses. The resulting relationships are used to predict concentrations of desired metabolites in related samples.

We collected spectra (between 408–1,092 and 1,108–2,492 nm) on samples of air-dried, ground leaves with an NIR Systems Model 6500 scanning Spectrophotometer fitted with a spinning cup module (Foss-NIR Systems, Laurel, MD, USA) housed in a room at 22–24 °C and at 55–60 % RH. Each sample was scanned twice or until the root mean square of two scans [stored as log (1/Reflectance)] was less than 2.0×10^{-4} and then the spectra were averaged. We conducted the chemical assays described later to determine foliar concentrations of N and AvailN measures on calibration sets selected to represent the population of all samples based on a Mahalanobis distance calculation of spectral variation.

We used the WinISI III software (FOSS Analytical–Laurel, MD) to manipulate the spectral data. We modelled the relationship between chemical concentrations and spectral characteristics of the leaf by modified partial least squares regression (MPLS; Shenk and Westerhaus 1991) following the standard principles described by the American Society for Testing and Materials (Anon. 1995). For each measure of interest we developed several models that differed in the wavelengths selected, the smoothing of the spectrum (i.e. the number of wavelengths averaged into one data point), the mathematical derivation of the spectrum and the type of correction for light scattering.

We selected final models based on five statistics. These were the standard error of calibration (SEC; N = 0.0201; AvailN = 0.0451), the standard error of cross-validation (SECV; N = 0.0295; AvailN = 0.0716), the coefficient of determination (R^2) between the spectra and the analytical values (the R^2 of calibration; N = 0.986; AvailN = 0.979), the proportion of variation explained by cross-validation (1-VR; N = 0.970; AvailN = 0.947) and the standardised SECV—the ratio of the standard deviation of the sample set to the SECV (N = 5.77; AvailN = 4.30). We sought models that gave both low values and agreement between values for SEC and SECV; similarly, we looked for agreement between R^2 and 1-VR but with values close to one, i.e. explaining most of the variance. Finally, models with standardised SECVs greater than three tend to give accurate predictions. Conversely, if less than two it is of little predictive value (Fontaine et al. 2002).

We used a A Leco Truspec Carbon/Nitrogen Determinator (St. Joseph, USA) calibrated with EDTA and corn flour to determine the N content of air-dried leaf using 200 ± 10 mg of sample.

We determined in vitro AvailN using the method of DeGabriel et al. (2008) as modified by Wallis et al. (2010) by incubating 800 ± 10 mg samples in ANKOM F57 filter bags (Macedon, USA) in a series of buffers and enzymes, at 35 °C with constant stirring. At the start, we incubated two bags per sample in 0.05 M Tris-Base buffer. After 24 h we washed the samples before drying them to constant mass and reweighing them (to obtain information on soluble material not used in this paper). We then digested the samples in pepsin (2 g/l 1:10,000 pepsin in 0.1 N HCl) for 24 h, followed by cellulase (6.25 g/l cellulase in acetate buffer, pH 4.8) for 48 h. Finally, after thorough washing, they were dried to a constant mass at 50 °C, weighed and the contents were analysed for N. We repeated the assay for samples in duplicate if the coefficient of variation for AvailN concentration exceeded 5 %.

High-performance liquid chromatography (sideroxylonals and other FPCs)

We extracted FPCs by sonicating single 50 mg foliage samples with a known mass (ca 4.0 g) of solvent (7 % water in acetonitrile containing 0.1 % trifluoroacetic acid and 0.3000 g/l of the internal standard 2-ethylphenol) for 5 min (Wallis and Foley 2005). The resulting mixture was filtered (0.2 um) directly into an autosampler vial and then we injected 20 µl onto a Wakosil 250 × 4 mm GL 3C18RS (SGE) column maintained at 37 °C with a flow rate of 0.75 ml/minute on a Waters Alliance Model HPLC. We eluted the FPCs under gradient conditions with 0.1 % TFA acid in water (A) and 0.1 % TFA in acetonitrile (B) as follows: 40 % A/60 % B for 5 min, linear gradient to 90 % B/10 % A at 60 min, held for 10 min and returned to starting conditions over 10 min. We measured the peak response at 275 nm. We quantified the specific FPCs present using authentic standards purified in our laboratory (or a generic equation for the unknown macrocarpals). The coefficient of variation between duplicate measurements is typically less than 3 %.

Gas chromatography-mass spectrometry (terpenes and waxes)

Terpene and wax compositions were characterised using fresh leaves from one tree of each species. Approximately 1 g (wet weight) of leaf discs (6 mm diameter) were punched from leaves and placed in 20 ml Econo glass vials each containing 10 ml of dichloromethane. Vials were capped and allowed to stand overnight before leaf discs were removed. 1 ml aliquots of original extract were evaporated before the waxes were reconstituted in 100 µl of dichloromethane. Terpenes were analysed using a Varian 3800 GC coupled to a Varian 1200 triple quadrupole mass spectrometer in electron ionisation mode. The range from *m/z* 35 to 350 was scanned four times every second. A Varian 1177 injector was used in split mode with a split ratio of 20:1 and a temperature of 220 °C, the transfer line was held at 290 °C and the ion source temperature was 220 °C. Injection volume was 1 ul. The samples were analysed using a Varian VF5-ms column

(30 m × 0.25 mm × 0.25 micron film). The carrier gas was helium at 1.2 ml/minute in constant flow mode. The column oven was programmed from 60 to 210 °C at 6 °C per minute then to 270 °C at 25 °C per minute with a final hold time of 5 min. Compounds were identified using a combination of commercial MS databases [National Institute of Standards and Technology (NIST) Chemistry WebBook; webbook.nist.gov/chemistry], specialised in-house essential oil MS databases and Kovats' retention indices (in-house databases used previously, see Steinbauer et al. 2004). Leaf waxes were analysed using the same column and instruments as above, but using a Varian 1079 Programmable Temperature Vaporizing injector. The injector conditions were an initial temperature of 30 °C, with a 0.3 min hold, then a rapid ramp to 275 °C at 200 °C per minute, with a 5 min hold at 275 °C, followed by cooling to 140 °C at 150 °C per minute. The flow rate was 3.5 ml/minute of helium in constant flow mode. Injection volume was 1 ul. The column oven was programmed from 60 °C (1 min hold) to 220 °C at 30 °C per minute, then to 310 °C at 10 °C per minute with a final hold of 8 min. The transfer line was held at 310 °C and the ion source temperature was 230 °C. The range from *m/z* 40 to 550 was scanned every 0.3 s. Leaf waxes were identified through a combination of Kovats' indices, NIST database MS, literature MS (for β-diketones) and from first principles interpretation of spectra in the case of the benzoate esters.

Statistical analyses

The metabolite data were transformed [$\log_{10}$ (concentration + 1)] before analyses. Multivariate abundance analysis [package 'mvabund' (Wang et al. 2013)] in the statistical computing environment R (R Core Team 2014) was used to examine the variability in composition of metabolite concentrations in leaves between species, months, trees and the interaction between species × month; univariate test statistics were used to identify which variables had the greatest influence on metabolites. Linear mixed effects models [package lme4 (Bates et al. 2014), R statistical computing environment (R Core Team 2014)] were used to investigate the changes in concentrations of metabolites in consecutive harvests. For each species, we built a model for each of the four metabolites—N, AvailN, total sideroxylonals and total FPCs. We specified month as the fixed effect. Since we made repeated measures of metabolite concentrations on each tree, tree was included as a random factor in all models. Statistical significance of variables was calculated using the package lmerTest (Kuznetsova et al. 2014). Significance of month was based on *P* values from a Type III *F*-tests employing the Satterthwaite approximation for degrees of freedom. We conducted post hoc tests, corrected for family-wise error using the Tukey method, to test for differences in metabolite concentrations between harvest periods [conducted using package "lsmeans" (Lenth and Hervé 2014)].

We used linear mixed effects models also to determine the influence of key phenological and climatic variables on the quality of leaves. We selected N and total sideroxylonals as the metabolites for analysis. These two variables were not highly correlated (Spearman's rho <0.1 for three species and <0.5 for one), indicating that different variables affect their concentrations (see Supplementary material 1 for correlations between metabolites). Models included four explanatory variables: flushing, flowering, mean maximum temperature (at time minus 2 months) and total rainfall (at time minus 2 months). Flushing and flowering were each represented as categorical variables with two levels: flushing/flowering or not flushing/flowering. A lag time of 2 months was chosen to relate temperature and rainfall variables to metabolite concentrations because "flushing" is linked to temperature (Specht 1981) and was initiated and ceased between successive leaf harvests. We did not know the relatedness of trees and as a consequence could take no account of genotypic variation in tree flushing. Explanatory variables were mostly correlated at Spearman's rho <0.7, indicating that models were unlikely to be detrimentally affected by co-linearity of variables (Dormann et al. 2013); correlation coefficients were all >0.7 in the case of *E. incrassata* (see Supplementary material 2). Tree was included as a random factor in all models. Statistical significance of variables was again calculated using Type III *F*-tests employing the Satterthwaite approximation for degrees of freedom.

Results

Formylated phloroglucinol compounds in the four eucalypt species

The four eucalypts produced similar FPCs, namely sideroxylonals A, C and very low concentrations of B and macrocarpals A, B, G and eucalyptone and lower concentrations of the unknown macrocarpals that elute at 35 and 47 min (Moore et al. 2004a). We also detected grandinol in *E. gracilis*. Experience suggests that the different FPCs have similar effects on herbivores and so we combined them as total sideroxylonals and total other FPCs.

Foliar quality of species and individual trees through time

Multivariate abundance analysis revealed significant differences in the composition of metabolites between

eucalypt species ($df = 3$, Likelihood Ratio (LR) = 619.9, $P < 0.001$), month ($df = 5$, LR = 57.3, $P = 0.003$) and among individual trees ($df = 9$, LR = 201.3, $P < 0.001$) as well as a significant species × month interaction ($df = 15$, LR = 160.6, $P < 0.001$). For N, AvailN and total sideroxylonals, the ordering of LRs from the univariate tests was: species (highest), tree, species × month interaction and month (lowest) (Table 1). In the case of total other FPCs, the ordering of LRs from the univariate tests was: tree (highest), species × month interaction, species and month (lowest).

Table 1 Univariate results from multivariate abundance analysis

	Source	Likelihood ratio	*P*
N	Species	155.7	0.001
	Month	13.0	0.056
	Tree	36.3	0.002
	Species × month	24.5	NS
AvailN	Species	367.6	0.001
	Month	4.5	NS
	Tree	42.1	0.001
	Species × month	25.8	NS
Total sideroxylonals	Species	30.8	0.001
	Month	19.1	0.006
	Tree	26.8	0.001
	Species × month	24.1	NS
Total other FPCs	Species	65.9	0.001
	Month	20.8	0.006
	Tree	96.2	0.001
	Species × month	86.2	0.001

Values >0.1 are not shown (*NS* non-significant)

Eucalyptus gracilis

This species produced flush foliage between January and March 2009 (Austral summer/early autumn) but flowered between July and September 2008 (Austral winter/early spring; Table 2). The mean N content of leaves of *E. gracilis* varied from 1.25 % (November 2008) to 1.40 % (January 2009) (Fig. 2a). Across all harvests, *E. gracilis* had the lowest AvailN content of the four species (Fig. 2b). Significant changes in N occurred between the September–November (decrease) and November–January (increase) harvests (Table 3). Significant changes in AvailN occurred between the July–September (increase) harvests (Table 3). No post hoc tests of total sideroxylonals concentrations were statistically significant (Table 3; Fig. 3a). Significant changes in total other FPCs occurred between the January and March (decrease) harvests (Table 3; Fig. 3b). Nitrogen was affected by all four explanatory variables, in particular by temperature and rainfall (Table 4). Total sideroxylonals concentrations were only affected by flowering (Table 4).

Eucalyptus socialis

Most *E. socialis* trees (9/10) produced flush foliage between January and March 2009 (Table 2). One tree flowered in November 2008 and three flowered in January 2009 (Austral spring/summer). The mean N content of leaves of *E. socialis* varied from 1.28 % (November 2008) to 1.42 % (March 2009) (Fig. 2a). Across all harvests, *E. socialis* had the highest AvailN content of the four species (Fig. 2b). Significant changes in N occurred between the November–January (increase) harvests (Table 3). No post hoc tests of AvailN were statistically significant (Table 3).

Table 2 Climatic conditions (for Ouyen Post Office) relative to flushing and flowering phenology of four mallee eucalypts (proportions indicate number of trees out of ten flushing and flowering on a given date when leaves were harvested)

	May (2008)	July (2008)	September (2008)	November (2008)	January (2009)	March (2009)	May (2009)
Climatic data							
Total rainfall	20.0 mm	35.5 mm	10.6 mm	46.6 mm	0 mm	6.9 mm	19.8 mm
Mean max. temp.	19.2 °C	14.6 °C	10.6 °C	26.4 °C	35.5 °C	28.6 °C	18.8 °C
Tree responses							
Flushing							
E. gracilis	–	0/10	0/10	0/10	10/10	10/10	0/10
E. socialis	–	0/10	0/10	0/10	9/10	9/10	0/10
E. dumosa	–	0/10	0/10	0/10	10/10	10/10	0/10
E. incrassata	–	0/10	0/10	0/10	10/10	10/10	0/10
Flowering							
E. gracilis	–	10/10	9/10	0/10	0/10	0/10	0/10
E. socialis	–	0/10	0/10	1/10	3/10	0/10	0/10
E. dumosa	–	0/10	0/10	0/10	1/10	9/10	0/10
E. incrassata	–	0/10	0/10	10/10	0/10	0/10	0/10

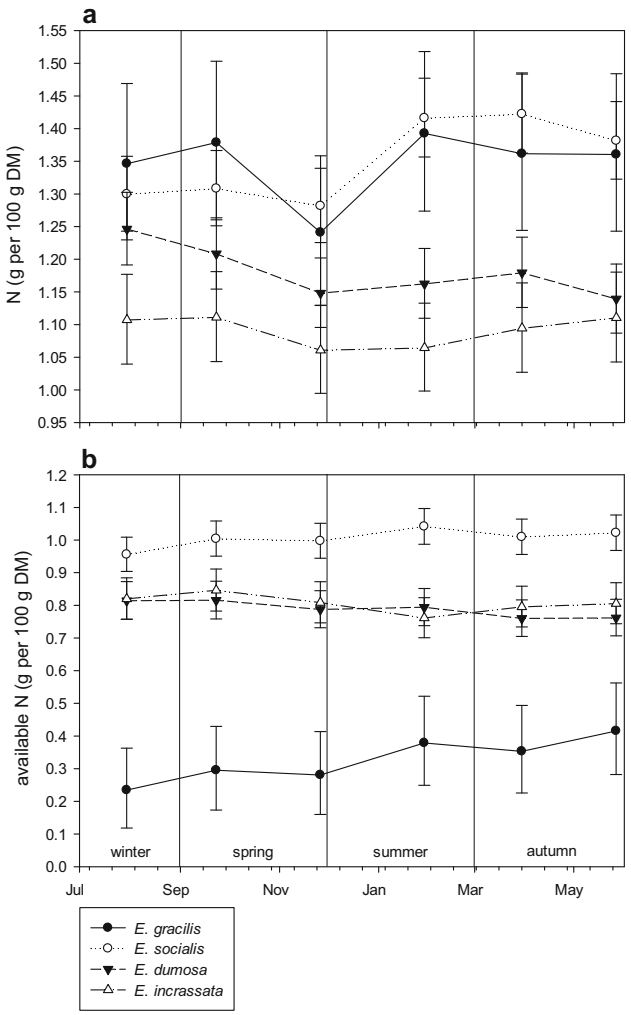

Fig. 2 Temporal variability between July 2008 and May 2009 in foliar nitrogen of four mallee eucalypt species. **a** N and **b** AvailN. Data are geometric means ± 95 % confidence intervals; *error bars* are asymmetric because data are log normal

Significant changes in total sideroxylonals occurred between the July–September (decrease), September–November (increase) and January–March (decrease) harvests (Table 3; Fig. 3a). Significant changes in total other FPCs also occurred between the July–September (decrease), September–November (increase) and January–March (decrease) harvests (Table 3; Fig. 3b). Nitrogen and total sideroxylonals were positively and negatively affected by temperature, respectively (Table 4).

Eucalyptus dumosa

This species produced flush foliage in January and March and most (9/10) trees flowered in March; one tree began flowering in January (Table 2). The mean N content of leaves of *E. dumosa* varied from 1.14 % (May 2009) to 1.25 % (July 2008) (Fig. 2a). Concentrations of AvailN in *E. dumosa* leaves were very similar to those in *E. incrassata* (Fig. 2b). No post hoc tests of N or AvailN were statistically significant (Table 3). Significant changes in total sideroxylonals occurred between the November–January (decrease) harvests (Table 3; Fig. 3a). Significant changes in total other FPCs occurred between the November–January (increase) and January–March (decrease) harvests (Table 3; Fig. 3b). Nitrogen was positively affected by rainfall (Table 4). Total sideroxylonals were negatively affected by flushing and temperature (Table 4).

Eucalyptus incrassata

This species produced flush foliage in January and March but flowered in November (Austral spring; Table 2). Across all but the March–May harvests, *E. incrassata* had the lowest N content of the four species; concentrations varied from 1.06 % (November 2008) to 1.11 % (September 2008) (Fig. 2a). Concentrations of AvailN in *E. incrassata* leaves were very similar to those in *E. dumosa* (Fig. 2b). No post hoc tests of N or AvailN were statistically significant (Table 3). Significant changes in total sideroxylonals occurred between the November–January (increase) and January–March (decrease) harvests (Table 3; Fig. 3a). Significant changes in total other FPCs also occurred between the November–January (decrease) and January–March (increase) harvests (Table 3; Fig. 3b). Nitrogen was not affected by any explanatory variable but concentrations of total sideroxylonals were positively affected by rainfall (Table 4).

Comparisons of mallee terpenes and waxes

The four mallees have similar terpene and wax compositions. Qualitatively (*U*), *E. dumosa* has the most unique terpenes while *E. gracilis* has the least but quantitative (*Um*) differences are small (Table 5). In terms of waxes, *E. socialis* has the most unique compounds and *E. incrassata* the least but, again, qualitative differences are small.

Discussion

The observation that foliar quality declines as leaves age is common in plant ecology. Expanding leaves are high quality modules because they are sinks for nitrogenous metabolites and may be relatively poorly defended by low concentrations of secondary metabolites and/or the weak physical integrity of their cell walls. By the time a leaf is fully expanded, there has been a reversal in these characteristics resulting in a decline in their quality for herbivores (e.g., Edwards and Wanjura 1990; Steinbauer et al. 1998).

Table 3 *P* values for post hoc tests on pairwise contrasts of consecutive harvest periods (adjusted for family-wise error using Tukey method) from linear mixed effect models

	Jul *versus* Sep	Sep *versus* Nov	Nov *versus* Jan	Jan *versus* Mar	Mar *versus* May
E. gracilis					
N	NS	<0.001	<0.001	n.s.	NS
AvailN	0.046	NS	<0.001	n.s.	0.071
Total sideroxylonals	NS	NS	NS	NS	NS
Total other FPCs	NS	NS	NS	0.011	NS
E. socialis					
N	NS	NS	<0.001	NS	NS
AvailN	0.065	NS	NS	NS	NS
Total sideroxylonals	<0.001	0.021	NS	0.002	NS
Total other FPCs	<0.001	0.022	NS	0.019	NS
E. dumosa					
N	n.s.	NS	NS	NS	NS
AvailN	NS	NS	NS	NS	NS
Total sideroxylonals	NS	NS	<0.001	NS	NS
Total other FPCs	NS	NS	<0.001	<0.001	NS
E. incrassata					
N	NS	NS	NS	NS	NS
AvailN	NS	NS	NS	NS	NS
Total sideroxylonals	NS	NS	0.025	0.003	NS
Total other FPCs	NS	NS	<0.001	0.009	NS

Values >0.1 are not shown (*NS* non-significant). Values between 0.05 and 0.1 are included to highlight non-significant trends

Taken in conjunction with the findings of Macauley and Fox (1980), who showed that concentrations of total phenols and condensed tannins increased during winter, our findings show that although all expanded leaves attain relatively constant N content, they can differ substantially in their content of antifeedant and/or antibiotic PSMs and thus may be of highly variable foliar quality.

We found concentrations of N to be remarkably stable during our study. Mature eucalypt leaves are sources of N for expanding leaves. Consequently, we expected N and AvailN to decline when trees were flushing and, possibly also, when flowering. The phenology of individual trees only affected concentrations of N in leaves of *E. gracilis*; as expected flushing had a negative effect on concentrations of N but flowering had a positive effect on N (Table 4). In a study of *E. globulus*, a species endemic to mesic, coastal habitats in Tasmania and Victoria, Wendler et al. (1995) found that the magnitude of declines in foliar N of mature leaves during the production of new leaves depended on nutrient status; leaves of seedlings with access to high soil N lost less N (10 %) than did leaves of seedlings with access to low soil N (44 %). The trees we studied were growing on a nutrient poor soil type but the species and leaf type (adult morphology) are presumably better adapted to conservation of N than is *E. globulus* with its fast expanding juvenile leaves. Consequently, it might be unrealistic to expect changes in foliar N of the magnitude reported by Wendler et al. (1995). In addition, the quantity of flush foliage produced in 2008–2009 may have been reduced by the drought so the drain on foliar N may have been less than in non-drought seasons. Further research to address these possibilities would help resolve the relative importance of the effects of temperature and rainfall on N, which our analyses indicate is important for *E. gracilis* (temperature and rainfall, positive effects), *E. socialis* (temperature, positive effect) and *E. dumosa* (rainfall, positive effect) (Table 4).

In contrast to the relatively small changes in N concentrations with season, concentrations of PSMs showed pronounced fluctuations, being affected by both tree phenology and climate. While it is recognised that the composition of PSMs in leaves and other tissues (e.g., bark) of trees fluctuates between seasons, responses are often species specific and have been most frequently attributed to changes in plant growth (Lindroth et al. 1987; Salminen et al. 2004; Förster et al. 2008; Virjamo and Julkunen-Tiitto 2014). Recently, researchers have highlighted that climatic variables can also play an important role. For example, leaves produced by frost-defoliated aspen had two- to three-fold higher concentrations of phenolic glycosides and condensed tannins than before the defoliation occurred and concentrations remained high into

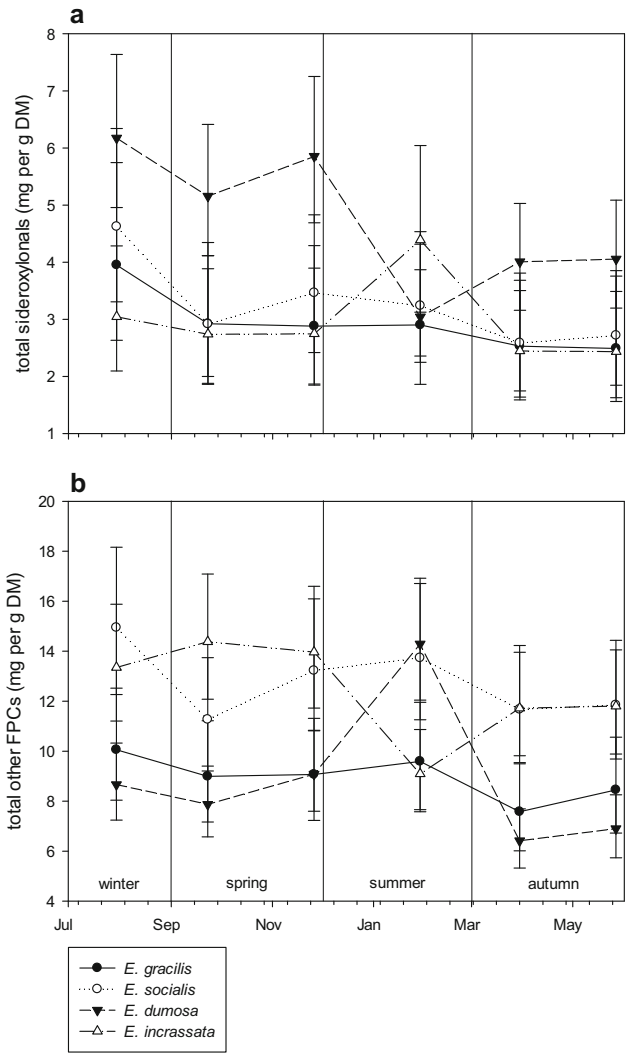

Fig. 3 Temporal variability between July 2008 and May 2009 in foliar plant secondary metabolites of four mallee eucalypt species. **a** Total sideroxylonals and **b** total FPCs. Data are geometric means ± 95 % confidence intervals; *error bars* are asymmetric because data are log normal

the subsequent growing season (St Clair et al. 2009). Our flushing/flowering and temperature/rainfall data did not exhibit strong co-linearity indicating that the effects of these different mechanisms may act independently.

Fluctuations in sideroxylonals and other FPCs with season are to be expected because the concentrations of terpenes may fluctuate seasonally and there is a close affinity in the biosynthetic pathways of the two groups of compounds (Eschler et al. 2000; Wallis et al. 2003; Moore et al. 2004a). Brooker et al. (1988) found that the terpene composition of eucalypt leaves exhibited seasonal fluctuations but did not measure sideroxylonals and other FPCs. Our results show that tree phenology is associated with fluctuations in PSMs in leaves of *E. gracilis* (sideroxylonals and flowering, positive effect) and *E. dumosa* (sideroxylonals and flushing, negative effect) (Table 4). We hypothesised that flowering could influence the expression of eucalypt PSMs because, unlike perennial species with non-woody reproductive structures, the hypanthium and operculum of developing inflorescences are probably of comparable functional significance to expanding leaves, i.e., they are sinks for photosynthate (see Clark and Dallwitz 1975) and are locations where terpenes are synthesised. In some perennial species, changes in the expression of PSMs with changes in vegetative phenology (e.g., bud burst) have been attributed to the disappearance of precursors necessary for their biosynthesis (Virjamo and Julkunen-Tiitto 2014). Species specific effects of tree phenology on sideroxylonals and other FPCs presumably reflect demands on precursors needed for PSM synthesis in expanding leaves and/or young inflorescences.

Yen (1989) presented unpublished data on leaf area losses of 1,000 leaves of each of three mallee eucalypts in the Big Desert, Victoria. Losses for adult leaves were 20.81 % for *E. dumosa*, 18.13 % for *E. incrassata* and 10.37 % for *E. foecunda* Schauer measured in March 1980. Yen (pp 295) suggested that such differences were not very pronounced, despite marked differences in leaf shape. While, Yen's (1989) findings indicate comparable levels of leaf damage at the time of measurement, his data do not reveal the timing of the herbivory. Our findings confirm that although expanded leaves are broadly comparable in primary and secondary metabolites (latter including sideroxylonals, other FPCs, terpenes and waxes), there are significant fluctuations in PSMs such that expanded leaves are not all of equal foliar quality all the time and consequently that damage to leaves is likely to occur inconsistently during the times of the year when herbivorous insects are most active rather than at the same rate each year.

There is clearly a need for more research to resolve the mechanisms which link climate and PSMs and also subsequent effects on insect survival and performance. However, the implications of our findings are large in light of anthropogenic climate change which is expected to result in greater extremes of temperature and rainfall in many regions around the world. The impact of elevated CO_2 on the nutritional quality of eucalypt leaves has been studied in two species (Lawler et al. 1997; Gleadow et al. 1998; Murray et al. 2013). Nevertheless, rising atmospheric CO_2 concentrations are but one aspect of climate change and the impacts of extremes of temperature and rainfall, combined with changes in tree phenology and leaf longevity, on the nutritional quality of eucalypt leaves remain to be studied. Moore et al. (2004b) found that 1,8-cineole (a monoterpene) and sideroxylonal concentrations in leaves of *Eucalyptus microcorys* were higher at colder sites which contrasts with increased synthesis of phenolic compounds

Table 4 Summary of estimated effect sizes for phenological and climatic variables associated with changes in foliar quality of four mallee eucalypts

Response variables	Explanatory variables	Coefficient	SE	*P*
E. gracilis				
N	Flushing	−0.0080	0.0040	0.054
	Flowering	0.0055	0.0029	0.058
	Mean maximum temperature T-2	0.0013	0.0002	<0.001
	Total rainfall T-2	0.0004	0.00008	<0.001
Total sideroxylonals	Flowering	0.0767	0.0355	0.036
E. socialis				
N	Mean maximum temperature T-2	0.0011	0.0002	<0.001
Total sideroxylonals	Mean maximum temperature T-2	−0.0029	0.0017	0.088
E. dumosa				
N	Total rainfall T-2	0.0003	0.0002	0.072
Total sideroxylonals	Flushing	−0.1030	0.0580	0.082
	Mean maximum temperature T-2	−0.0068	0.0026	0.011
E. incrassata				
N	–	–	–	–
Total sideroxylonals	Total rainfall T-2	0.0047	0.0019	0.016

Table 5 Terpene and wax relatedness of four mallee eucalypts

	D	*U*	*Um*
Terpenes			
E. gracilis	14	1.024	0.058
E. socialis	16	1.104	0.054
E. dumosa	19	1.421	0.045
E. incrassata	18	1.389	0.051
Waxes			
E. gracilis	24	1.261	0.043
E. socialis	26	1.423	0.060
E. dumosa	19	1.404	0.055
E. incrassata	17	1.078	0.056

D number of compounds identified, *U* uniqueness, *Um* modified uniqueness

Higher values of *U* indicate species has a greater number of unique compounds compared to the other species; *Um* takes account of the relative contribution of each compound to the overall terpene or wax composition. Terpene and wax compositions are summarised in Supplementary materials 3 and 4, respectively

with high-temperature stress (Wahid et al. 2007). McKiernan et al. (2014) found that decreased water availability had little influence on terpene, sideroxylonal A and macrocarpal G concentrations. Based on these findings, we suggest that particular focus should be given to the impact of temperature on eucalypt leaf quality and subsequent effects on insect herbivores. We suggest that explaining fluctuations in leaf quality arising in response to extreme environmental conditions will help explain temporally and spatially sporadic insect communities on mallee eucalypts.

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