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## Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials

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**Abstract** Traditional approaches to the question of the effects of plant secondary metabolites on the feeding choices of folivores of *Eucalyptus* have focused on the tree species level, although numerous field studies of foraging behaviour have identified selection at the level of the individual trees. Attempts to relate these decisions to deterrence resulting from secondary leaf chemistry have been inconclusive because assays used have focused on broad groups of compounds such as “total” phenolics. In this study we have conducted no-choice feeding trials with two arboreal mammalian folivores, the common ringtail possum (*Pseudocheirus peregrinus*) and the koala (*Phascolarctos cinereus*), to measure deterrence of individual trees of two species of *Eucalyptus*, *E. ovata* and *E. viminalis*. Average daily intakes of *E. ovata* foliage by common ringtail possums ranged from 2.5 to 50 g kg<sup>-0.75</sup> body mass. Koala intakes of foliage from the same individual trees ranged from 22.4 to 36.3 g kg<sup>-0.75</sup> body mass. When fed foliage from different individual *E. viminalis* trees, common ringtail possums ate between 1.26 and 6.28 g kg<sup>-0.75</sup> body mass while koalas ate from 14.3 to 45.9 g kg<sup>-0.75</sup> body mass. Correlative analyses showed no relationships between feeding and several measures of nutritional quality, nor

with total phenolics or condensed tannins. They did, however, identify two groups of plant secondary metabolites that may cause deterrence: terpenes, and a defined group of phenolic compounds, the diformylphloroglucinols (DFPs). Further bioassay experiments with common ringtail possums showed that only the DFPs could cause the effects seen with the foliage experiments at concentrations similar to those found in the leaves. We argue that, when in sufficiently high concentrations, DFPs determine the level of food intake by these animals irrespective of other questions of nutritional quality of the leaves.

**Key words** Intraspecific variation · *Eucalyptus* · *Pseudocheirus peregrinus* · *Phascolarctos cinereus* · Deterrence

### Introduction

For arboreal folivores, the quantity of potential food available is seldom limiting, yet it is widely recognised that factors such as low protein content and the presence of plant secondary metabolites (PSMs) can potentially limit the food choices of herbivores (Cork and Foley 1991; Ganzhorn 1992). Although the effects of PSMs on feeding have been widely investigated, studies of mammalian arboreal folivory have rarely examined food choices at a scale finer than the plant species. Many studies have shown that mammalian herbivores discriminate between individuals within a taxonomic species (e.g. Glander 1978; Milton 1978; Hindell et al. 1985; Hindell and Lee 1987; Pahl 1987; Pahl and Hume 1991; Snyder 1992; Ernest 1994) as do herbivorous birds (Guglielmo et al. 1996) yet the plethora of studies that have attempted to relate food choices by arboreal mammalian folivores to leaf chemistry ignore this finer scale (e.g. Braithwaite et al. 1983; Oates et al. 1990; Ganzhorn 1992; Cork and Catling 1996).

There is then an important level of variation in the feeding ecology of arboreal folivores on which we have

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as yet very little information. This prevents an understanding of the foraging decisions made by individual animals and this constraint cannot be addressed by the larger, landscape scale studies which to date have been unable to address questions of intraspecific variations in leaf chemistry (e.g. Braithwaite et al. 1983; Oates et al. 1990; Ganzhorn 1992). The difficulty lies in deciding what aspects of the primary and secondary chemistry should be measured. Subtle variations in chemical structure of reputed toxins/deterrents can have major effects on the palatability of plants for herbivores (Clausen et al. 1986) but these variations cannot be captured by crude measures such as total phenolics or phenol:protein ratios (e.g. Cork and Catling 1996) and in recognition of this problem, measurement of deterrent compounds is occasionally omitted altogether (e.g. Yeager et al. 1997).

Australian temperate forests offer excellent opportunities to investigate and resolve these issues because a single tree genus, *Eucalyptus*, dominates more than 90% of forests and woodlands (Landsberg and Cork 1996). Thus there should be sufficient similarities in chemical constituents that intensive studies of a few species can be made more widely applicable. *Eucalyptus* foliage is eaten by several folivorous marsupials that vary in their abilities to meet their needs solely from foliage. However, a number of studies of food choice in these animals have been unable to show clear relationships between levels of feeding intensity and the content of either primary or secondary metabolites (Cork and Pahl 1984; Cork and Sanson 1990; Zoidis and Markowitz 1992; Hume and Esson 1993).

With the lack of firm evidence of the role of plant secondary metabolites (PSMs) in food choice of mammals feeding on eucalypts, it is apparent that new approaches must be adopted to investigate this issue. In the larger project of which this study is a part, we have adopted a varied approach which uses both correlative and bioassay experiments. An initial bioassay-guided fractionation led us in a different direction to that of previous studies (Pass et al. 1998). We have identified a more specific group of phenolic compounds, the diformylphloroglucinols (referred to hereafter as DFPS), which show uniformly high antifedant activity to arboreal marsupials.

In this paper we focus on demonstrating the enormous degree of intraspecific variation in the palatability of *Eucalyptus* foliage for common ringtail possums (*Pseudocheirus peregrinus*). We then show that this variation is dependent not only on the chemistry of the foliage but also on the physiology of the consumer by comparing intake of the same individual trees by koalas (*Phascolarctos cinereus*) and common ringtail possums. We go on to show by correlative experiments what chemical groups control this variation and demonstrate by selected bioassay that a chemically defined compound can recreate the patterns observed in natural diets.

## Methods

### Animals

This research was approved by the Animal Experimentation Ethics Committees of James Cook and Monash Universities and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Common ringtail possums (*P. peregrinus*) were caught by hand in woodland dominated by *Leptospermum laevigatum* near Melbourne and maintained initially on a mixture of foliage from *E. ovata* and *L. laevigatum*. The proportion of *E. ovata* was gradually increased until the animals were eating *E. ovata* foliage solely. The common ringtails were housed individually in metabolism cages 0.6 m wide × 0.6 m high and 0.75 m long in a room with a 12:12 h light:dark cycle at temperatures ranging between 16°C at night and 20°C during the day. Daytime light was provided by fluorescent lighting and night light by a 40-W red incandescent bulb. Common ringtail possums are strictly nocturnal and so food was offered at dusk and removed after sunrise.

Koalas (*Ph. cinereus*) were caught by hand in woodland dominated by *E. viminalis* and *E. ovata* on French Island near Melbourne. They were housed in outside enclosures measuring 3 m wide × 3 m long × 2.5 m high and subject to natural light:dark cycles (approximately 12:12 h). Temperatures ranged between a low of 13°C at night and a high of 26°C during the day. They were maintained on a mixture of *E. ovata* and *E. viminalis* foliage.

### Experimental design

All experiments were carried out using Latin square designs, in which each animal was fed each treatment once over the course of the experiment and each treatment was applied to only one animal each night. This design allowed the measurement of, and correction for, carryover effects between treatments. Comparisons between trees within each Latin square experiment were analysed by ANOVA, as described in Ratkowsky et al. (1993). A term for carryover effects of the treatments was initially included, but was found to be non-significant in all cases and omitted from the final analysis.

### Detergency of foliage of individual trees to feeding by ringtail possums

Our initial experiment examined the range of individual variation in detergency in *E. ovata*. A number of individual trees were chosen on the basis of observational and anecdotal data on the herbivory levels in the field and on preliminary screening by offering to captive ringtail possums. Individual trees were chosen to represent a broad spectrum of detergency from highly preferred to strongly deterrent. All trees were mature, ranging between 6 m and 15 m and had foliage of apparently similar high quality, in terms of abundance of young growth, and greenness, softness and lack of damage to leaves.

Detergency was measured as the amount of foliage that each individual animal would voluntarily eat in a no-choice situation for each feeding period. That is, when leaf from an individual tree was offered there was no choice but to eat the leaf or to go hungry. Dry matter intakes were calculated in all cases using appropriate controls for evaporation. We chose in these experiments to adopt a no-choice protocol as we are interested in the limits to the animals' ability to ingest a diet. Adoption of choice tests would likely exaggerate the level of detergency, especially if a highly palatable alternative was offered.

Eight common ringtail possums were offered leaf from eight different individual *E. ovata* using trees collected from a variety of sites. Animals were offered ad libitum quantities of leaf from a single tree each night, plus 27.5 g of 10% (w/v) aqueous glucose, so that those animals that fed little had some energy intake to meet

maintenance requirements and to reduce carryover effects between treatments. Treatments were applied over 8 consecutive nights in June 1996.

#### Comparison of ability of koalas and ringtail possums to ingest foliage of deterrent trees

In these experiments we measured the intake by both common ringtail possums and koalas of leaves of the same individual trees of two *Eucalyptus* species: *E. ovata* and *E. viminalis*. Again all trees were mature and had foliage of similarly high apparent quality.

#### Ringtail possums

Six common ringtail possums were fed leaf from the six different individual trees of *E. ovata* and *E. viminalis* in separate experiments with a protocol slightly modified from that described above. Animals were offered ad libitum quantities of leaf from a single tree each night and this was then removed at 0500 hours and replaced with leaf from a very highly palatable form of *E. ovata* which remained in the cage until approximately 0800 hours (after sunrise). Treatment days were separated by a single day on which only the palatable *E. ovata* was offered. This was considered to be the best method of ensuring the welfare of the animals and of reducing carryover effects between treatments. These experiments were conducted in November 1996.

#### Koalas

Foliage from five different individual trees of the six from each of the two species used in the ringtail experiment were offered to koalas in separate experiments. As only five koalas were available one tree of each species was omitted to balance the Latin square design.

On each treatment night each koala was offered ad libitum quantities of leaf from a separate individual tree. As koalas are mainly nocturnal feeders treatment periods began at approximately 1730 hours and finished at approximately 0700 hours, at which time the treatment leaves were removed and a highly palatable form, of the same species as used in that experiment, offered. This was then removed at approximately 1230 hours and the next treatment begun at 1730 hours that same day.

#### Analysis of foliage

On each day of the experiment, a control to account for evaporative loss from each bunch of foliage was kept in similar conditions to that fed to the animals. On the morning following each treatment, these controls were sampled in a manner considered to represent the sampling of those leaves by the animals (e.g. samples of the food eaten by koalas included petioles and some thin twigs, whereas for ringtail possums, the leaf samples omitted petioles and included mostly young leaves).

Three samples were taken on each day: one for conversion of wet matter intake to dry matter intake, one for terpene analysis and one for other chemical analyses. The samples taken for terpene and general chemical analyses were bulked and a subsample taken for each analysis. The sample for general chemical analysis was subsampled again for DFP analysis and the remainder freeze-dried and ground to pass a 1 mm sieve.

Dietary fiber was extracted in neutral detergent solution (Van Soest et al. 1991) omitting sodium sulphite. Subsamples of the residue were then hydrolysed in two ways: (1) 12 M sulphuric acid for 1 h at 25°C then 1 M sulphuric acid for 2 h in a boiling water bath (conditions were found optimal for liberating monosaccharides from cellulose); (2) hydrolysis in the 1 M sulphuric acid only, to liberate non-cellulosic monosaccharides (Englyst and Cummings 1988; Hoebler et al. 1989). The monosaccharides were determined

colorimetrically using dinitrosalicylic acid (Englyst and Cummings 1989). Lignin was determined as the washed and dried residue remaining after acid hydrolysis.

Cyanogenic glycosides were measured by crushing leaves with a small amount of  $\beta$ -glucosidase (Sigma) in sealed apparatus containing a separate well containing 1 M NaOH into which the cyanide dissolves. The cyanide content of the NaOH solution is then assayed colorimetrically (Lambert et al. 1975). Total phenolics and condensed tannins were extracted from dry, ground foliage samples in 50% acetone:water and assayed by the Folin-Ciocalteu method. Results are expressed in milligram quebracho equivalents (Cork and Krockenberger 1991). Terpenes were extracted in sealed vials of hexane at 60°C and then analysed by gas-liquid chromatography to identify and quantify individual terpenes (Edwards et al. 1993). The concentration of total nitrogen was measured by semi-micro Kjeldahl digestion using selenium as a catalyst.

Diformylphloroglucinols (DFPs) were extracted overnight using a Soxhlet apparatus with a 20:80 (v/v) mixture of acetone and light petroleum spirit (40–60°C boiling point). Total DFP content was estimated by comparing the integration of the total aldehyde proton peak to the peak area for an internal standard (*m*-dinitrobenzene). This gives an indirect measure of the number of moles of DFPs as the majority of identified DFPs in these species contain two aldehyde groups per molecule (D. Pass, B. Eschler and W. Foley, unpublished work). Other aldehydes in the extracts contribute only a small proportion of the total aldehyde proton peak (B. Eschler, unpublished work). Hence these data are expressed as moles DFP per gram dry leaf rather than as a percentage of weight due to uncertainty about the molecular weights.

#### Correlative analysis of leaf experiments

Relationships between mean food intake for each tree and leaf characteristics were investigated using a combination of exploratory graphical analysis and linear regression. Separate analyses were carried out for each *Eucalyptus* species in each experiment, due to differences in protocol between the *E. ovata* experiments with common ringtail possums (see above) and because of likely qualitative differences in DFP composition between species that could not be investigated with the assay in its current form. The relationships between leaf chemical components and food intake were analysed separately for each component using graphical analysis and pairwise correlations, rather than as more complex multiple regression analyses, because of insufficient degrees of freedom.

#### Bioassay of compounds potentially deterrent to ringtail possum feeding

Two bioassay experiments, in which isolated compounds were added to a basal diet, were conducted to test whether the relationships identified in the preceding section were of a causal nature. The basal diet consisted of (% wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% lucerne hay (ground to pass a 2 mm screen), 5.5% ground rice hulls, 4.7% ground Weetbix (a wheat-based breakfast cereal) and 1.6% acid casein. All animals maintained body mass on this basal diet. This diet contained 32% dry matter and this dry matter contained 97% organic matter, 1.9% N, 6.0% cellulose, 6.4% hemicellulose and 9.9% acid lignin.

Experiments were conducted using a similar protocol to the second round of leaf experiments with ringtail possums. The basic design was again a 6 × 6 Latin square with treatment days alternating with non-treatment days on which only the basal diet was offered. On treatment days animals were offered the basal diet at 1800 hours to which was added one of six concentrations of the suspected deterrent compound and this was removed and replaced by untreated basal diet at 0500 hours.

To determine the dry matter intake of the animals the dry matter content of the diet offered was determined by subsampling the food offered and the dry weight of refusals determined by drying for 24 h at 80°C.

## Cineole

Cineole is the major component of the terpenes of a variety of *Eucalyptus* species including *E. viminalis*, though not *E. ovata* (Boland et al. 1991; I.R. Lawler, unpublished work) and has been investigated as a deterrent in previous studies of mammalian feeding (e.g. Krockenberger 1988; Reichardt et al. 1990). Thus it was chosen to investigate whether terpenes could cause the effects seen in the leaves from previous experiments. The concentrations of cineole, as a percentage of the dry weight of the diet were: 0, 1.67, 2.67, 4.0, 6.67 and 11.67%.

## Macrocarpal G

Through the process of bioassay-guided fractionation described in the introduction (Pass et al. 1998) macrocarpals, a sub-group of diformylphloroglucinols (see discussion), were identified as the likely cause of unpalatability in *E. ovata*. Macrocarpal G is the predominant macrocarpal found in the resistant *E. ovata* used in the experiments of Pass et al. (1998) and was extracted and purified from those trees for addition to the artificial diet in the following concentrations, as moles  $\times 10^{-5}$  g $^{-1}$  dry weight: 0, 0.73, 1.47, 2.94, 4.41 and 5.87.

Macrocarpal G was isolated by fractionation of a crude phenolic extract of *E. ovata* foliage by repeated vacuum-assisted silica gel chromatography (90:10 (v/v) dichloromethane:methanol) which resulted in a polar and non-polar fraction. The dominant component of the polar mixture had previously been identified as macrocarpal G (Pass et al. 1998) and was isolated by chromatography on Sephadex LH-20 and reverse phase HPLC (97:2.5:0.5 (v/v/v) acetonitrile:dichloromethane:acetic acid). Comparison of the  $^{13}\text{C}$  NMR data (in d4-methanol) with published values (Yamakoshi et al. 1992) confirmed the earlier identification.

## Results

### Deterreny of individual trees to feeding by ringtail possums

Our initial experiment clearly showed that there is great variation in the deterreny of individual *E. ovata* trees to feeding by common ringtail possums ( $P < 0.001$ , Fig. 1). The amount of foliage that ringtail possums could ingest from these trees ranged from 14.9 to 50.1 g dry matter kg $^{-0.75}$  body mass. It should be noted that two even more highly deterrent individuals of *E. ovata* were omitted from this experiment due to concern for the safety of the animals with the protocol in its initial form. One of these trees (9) was then included in the second set of experiments with *E. ovata*.

### Comparison of ability of koalas and ringtail possums to ingest leaves of deterrent trees

As in the first experiment, there were clear differences between individual trees, within both *Eucalyptus* species, in the amount of foliage that could be eaten by ringtail possums (Figs. 2 and 3,  $P < 0.001$  for all experiments). The range of intakes of *E. ovata* was even greater than observed in the initial experiments, due to the inclusion of tree 9 which the possums almost totally refused to eat. However, while there were differences in intakes between

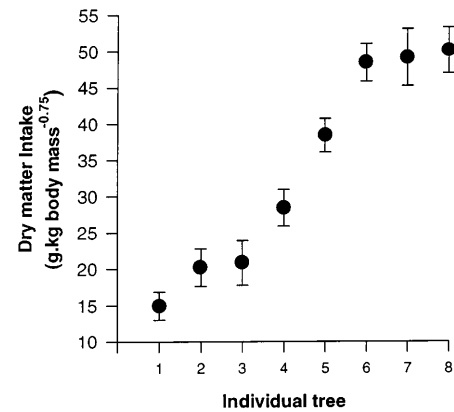


Fig. 1 Voluntary intake of *Eucalyptus ovata* foliage by common ringtail possums fed foliage from individual trees in no-choice experiments. Data are means  $\pm$  SE for 8 animals

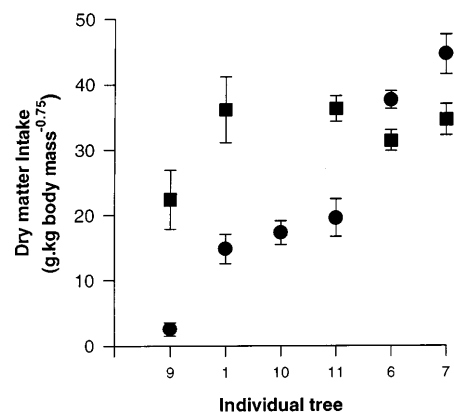


Fig. 2 Voluntary intake of *E. ovata* foliage by common ringtail possums (●) and koalas (■) fed foliage from individual trees in no-choice experiments. Data are means  $\pm$  SE for six animals for ringtail possums and five animals for koalas

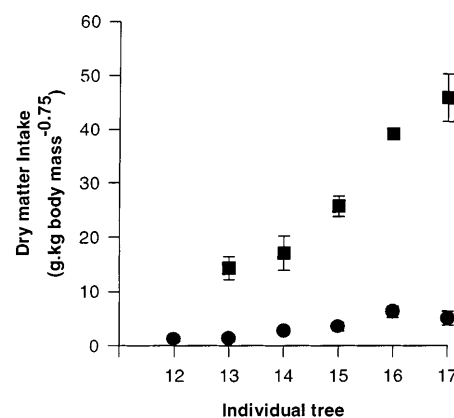
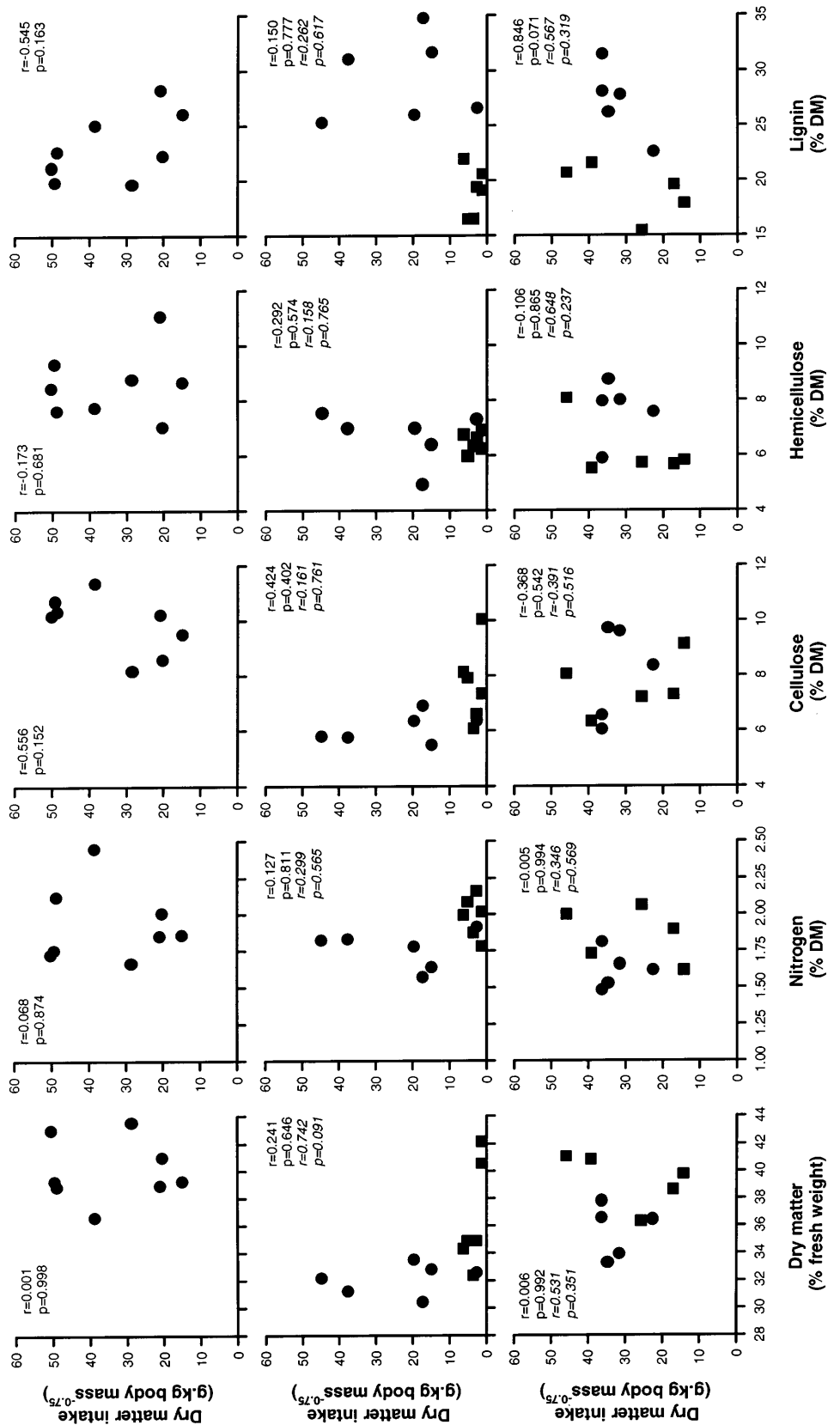


Fig. 3 Voluntary intake of *E. viminalis* foliage by common ringtail possums (●) and koalas (■) fed foliage from individual trees in no-choice experiments. Data are means  $\pm$  SE for six animals for ringtail possums and five animals for koalas

trees, the ringtail possums ate very little of any of the *E. viminalis* foliage, suggesting that all the trees used contained compounds sufficient to deter feeding by ringtail possums (Fig. 3).

**Fig. 4** Relationships between mean intakes of foliage from experiments shown in Figs. 1–3 and nutritional characteristics of the leaves. *Top row*: ringtail possum data shown in Fig. 1. *Second row*: intakes by ringtail possums of *E. ovata* (●) and *E. viminalis* (■). *Bottom row*: intakes by koalas of *E. ovata* (●) and *E. viminalis* (*italics*) (DM dry matter, DFP diformylphloroglucinol)



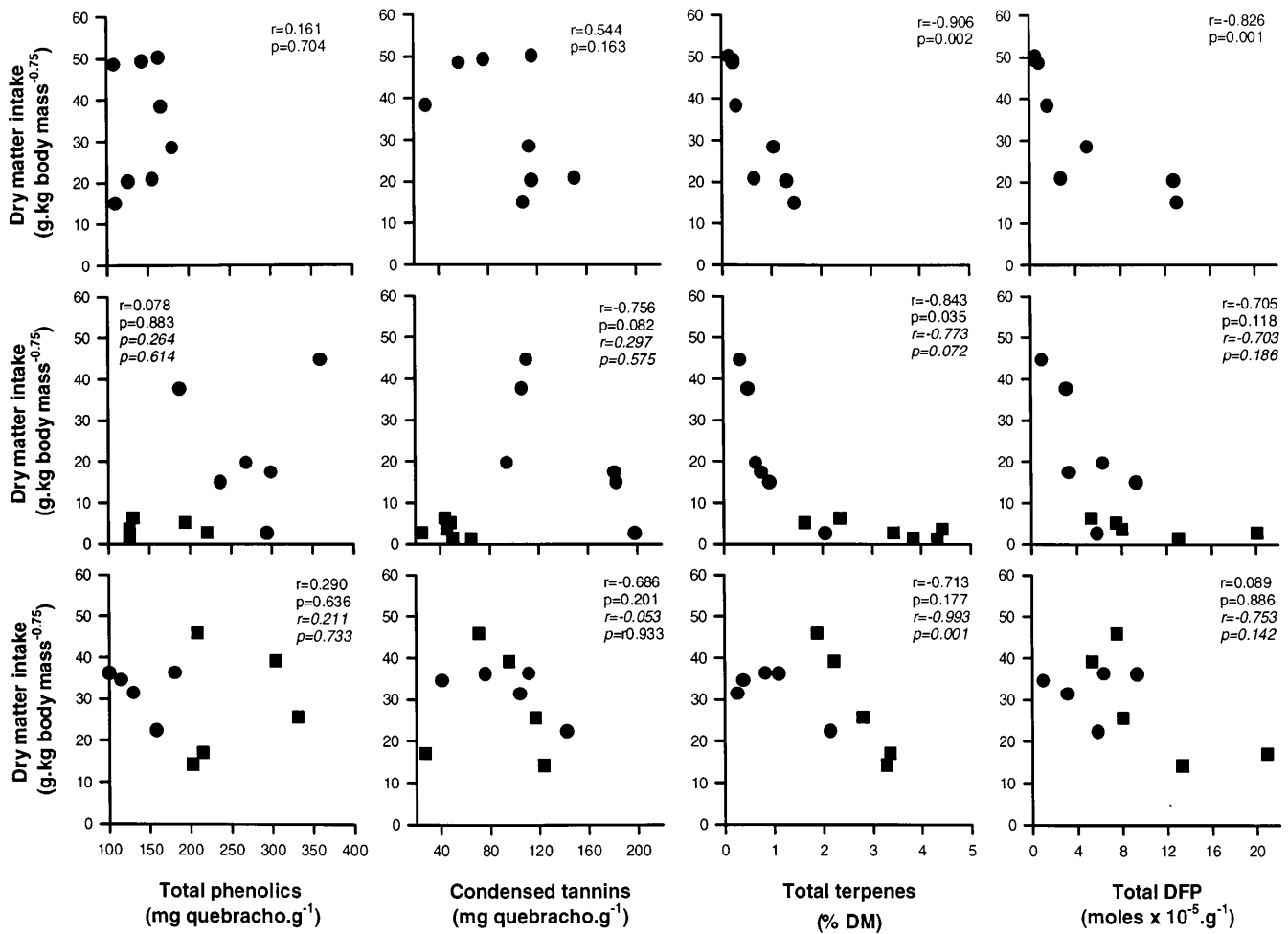


Fig. 5 Relationships between mean intakes of foliage from experiments shown in Figs. 1–3 and reputed deterrent compounds of the leaves. Legend as for Fig. 4

In contrast, koalas were able to ingest large amounts of leaf which the possums found inedible. The significant difference in intakes between individual *E. ovata* trees ( $P = 0.042$ ) was due to only one tree (tree 9, Fig. 2) which was found to be less palatable to koalas. In contrast, they reduced intakes on three of the *E. viminalis* trees, to as low as 31% of the highest intakes (Fig. 3,  $P < 0.0001$ ). Interestingly, the *E. ovata* tree on which koala intakes were reduced was also the least palatable to ringtail possums, while the two *E. viminalis* trees on which koala intakes were highest coincided with the highest intakes by ringtail possums (though the possums' intakes were still very low).

When the data for both *Eucalyptus* species are considered together, ranked in order of ringtail possum intake, there is an indication of a threshold of deterrence which is greater for the koala than the ringtail possums (Figs. 2 and 3). That is, voluntary food intake by koalas is only decreased at a level of deterrence well beyond that at which ringtail possum food intake is reduced.

#### Correlates of deterrence to feeding by ringtail possums and koalas

The relationships between food intake and a variety of measures of leaf nutritional quality and possible deterrent compounds are summarised in Figs. 4 and 5. None of the measures of nutritional quality made here consistently correlated with food intake. Only for dry matter of *E. viminalis* fed to ringtail possums and lignin in *E. ovata* fed to koalas was there any apparent correlation (Fig. 4). Similarly, there was no relationship between intake by either folivore and total phenolics or condensed tannins (Fig. 5). There was also no relationship between the measure of total phenolics and total DFPs (I.R. Lawler, unpublished work). This is notable as the DFPs are clearly important phenolic compounds (see below), but are not accounted for in this assay. In fact, when pure samples of four different DFP compounds (macrocarpal G, sideroxylonal, euglobal III and jensenone) were subjected to the same extraction and colorimetric assay, only jensenone, the most polar of the group, showed any color and this was not in relation to the amount of compound in the sample (I.R. Lawler, unpublished work). Only one *E. viminalis* and no *E. ovata* contained trace amounts of cyanide, thus cya-

nide did not appear to contribute to the deterrence of the avoided trees.

The only apparently consistent relationships were between food intake by the animals and the terpene or DFP content of the leaves (Fig. 5). In general, an increase in either the DFP content or the terpene content of the diet coincided with a decrease in food intakes by both koalas and ringtail possums. As there was only one *E. ovata* on which koalas ate significantly less leaf there can be little correlation expected between feeding and any leaf characteristic. The correlation between terpene concentration and feeding was always slightly better than any individual compound that makes up the total terpene fraction (I.R. Lawler, unpublished work).

#### Bioassay of potential deterrents to ringtail possums

Both cineole ( $P < 0.001$ , Fig. 6) and macrocarpal G ( $P < 0.001$ , Fig. 7) deterred feeding by common ringtail possums. Cineole decreased food intake at concentrations above approximately 2.6% of the dry weight of the diet and at 11.7% the food intake was reduced to approximately 30% of control diets. Similarly, intakes were decreased at concentrations of macrocarpal G above approximately  $0.73 \times 10^{-5}$  moles  $g^{-1}$  dry weight and at  $5.87 \times 10^{-5}$  moles  $g^{-1}$  dry weight the intakes were reduced to approximately 20% of controls.

These results can only be interpreted usefully in comparison to the concentrations of each compound found in resistant leaves. The cineole concentration required to reduce food intake significantly was much greater than was found in leaves on which intake is greatly reduced (Fig. 5). For example, food intake in animals fed the most resistant *E. ovata* tree, tree 9, was

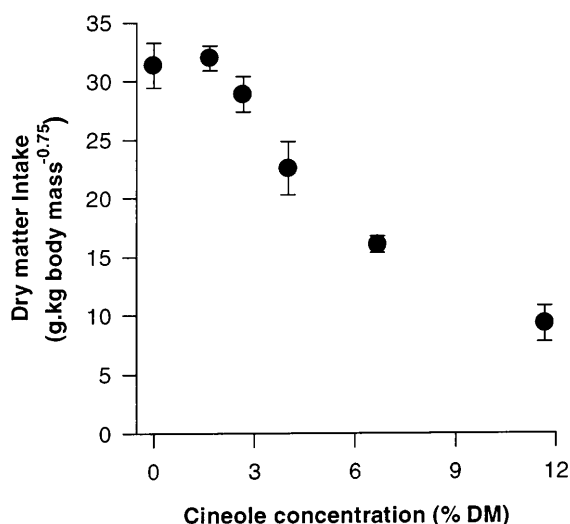


Fig. 6 Relationship between voluntary intake of an artificial diet by common ringtail possums in no-choice experiments and the concentration of cineole added to the diet. Data are means  $\pm$  SE for six animals

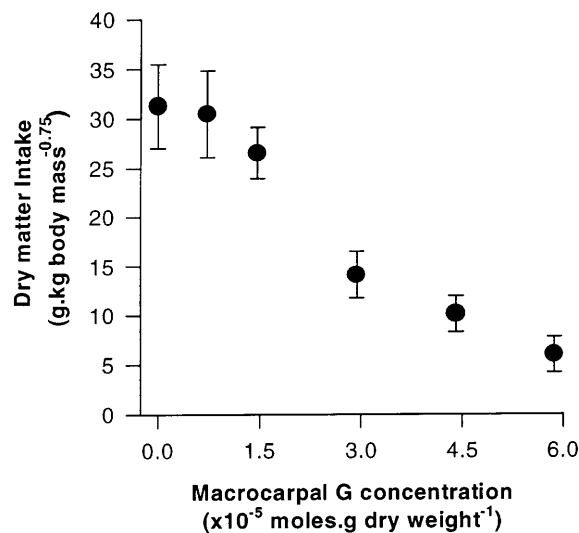
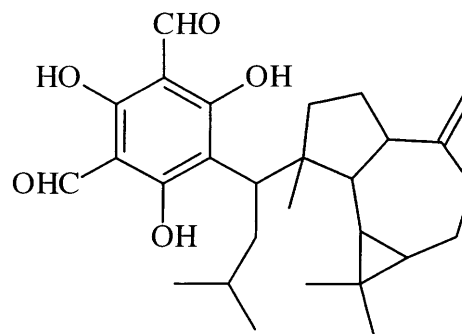


Fig. 7 Relationship between voluntary intake of an artificial diet by common ringtail possums in no-choice experiments and the concentration of macrocarpal G added to the diet. Data are means  $\pm$  SE for six animals



Macrocarpal G

Fig. 8 Structure of macrocarpal G

2.5 g  $kg^{-0.75}$  body mass (over 90% less than the highest leaf intakes by the same animals) while its terpene content was only 0.77% of the wet weight. This is substantially lower than the threshold for reduced intake with pure cineole. In contrast, the decreasing intakes of the diet with macrocarpal G added, occur at concentrations corresponding closely to those found in leaves (Figs. 5 and 7). Thus, it would appear that DFPs are the major group of compounds causing deterrence to herbivory in the leaves used in these experiments.

#### Discussion

These experiments have shown that there are strong intraspecific differences in the susceptibility of two

*Eucalyptus* species to vertebrate herbivory and furthermore, that susceptibility varies among mammalian species. Several field studies of feeding in folivorous marsupials identified resistant trees within an otherwise palatable species but no previous work has provided any convincing explanation of the basis of the food choices. This is because earlier studies used taxonomic divisions as the unit of analysis or because they have not used bioassay procedures to direct the chemical analyses. Clearly, for vertebrate herbivores of *Eucalyptus*, an important component of habitat patchiness is manifest at the level of the individual tree and not at the plant species level.

Correlative studies and the roles of diformylphloroglucinols and terpenes as feeding deterrents

DFPs are clearly the major cause of variable feeding by common ringtail possums on the *E. viminalis* and *E. ovata* trees examined in this study. Previous studies (McArthur and Sanson 1991; Foley 1992) have shown little variability in the digestibility of *E. ovata* foliage by common ringtails. In contrast, the differences in feeding demonstrated here suggests that variation in the level of food intake is the most important contribution to the nutritional quality of *Eucalyptus* foliage for folivorous marsupials. DFPs have been identified in all but one species of *Eucalyptus* examined to date (Ghisalberti 1996; D.M. Pass, personal communication) and the intraspecific differences we have found in feeding on these two species are likely to be a feature of other species as well.

The value of combining correlative analyses of feeding with bioassays was demonstrated by the differences between cineole and macrocarpal G in these experiments. The evidence from the correlative studies favoured terpenes over DFPs, as the proximate cause of the feeding behaviour we observed. However, the deterrent effects of cineole were only apparent at concentrations significantly higher than those found in intact foliage and significantly higher than that which has been measured in a wide range of *Eucalyptus* species and individuals (Southwell 1978; Boland et al. 1991). In a separate study, cineole proved deterrent when animals were given a choice of a cineole-rich or control diet, but a 0.8% cineole diet was eaten avidly when no choice was provided (Pass et al. 1998).

If, as our data show, the deterrent effects of the terpene fraction are small compared with DFPs then we need an alternative explanation for the strong correlation between foliage intake and the concentration of terpenes. Accordingly, we hypothesise that the terpenes may act as a cue to the concentration of the ultimate deterrent in the foliage. To humans, terpenes have a strong smell and taste whereas the DFPs have no odour (I.R. Lawler and W. Foley, personal observation). Both koalas and ringtail possums appear to smell leaves carefully before ingestion (Zoidis and Markowitz 1992;

I.R. Lawler, personal observation). Gently shaking a branch is sufficient to release detectable quantities of *Eucalyptus* terpenes (Rasmussen 1970) Furthermore, since DFPs consists of terpene side chains attached to phenolic moieties (Ghisalberti 1996) (Fig. 8) there may be a correlation between the concentration of at least some DFPs and some terpenes, especially if the production of DFPs is substrate-limited (Ghisalberti 1996).

Studies in other species have clearly shown that mammals can learn to associate distinctive flavours with the presence of PSMs (Provenza et al. 1990; Kyriazakis et al. 1997). Hence common ringtail possums may develop a conditioned aversion to volatile terpenes (or cineole in particular) based on the consequences of ingesting DFPs such as macrocarpal G. Evidence for such a feedback mechanism can be seen in the pattern of ingestion of macrocarpal G (Fig. 7).

In our experiments, animals did not simply avoid macrocarpal G when it was added to an artificial diet, but they regulated its intake such that they did not ingest more than approximately 200 mg. The pattern of regulation was similar to that seen when both common ringtails and brushtails were fed diets containing jensenone, a simple DFP closely related to macrocarpal G (I.R. Lawler, D. Pass and W. Foley, unpublished work). Common ringtail possums regulated ingestion to limit jensenone intake to very similar molar quantities as seen here for macrocarpal G. Jensenone is a powerful anti-feedant for both brushtail and ringtail possums and its action is mediated through the release of 5HT<sub>3</sub> (serotonin). Injections of the serotonin antagonist, ondansetron, led to significantly greater intakes of jensenone in common brushtails than controls. Since serotonin is a potent stimulator of nausea and emetic responses, we interpreted these results as support for the arguments of Provenza et al. (1994) that mammals learn to control their intake of PSMs through a feedback from nausea or gastrointestinal illness. The similarities between the structure of jensenone and macrocarpal G and the pattern of intake of macrocarpal G in these experiments suggest that a similar mechanism could be advanced to explain why macrocarpal G is such an effective anti-feedant. Clearly further experiments exploring the interrelations between volatile terpenes, DFPs and feeding deterrence are needed to evaluate these possibilities.

Comparisons between koalas and common ringtail possums

Our experiments show that koalas can feed on a greater range of individual *E. ovata* and *E. viminalis* trees than can common ringtail possums. Since so much has been written about the supposed restricted nature of koala diets (e.g. Hindell and Lee 1987; Zoidis and Markowitz 1992), it is surprising that these are the first data to directly compare feeding in koalas with another species of marsupial folivore. Koalas have been supposed to



have highly specialized and restricted food choices but on the basis of their wide distribution and diversity of habitats occupied, this has been questioned (Norton and Neave 1996). Certainly, koalas feed almost exclusively within *Eucalyptus* yet our data suggests that a large proportion of the individuals of at least the two species we studied could be eaten sufficiently for the animals to maintain themselves. In contrast, common ringtails could not maintain themselves on any of the *E. viminalis* that was offered and only about 50% of the *E. ovata*. We argue that this is evidence that food choice by koalas is far wider than previously realized and that ringtail possum selection of *Eucalyptus* is narrower than supposed for a species that has been widely described as a specialist folivore.

We interpret the differences in feeding as a result of differences in the capacity of the two marsupial species to tolerate or biotransform and excrete DFPs. Given how little we know about the metabolism of DFPs in mammals (or any PSM for that matter) at present it is difficult to be certain how this difference is effected. However, the ten-fold difference in body size between the two marsupial species is unlikely to be important since there are strong theoretical and empirical reasons for believing that small species should be able to biotransform and excrete a given toxin load more rapidly than larger species – largely as a consequence of their greater mass-specific metabolic rate (Freeland 1991). However why there should be such a marked difference between related species must await more detailed pharmacological studies. One study suggests little difference in a standard liver clearance tests between brushtail possums and koalas (Pass and Brown 1990) but the usefulness of these tests for gauging the effects of specific PSMs is unknown. For now, we know that DFPs are absorbed rapidly from the stomach, but we have not been able to detect their metabolites in either faeces or urine of either ringtail or brushtail possums (McLean, Brandon and W. Foley, unpublished work). Differences in the intake of PSMs among different breeds of goats feeding on juniper have been attributed to differences in their abilities to biotransform and excrete PSMs (Pritz et al. 1997) but again details of specific pathways are lacking. These issues need to be addressed if we wish to ascribe a significant role to metabolic biotransformations in plant-mammal interactions.

## Conclusion

The data presented here clearly show that the DFPs are a major determinant of the intake of *Eucalyptus* foliage by marsupial folivores and that the concentrations of these compounds vary significantly between individual trees within species. This is the first study to show such effects in this system and it is now important that measures of these compounds be made in a field study of food choices of these animals where the chemistry of foliage of individual trees is assessed. Currently, the DFPs are the most likely explanation for the frequently

reported intraspecific preferences recorded in field studies of arboreal marsupial folivores of *Eucalyptus*.

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