Errata for thesis by Ivan Robert Lawler

“Variation in Marsupial Folivory Between and Within *Eucalyptus* species: The Roles and Actions of Plant Secondary Metabolites”

p. 10, para 2, line 1: “receives ~~may receive~~”
p. 30, reference Pass and Foley: spelling of taste
p. 35, para 3, last sentence: Full stop at end “Esson 1993).”
p. 41, para 4, last sentence: Full stop at end “with *E. ovata*.”
p. 51, para 2, last sentence: Full stop at end “(Fig. 7).”
p. 63, para 1, line 2: “test and~~s~~ control.”
p. 73, para 3, line 10: “it must have still be**en** cleared…”
p. 99, para 1, line 3: spelling of oc**c**ur
p. 104, para 2, line 6: spelling of lipophil~~l~~icity
p. 117, para 1, line 5, “body mass$^{-0.75}$.d^{-1}…”
p. 118, para 3, last line: “ a significant cost **presumably** must accompany…”
p. 155, para 1, line 2: spelling of her**e**

VARIATION IN MARSUPIAL FOLIVORY BETWEEN AND WITHIN *EUCALYPTUS* SPECIES: THE ROLES AND ACTIONS OF PLANT SECONDARY METABOLITES

Common ringtail possum (*Pseudocheirus peregrinus*) browsing on *Eucalyptus sideroxylon*. Illustration by Lois Genis

A thesis submitted for the degree of Doctor of Philosophy of the Australian National University

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DECLARATION

The research described in this thesis is my own original work except where otherwise acknowledged. In all cases I am the senior author and principal contributor to the development of ideas, experimental design and execution and ecological interpretation. Chapters 1 and 7 are entirely my own. All remaining chapters are co-authored with my supervisor, William Foley, and Bart Eschler. William Foley contributed the original idea for this project through his role in identifying the role of diformylphloroglucinol compounds in deterring marsupial folivory. He also secured funding for this project and assisted in field work and discussion of ideas. Bart Eschler was the principal chemist in our research group, developing the methods to identify and quantify the chemical compounds of interest and extracting and purifying sufficient compound for bioassay experiments. In chapters 3 and 4, Georgia Pass and Jesicca Stapley respectively, replicated my experiments with ringtail possums with brushtail possums and their data is included to extend the generality of discussion and conclusions. David Pass and Kath Handasyde are co-authors of Chapter 2 as they contributed purified macrocarpal G and expert assistance with captive koala husbandry respectively. Darren Schliebs is a co-author of Chapter 5 as both he and Bart Eschler were responsible for the production of synthetic analogues of jensenone and torquatone.

I Lawler

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This thesis could not have been done without the foresight and perception of my supervisor, Bill Foley. Throughout my time conducting the research for, and writing, this thesis his support has been unstinting and valuable (extending even to slogging through sodden paddocks while ill to collect foliage while I stayed in the comfort of a climate-controlled animal house), while also being respectful of my commitments outside of academia. Throughout my long association with Bill (since undergraduate study) I have come to respect his opinions and value to his ability to see the application to our study of research in obscure fields. I have also come to view him as a trusted and valued friend and I owe him my gratitude for making this study not only possible, but rewarding and enjoyable.

While this work focuses on the ecological effects of the secondary chemistry of Eucalyptus leaves, the amount of work on development of the methods and compounds used in my experiments has been enormous: a four year postdoctoral appointment, a PhD, a short term postdoctoral position and a 6 month laboratory technician appointment have all been necessary to bring our knowledge of the chemistry of these compounds to the level that it can be used in an ecologically meaningful way. There is no way known that I could have developed the methods for identification, quantification and isolation of large quantities of these compounds on my own. Therefore I am indebted to Bart Eschler, Darren Schliebs, Dave Pass and Bruce Clarke for their contributions to this thesis.

Steve Cork of CSIRO Division of Wildlife and Ecology provided me with valuable guidance in determining the directions of the research, and allowed me the frequent use of his freeze-drying machine. Gordon Sanson enabled us to use the facilities and environment of Monash University for my experiments in Victoria. Kath Handasyde of Melbourne University gave expert (and much needed) advice on koala husbandy, and also identified for us some of the trees used in the experiments.

I have endeavoured always to employ suitable statistical rigour in the design and analysis of my experiments. While I take full responsibility for the end results and interpretation, I have been guided by expert advisers, including Glenn De'ath of James Cook University and David Ratkowsky of University of Tasmania. In the latter stages I received valuable advice and assistance from Ross Cunningham and Christine Donelly of the Statistical Consulting Unit. The assistance and patience of these people was always greatly appreciated.

Throughout my time as a PhD student, I have conducted my studies independently, but always felt I was a member of a team. All of these people worked on varying aspects of the larger project, and all contributed to the interchange of ideas and data which served to make it an effective research environment. Not only that, you have all been good fun to work with and supportive of my endeavours. So, I thank "The Foley team": Bart Eschler, Dave Pass, Georgia Pass, Jess Stapley, Michelle Watson, Ben Moore, Darren Schliebs, Bruce Clarke, Miranda Ebbers, Mamoru Matsuki and Ian Wallis for creating an enjoyable and supportive team spirit.

Others have helped me with laboratory analysis and again I extend my thanks to Mandy Yialeloglou of the CSIRO Division of Wildlife and Ecology and Michelle Larsen and Deanne Haffner of James Cook University.

None of these experiments could have been done of course, if I had not captured possums and koalas. I must acknowledge the permission of the National Parks and Wildlife agencies of Queensland, New South Wales, Australian Capital Territory and Victoria. Staff of the Victorian department also assisted in koala capture (well actually they did it all and then gave them to me, but I rode on the truck). The Australian Army allowed access to their training areas for possum collection. A surprising number of people helped me to catch possums. These included Dave Pass, Steve and Yvette Williams, Simon Ward, Stephen Lawler, Paul Marshall, Shelly Watson, Ben Moore, Geordie Torr, Matthew Pope and Russel Kirkwood. I hope I haven't forgotten anyone. My wife Karen must get full marks for commitment in this endeavour, helping to catch possums while 8 months pregnant, and then looking after them for me for several weeks. Natasha McLean and Robert Bednarik assisted in koala husbandry.

I have been fortunate in having worked at two universities and having thus been able to rely on the expertise of those in two workshops as required. In both cases, those involved, Rob Gegg at JCU and Bruce Barrie and Allan Muir of ANU have provided able and enthusiastic assistance often and at very short notice and it has always been appreciated.

Other individuals have helped with various aspects of the project and I thank them all. More specific acknowledgements are given in the appropriate section at the end of each Chapter.

Of course, a body of work such as this can not be completed without the support of family. My parents, Ted and Gillian, have given me intellectual, emotional (and financial) support since undergraduate study. Most especially, I thank my own blossoming family. My wife Karen has not only been supportive and encouraging, but has been always willing to put in the effort when asked (see above). And my two little girls, Tahni and Kenyah, I love more than anything, and I thank them for teaching me about what really matters.

PREFACE

This thesis is written as a series of five papers (Chapters 2 to 6), plus a general introduction (Chapter 1) and a general discussion (Chapter 7) of my findings and ideas for future research directions. This approach maximises brevity, but some replication of methods and references is inevitable. To enhance the speed of submission, there is some variation in the referencing style used, as specified by the journal to which the work was submitted.

Other publications resulting from this research are included as appendices. This includes two short notes not sufficient for full research papers, two review articles and a research paper on which I am not the principal author.

ABSTRACT

The feeding of arboreal marsupial folivores on *Eucalyptus* foliage is well known to be highly selective. That these animals show preferences between *Eucalyptus* species, and between individual trees within species, has been recorded often in studies of both free-ranging and captive animals. The presence of toxic or deterrent plant secondary metabolites (PSMs) is often invoked as the cause of avoidance of otherwise nutritious foliage, however no clear trends have yet become apparent. In this study I have made significant progress in identifying the key PSMs in *Eucalyptus* which deter feeding by marsupial folivores. The main study animal has been the common ringtail possum (*Pseudocheirus peregrinus*) with some comparative data on the koala (*Phascolarctos cinereus*) feeding on natural leaf diets, and the inclusion also of complementary data for common brushtail possums (*Trichosurus vulpecula*) fed artificial diets with purified PSMs extracted from *Eucalyptus* foliage. Progress has been made by departing from the conventional species-based approach and focussing directly on the extensive variation in foliar secondary chemistry between individual trees within each of four species of *Eucalyptus*. This has allowed me to avoid confounding the quantitative effects of particular PSMs within species with qualitative differences in the identity of PSMs between species. The discovery, immediately prior to the commencement of this research, of a well-defined group of PSMs, the diformylphloroglucinol compounds (DFPCs), which act as marsupial folivore deterrents in *E. ovata*, has been fundamental to the work described below.

The initial species investigated in this study were *E. ovata* and *E. viminalis*. The amount of foliage animals would consume in a single night of feeding was recorded in no-choice experiments. Substantial variation was recorded between individual trees within each *Eucalyptus* species in the amount of foliage that the animals would eat. Daily dry matter intakes of ringtail possums ranged from 2.5 to 50.0 $g.kg^{-0.75}.d^{-1}$ on *E. ovata* and 1.26 to 6.28 $g.kg^{-0.75}.d^{-1}$ on *E. viminalis* foliage. Dry matter intakes of koalas ranged from 22.4 to 36.3 and from 14.3 to 45.9 $g.kg^{-0.75}.d^{-1}$ on foliage of the same *E. ovata* and *E. viminalis* trees fed to ringtail possums. Correlative analyses of food intakes against a range of nutritional measures and assays of PSMs showed that terpenes and DFPC compounds contributed to feeding deterrency. Further bioassay experiments with a purified DFPC (macrocarpal G, the predominant DFPC in *E. ovata*) showed that only DFPCs could cause the differences seen in food intakes. Terpenes added to the diet reduced food intakes of possums, but only at concentrations substantially higher than found in resistant foliage. Food intakes of koalas were considered to be determined by the same chemical factors, but their threshold for the intake of deterrent PSMs was greater than that of ringtail possums.

There was some difficulty with correlative analysis of the role of DFPCs in the initial study, because the few individual trees used provided insufficient degrees of freedom in statistical analyses. There was also no precise quantitative assay for the particular active DFPCs in these *Eucalyptus* species. The role of DFPCs in determining intraspecific variation in deterrency to marsupial folivores was therefore again investigated with these shortcomings addressed. An HPLC method had been developed to enable quantitative assays of sideroxylonals, another sub-group of DFPCs expected to show high anti-herbivore activity. Two *Eucalyptus* species were chosen as the focus of these experiments, because they were known to have sideroxylonals as the predominant DFPCs, and because ringtail possums were known to exhibit strong preferences between individual trees with each species. I conducted three no-choice experiments, in which I measured the dry matter intakes of ringtail possums fed the foliage of 24 individual trees of *E. polyanthemos*, 12 additional individuals of *E. polyanthemos* and 12 individuals of *E. sideroxylon*. With the improved assay and expanded experimental design, it was possible to show that a single PSM, sideroxylonal, could explain >80% of the variation in intake (from 3.28 to 44.0 $g.kg^{-0.75}.d^{-1}$) between the 36 *E. polyanthemos* trees. Food intakes by ringtail possums between 12 individual *E. sideroxylon* trees varied from 2.4 to 67.0 $g.kg^{-0.75}.d^{-1}$ and the majority of this variation could again be attributed to foliar sideroxylonal concentrations. Foliar terpene concentrations also correlated negatively with feeding but again bioassays showed that only sideroxylonal added to the diet could recreate this effect at concentrations similar to those found in deterrent leaves.

Near infrared reflectance spectroscopy (NIRS) was investigated as a means to measure foliage chemistry more rapidly and inexpensively than is possible with HPLC. It was found to be applicable to the measurement of foliar sideroxylonal concentrations (r^2 of relationship between values estimated by laboratory analysis and NIRS = 0.911) and also was able to be calibrated directly against intake of foliage of the 36 *E. polyanthemos* trees in the experiments described above by common ringtail possums (r^2 of observed vs. estimated by NIRS = 0.917). I used NIRS analysis to conduct a field study of small scale variation in foliar sideroxylonal concentrations and deterrency to ringtail possums of individual *E. polyanthemos* trees. Within an area considered small enough to be encompassed by the home range of a single ringtail possum, foliar sideroxylonal concentrations between individual trees varied from zero to 12.6 mg.g dry matter^{-1} and estimated daily intakes by ringtail possums of foliage of each tree ranged from 0 to 42.8 $g.kg^{-0.75}.d^{-1}$. Assessment of habitat quality thus requires assessment of leaf chemistry at least at the level of individual trees within species, and NIRS offers the analytical capacity to cope with the increased sampling intensity. The approximately normal frequency distribution of foliar sideroxylonal concentrations between trees may indicate a multi-locus genetic basis for their production.

The mechanisms underlying the observations described above where also investigated. These investigations used jensenone, a simple DFPC which was more readily isolated in sufficient quantities for the experiments, and deterred feeding at molar concentrations similar to the more complex DFPCs (macrocarpals, sideroxylonals). It was reasoned that if possums reduce their food intakes in response to increasing concentrations of DFPCs then there should be some post-ingestive feedback on which to base this regulation. Administration of a drug (ondansetron) which antagonises the $5HT_3$ receptors for serotonin enabled both ringtail and brushtail possums to increase their intakes of a diet treated with a simple DFPC, though it did not completely abolish the effect on food intake of either species. At least part of the mechanism by which regulation of a diet containing DFPCs is achieved is via post-ingestive feelings of nausea associated with the diet.

Because food intake was negatively correlated with foliar terpene concentrations (above), but terpenes failed to elicit this response when added to an artificial diet, an alternative role for these compounds was suggested. There are significant similarities between DFPCs and terpenes, as the molecular structure of the former includes at least an isoprene side chain, and more commonly a mono- or sesqui- terpene. It was reasoned that the concentrations of the two groups of compounds were therefore likely to be correlated (as was found to be the case - r^2 between sideroxylonal and total terpenes in the *E. polyanthemos* and 12 *E. sideroxylon* trees described above = 0.92) and that the more prominent odour and taste of the terpenes may be used as a pre-ingestive cue to the DFPC content of leaves. I tested the hypothesis that the reduction in food intake of ringtail possums when terpenes were added to an artificial diet (described above) was due to a conditioned flavour aversion, arising from the association of their taste and odour with the post-ingestive nausea induced by DFPCs. Wild-caught possums showed an aversion (i.e. reduced food intakes) to 1,8-cineole, a major *Eucalyptus* terpene, when added to a diet. This aversion could be significantly reduced by daily increasing the concentrations of cineole in the diet over a 12 day period. Exposure to a diet treated with both cineole and jensenone in corresponding amounts was able to recondition the aversion. Animals that had previously had their aversion to cineole reduced subsequently showed an aversion that could not be distinguished from that of animals not exposed to the increased dietary cineole. This was considered strong support for the role of terpenes as cues to foliar concentrations of the related DFPCs, rather than as toxins in their own right.

The final facet of this investigation was a study of the importance of specific molecular features of the DFPCs conferring the ability to deter marsupial feeding. This was done with a structure/activity study testing the necessity for aldehyde and/or phenol groups attached to the aromatic ring. I compared the reduction in food intake by ringtail possums in response to addition to the diet of four compounds varying in these structural features. Jensenone has two aldehyde groups *meta-*, one phenol *para-* and two phenols *ortho-* to the isoprene sidechain. A related compound (torquatone) extracted from *E. torquata* lacks both these types of functional groups. From each of the parent compounds an intermediate was synthesised that had either the phenol groups (acetyl-jensenone) or the aldehyde groups (demethyl-torquatone) capped. The results were inconclusive due to the limited range of structural variations that could be tested. Ringtail possums reduced their food intakes substantially in response to jensenone at low concentrations. Approximately twice the concentration of acetyl-jensenone was required to reduce food intakes of possums by similar amounts. The activity of torquatone was an order of magnitude lower, while demethyl-torquatone showed little activity at all. The results appear to indicate that the aldehyde groups attached to the aromatic are an important feature (high activity of jensenone and acetyl-jensenone) but that the phenol groups may be unimportant (low activity of torquatone and demethyl-torquatone). More pharmacological detail of the specific metabolic action of DFPCs may provide more valuable insights into the importance of particular molecular features. The importance of measuring concentrations of specific compounds, rather than functional groups is again emphasised: both jensenone and demethyl-torquatone are phenolic compounds, and yet their activity against ringtail possum feeding varied from extremely high to minimal.

I conclude that DFPCs are the major PSMs defending *Eucalyptus* foliage from marsupial folivores. Variation in foliar concentrations of DFPCs caused corresponding variation in intakes of marsupial folivores between individual trees within species as strong as any recorded for interspecific comparisons. While these data are for only four species of *Eucalyptus*, the DFPCs examined here, and a variety of structurally very similar compounds, occur across a wide range of other *Eucalyptus* species in concentrations likely to confer defence. Much has yet to be learned about their specific metabolic action, and the factors determining foliar concentrations. However, I argue that the capacity to measure foliar chemistry of large numbers of trees using NIRS, and to calibrate NIR spectra against animal food intakes, without the necessity to identify the underlying chemical causes, should be employed in detailed field studies of foraging as a high priority for future research.

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CHAPTER 1. INTRODUCTION - LITERATURE REVIEW

Perhaps the most fundamental of interspecific interactions is between an animal and its food. While the complexities of the relationships between a predator and its animal prey may be more intuitively obvious, the challenges faced by the herbivore in acquiring sufficient food to carry out its essential activities are no less daunting. Herbivores are limited in their range of food choices firstly by the poor quality of plant material as a food resource. Plant material typically contains large amounts of indigestible material (fibre, lignin) and low concentrations of nutrients such as nitrogen (Cork and Foley 1991). Thus while the food available to an herbivore might seem superabundant this is not the case (Ganzhorn 1992). Feeding choices of many herbivore species, particularly browsers (those eating dicotyledonous foliage) are further limited by the presence of mechanical (thorns, hairs) (Brooks and Owen-Smith 1994; Gowda 1996; Cooper and Ginnett 1998) or chemical defences (Palo and Robbins 1991). In many cases the concentration of a chemical defence compound may be the ultimate determinant of whether an herbivore can feed on a particular plant or plant part, irrespective of the nutritional quality of that food item (Clausen *et al.* 1986; Meyer and Karasov 1989; Jakubas and Gullion 1990). It is the chemical defences found in the foliage of trees of the genus *Eucalyptus*, that confer resistance to browsing by arboreal marsupials, that are the main focus of this thesis.

It has long been recognised that some types of chemical compounds have no apparent primary metabolic role but are produced by plants in significant quantities. These compounds are considered then to have "secondary" roles, one of which *may* be the protection of the plant from herbivore attack. Hence these compounds are referred to as plant secondary metabolites, or PSMs, and I use this term consistently throughout this thesis.

Over several decades, an immense amount of effort has been invested in the study of plant-herbivore interactions, and how this is mediated by PSMs. However, in many cases the picture remains unclear, principally because of the huge variety of compounds to be considered. A single leaf may contain numerous PSMs in each of several different types (e.g. phenolics, terpenes, alkaloids, cyanogenic glycosides) and this may be further complicated by the herbivore feeding on a range of unrelated plant species with qualitatively different PSM profiles. In this introduction I trace the development of the theory of the importance of PSMs in vertebrate herbivore feeding. I then briefly discuss the parallel development, and state of knowledge prior to this thesis, for marsupial folivory of *Eucalyptus*.

IMPORTANCE OF PLANT SECONDARY METABOLITES IN PLANT-HERBIVORE INTERACTIONS

Effects on Herbivores

For a PSM to be considered important in a plant-herbivore interaction it must be shown to have an effect on animal performance that confers a benefit to the plant. That benefit must be a reduced feeding pressure from the herbivore. Consequently it should be possible, with appropriate experiments, to measure the reduced animal performance. In the short term, such effects should manifest either as a reduced total food intake of the herbivore, or a reduced probability of choosing to feed on the particular plant when given a choice of alternatives (Freeland and Janzen 1974). The plant may also benefit if PSM ingestion causes death of the animal, or in the longer term reduced reproductive success. This latter may be particularly hard to assess.

An observation of a reduced preference for (or avoidance of) a food item by a free-ranging animal is an indication of a benefit conferred on the plant in that instance. However, it does little to tell us what is causing that effect, and how. If alternative foods (plant species, individuals or plant parts) are available in abundance then a number of alternatives may explain this effect. Where the reason for this effect cannot be explained in terms of nutrient concentrations or other impediments such as physical barriers, then PSMs are often invoked as the likely cause (note that this is frequently not substantiated (e.g. Glander 1978; Hindell *et al.* 1985; Pahl 1987; Hume and Esson 1993)). If the difference can be attributed to a PSM in this situation we can only say that it has deterrent properties. We cannot infer anything about whether this arises from some pre-ingestive irritant property or post-ingestive consequences such as digestibility reduction or toxicity. If, however, there is little alternative food available and the consequence of avoidance of that plant is to risk starvation, then we may say with more (but not absolute) confidence that the post-ingestive effects of the plant's PSMs are likely to be severe. I return to the topic of the specific effects of PSMs on herbivores in a later section.

If animal performance is negatively affected by a PSM then it may act at a range of scales, from variation in preferences shown by individual animals within their home ranges (Clausen *et al.* 1986; Jakubas and Gullion 1990; Snyder 1992) to variable population densities for the herbivore at larger scales (Braithwaite *et al.* 1983; Oates *et al.* 1990; Cork 1992; Ganzhorn 1992; Cork and Catling 1996). Thus there may be significant spatial heterogeneity in habitat quality for the herbivore that is not immediately apparent when viewing the site. I address this in the next section.

Heterogeneity of the Food Resource

Like most resources, plant material is not distributed uniformly across the landscape (Laca and Demment 1991). Even where this may superficially appear to be the case, herbivores may not forage uniformly, as they are affected by numbers of variables. They may respond to variation in species composition, variability in nutrient content between and within food species, three dimensional structure of the environment (e.g. koalas prefer large trees (Hindell and Lee 1987)) or external factors such as the presence of predators. They also respond to variation in concentrations of PSMs between and within food species (Hodar and Palo 1997).

A common approach to understanding of herbivore foraging is the use of mathematical models to understand the choices made by herbivores between two or more diets (Belovsky and Schmitz 1991, 1994; Illius and Jessop 1995; Elston *et al.* 1996; Farnsworth and Illius 1998). These models have only recently been modified to include plant defences (Belovsky and Schmitz 1991; Belovsky and Schmitz 1994; Illius and Jessop 1995).These models have generated some useful insights into the implications of PSMs for diet choice. They illustrate clearly the trade-offs inherent in diet choice: that in many cases the defence conferred on a plant by the presence (or high concentration) of a PSM is not absolute, that it depends on the balance between nutrient and PSM concentrations of that plant and of the plants surrounding it in nature. However, for toxic PSMs there are absolute limits to the animal's capacity to tolerate them. Where the concentration of a toxic PSM is sufficiently high (or the nutrient concentration sufficiently low) that the cost of ingesting the PSM exceeds the nutrient gain from that food item, then that plant ceases to be viewed as such by the animal.

All these models require some detailed knowledge of the role of the PSM to be constructed appropriately. In the optimal foraging models of Belovsky and Schmitz (1991, 1994) the distinction is made between quantitative and qualitative defences. The model of Illius and Jessop (1995) requires knowledge of the metabolic cost of detoxifying and excreting the PSM. Thus, the primary objective in attempting to understand the role of PSMs in any plant-herbivore interaction must be to first identify the important PSMs and, ideally, its (or their) mode of action. There are few generalisations that can be made about groups of compounds, an idea which I discuss in more detail below (following the next section).

Models of Allocation of Resources by Plants to Defence

Clearly the production of PSMs is a cost to the plant and there have been many attempts to develop models of the factors governing allocation of resources to defence. All these models have in common that they consider there to be a trade-off between defence and growth (Feeny

1976; Rhoades and Cates 1976; Coley *et al.* 1985; Gulmon and Mooney 1986; Herms and Mattson 1992). The early models of Feeny (1976) and Rhoades and Cates (1976) focussed on plant apparency. They reasoned that plants, or plant parts, whose occurrence was ephemeral and unpredictable, should be defended by qualitative defences which cost little as they could avoid specialist herbivores in time and defend themselves with low concentrations of toxic metabolites against generalist herbivores. Plants that were longer-lived and therefore more predictable in space and time would produce quantitative defences as they could not avoid discovery by specialist herbivores.

Although the rationale for the above hypothesis differs from the later resource availability hypothesis of Coley et al. (1985) and growth/differentiation hypothesis of Herms and Mattson (1992), its predictions are essentially similar. In these two later models, allocation to defence is dependent on the availability of resources (especially the ratio of carbon to nitrogen) and the requirements of growth and photosynthesis. Where nutrients (especially N) are limited, growth is more constrained than photosynthesis and excess carbon is available for investment in defence. Conversely, where nutrients are in good supply, growth increases by relatively more than photosynthesis and carbon is diverted from defence to growth (and investment in N-based defences may increase (Gleadow *et al.* 1998)). Thus long-lived ("apparent" *sensu* Feeny (1976) and Rhoades and Cates (1976)) plants that grow slowly invest more in carbon-based defence than do short-lived plants that grow rapidly. Similarly, within species, those individuals growing on poorer sites should invest more in carbon-based defence compounds.

These hypotheses have contributed to much to our understanding of plant allocation of resources to defence. However, there are significant examples of studies of plant-herbivore systems under varying conditions of resource availability that are not in accord with these hypotheses (Lincoln *et al.* 1986; Lincoln and Couvet 1989; Johnson and Lincoln 1990; Fajer *et al.* 1992; Kainulainen *et al.* 1998). There may even be agreement with the theory for one type of PSM but disagreement for another putative defensive compound within one plant species (e.g. terpenes vs phenolics) (Lawler *et al.* 1997). The allocation of resources to PSMs thus depends not only on resource availability but also the metabolic pathways involved (and particularly the likelihood of competition for substrate with primary metabolism) (Muzika 1993) and perhaps genetic control (Lincoln and Couvet 1989; Han and Lincoln 1994). It is perhaps significant that those studies that record agreement with resource availability hypotheses frequently measure tannins or total phenolics rather than terpenes (Lindroth *et al.* 1993; Lavola and Julkunentiitto 1994; Lawler *et al.* 1997; Penuelas *et al.* 1997; Gebauer *et al.* 1998) but see (Johnson *et al.* 1997). Phenolic compounds are a large proportion of the leaf carbon, and consist of a wide variety of compounds that may or may not play a role in defence (Zucker 1983; Ayres et al. 1997) or may have other functions such as protection from UV light (Robinson *et al.* 1993).

Such measures may be more an indirect measure of carbon excess, rather than allocation to defence *per se* and it is therefore not surprising to see a response to resource availability. Identification of particular active phenolic compounds (if any) and assessment of their specific responses to resource availability may yield further insights into this area. If we are to examine cost-benefit relationships in the allocation of resources to PSMs as anti-herbivore defence, we must be confident that we are measuring the benefit (increased resistance to herbivory) and the cost (increased production of the particular PSM conferring resistance) appropriately.

Hence I reiterate the concluding point of the previous section: in order to understand the importance of PSMs in plant-herbivore interactions the primary objective should ideally be first to identify the compound(s) of interest. This caveat holds whether we are considering models of feeding preferences, spatial heterogeneity of habitat, plant allocation of resources or co-evolution of defence and herbivore.

IDENTIFICATION OF IMPORTANT PLANT SECONDARY METABOLITES IN PLANT-HERBIVORE INTERACTIONS

Developing the understanding (and appropriate chemical assay techniques) to identify and quantify the compounds of interest in a plant-herbivore interaction is a daunting prospect in the face of the diverse array of PSMs to be found even in the foliage of a single tree species. For example over 60 compounds can be isolated from steam volatile extracts of *E. polyanthemos* (Boland *et al.* 1991). In light of this difficulty, and in order to try to develop more general rules about the effects of PSMs on herbivores, early studies attempted to group different compounds into classes according to similarities in structure (and, implicitly, function). Such categories include condensed tannins and "total" phenolics and the literature is replete with examples of studies attempting to relate such measures to vertebrate feeding (Cork and Pahl 1984; McArthur *et al.* 1993; Brooks and Owen-Smith 1994; Hjalten *et al.* 1994; Hartley *et al.* 1995; Ayres *et al.* 1997; Dearing 1997; Hodar and Palo 1997). However, while such studies have given some insights into broad trends (Braithwaite *et al.* 1983; Oates *et al.* 1990; Ganzhorn 1992; Cork and Catling 1996) they lack resolution and cannot explain finer scale discrimination shown by herbivores between plants (between closely related species, or between individuals within species) because of the variation in the structure and deterrent capacity between the many compounds within each class (Zucker 1983; Clausen *et al.* 1990; Waterman and Kool 1994; Ayres *et al.* 1997). In recent times it has come to be realised that interactions between a plant species and specific groups of herbivores (e.g. mammals) may be governed by one or a very few compounds of specific molecular structure, and that subtle changes in structure can have large effects on the effectiveness of the compound in deterring herbivore attack (Bryant *et al.* 1983; Reichardt *et al.* 1984; Clausen *et al.* 1986; Reichardt *et al.* 1990).

Without information on variation between plants in the specific PSMs deterring herbivores, both at the inter- and intra-specific levels, it will not be possible to fully describe other aspects of the biology of free-ranging folivores. Unless we can determine why otherwise nutritious plants are not fed upon, and develop the means to measure the appropriate attributes, we will not be able to understand such things as habitat quality and to relate it to population density and distribution, reproductive success or the foraging behaviour of individual herbivores.

Approaches to Identifying Important Plant Secondary Metabolites

Correlative Studies

One of the most common ways used to illustrate an effect of any diet constituent on animal performance is to use some form of correlative analysis to examine the relationship between the constituent and that measure. For example, numbers of studies have used multiple regression to illustrate a relationship between food intake by an herbivore and the concentration of PSMs in the diet (e.g. Southwell 1978; Hume and Esson 1993; Duncan *et al.* 1994). However, while this appears an intuitively sensible approach to the problem, the results of such studies in many cases are unconvincing. I suggest that this is because of two limitations in the purely correlative approach:

1. It assumes prior knowledge of the important factors affecting the measure of interest. A correlation can only be investigated if the two (or more) factors are measured (and measured precisely - see comments above regarding crude assays). It cannot be used to identify an unknown factor influencing the response variable.
2. Correlative studies cannot show cause and effect. A correlation between two factors may be identified, but may in fact be reflective of a correlation existing between the measured independent variable and an unidentified causative agent.

Thus this approach, in isolation, is insufficient to unambiguously assign a role to any particular PSM in a plant-herbivore interaction. However, in combination with the other approach to the problem, through bioassay experiments, the two aforementioned problems are overcome and the results of such a combined approach can be interpreted more confidently.

Bioassays

In this style of experiment cause and effect are directly illustrated. An extract, or pure PSM, is added to an artificial diet, and the animal's performance on that diet (e.g. food intake), relative to that of control animals on the matching untreated diet, is recorded. As the only difference between the two (or more) diets is the compound of interest its effect is directly observed. An extension of this approach, bioassay-guided fractionation, can be used also to overcome the first difficulty noted for correlative studies (Bryant *et al.* 1983; Sinclair *et al.* 1988; Reichardt *et al.* 1990; Pass *et al.* 1998). A plant considered to be resistant to herbivore attack due to an unidentified PSM is extracted with a broad-spectrum solvent. The resulting crude extract is tested using a bioassay experiment. If addition of the extract to the diet reproduces the deterrent effect, the extract is then refined (e.g. partitioning between water-soluble and water-insoluble fractions) and each fraction re-tested. Again, if one fraction is highly deterrent it is again fractionated and the fractions again tested. This process proceeds until a specific PSM (or well-defined group of PSMs) is identified as the major deterrent, without any *a priori* assumption about the nature of the PSM.

Having identified the potential deterrent, assays of this compound can then be used in correlative studies to confirm (or refute) its role in herbivore resistance in the natural diet. Similar reductions in food intake (or regulation of food intake such that intake of the PSM remains constant) between natural and artificial diets with the same concentration of the PSM lend strong support to its importance in the relationship.

FACTORS AFFECTING THE INTAKES OF PLANT SECONDARY METABOLITES BY HERBIVORES

Freeland and Janzen (1974) noted the difficulty in finding examples of herbivores that do not eat plants containing PSMs. It is now generally recognised that browsing herbivores can not avoid ingesting potentially toxic compounds, and that the ability to subsist on a foliage diet requires some means of assessing the toxin content of the diet and regulating the intake of that diet so as not to exceed a tolerable toxin load. To assign an important role to a particular PSM in a plant-herbivore relationship, and to understand it, we should ideally know the physiological effects of that compound (and the limits to the animal's ability to withstand those effects). In the many instances where detailed pharmacological information is unavailable it is not possible to fully understand the PSM's effects. Nevertheless, it is still possible to establish the animal's threshold tolerance for the compound and to develop some understanding of why it responds as it does to that compound.

Given that most herbivores are unable to completely avoid diets containing PSMs, to do so without incurring significant harm from overingestion they must respond to some form of feedback which signals to them that they should reduce, or cease, feeding. There are many forms of feedback that can arise from ingestion of a PSM and we can use them to provide some insight into the physiological effects of that compound. Feedback may be pre-ingestive or post-ingestive and a combination of these two types is usually used by the herbivore in regulating diet intake, often with the pre-ingestive feedback calibrated against the level of post-ingestive feedback. I discuss each type of feedback below.

Post-ingestive Feedback From Plant Secondary Metabolites

The internal effects of PSMs are many and varied. In order that I may summarise them I have divided them into three broad categories: digestibility reduction; toxicosis/emetic stimulation and disruption of acid-base balance.

Digestibility Reduction

One of the most widely studied groups of PSMs are the tannins, a broad group of polyphenolic compounds so named because of the ability to bind proteins such as occurs in the tanning of leather. Because of this capacity to bind with proteins, tannins have long been considered to act as anti-herbivore defences by reducing the digestibility of the plant material and hence its nutritive value to the herbivore (e.g. Foley and Hume 1987a; Robbins *et al.* 1987; Hagerman *et al.* 1992; McArthur *et al.* 1993; Chung-MacCoubrey *et al.* 1997; Dearing 1997). There are a variety of difficulties with ascribing this role to tannins in realistic ecological situations, such as the secretion of tannin-binding salivary proteins (TBSPs) by many specialist browsers (Mole *et al.* 1990; McArthur *et al.* 1995) or the possibility that tannin-protein complexes may breakdown in the gut (McArthur and Sanson 1991), full discussion of which is beyond the scope of this review. Nevertheless, there are circumstances in which reduced digestibility doubtless is the case (Robbins *et al.* 1987; McArthur and Sanson 1993a; Chung-MacCoubrey *et al.* 1997; Dearing 1997), and the animal therefore must receive some feedback from reduced digestibility. Interestingly these are not specific to the tannins themselves, but more likely manifest as nutritional inadequacies of the diet.

The most intuitively obvious feedback which would be received from reduced digestibility is increased gut fill. That is, if particles in the diet are not broken down then they must increase the bulk of material in the gut and the herbivore may be expected to experience feelings of "fullness". This may then be interpreted by the herbivore in the same manner as a diet that is simply high in fibre. However, the particular digestive physiology of the herbivore may determine whether the herbivore experiences such sensations. For example, several hindgut fermenters selectively retain fine particles for fermentative digestion while preferentially expelling larger particles (Sakaguchi and Hume 1990). In these cases, large tannin-protein complexes would be expelled rapidly, without constraining the rate of food intake.

An alternative form of feedback received by the herbivore would be the perception of reduced nutritive value through reduced digestibility of protein. Again this is unlikely to be perceived by the herbivore as a direct consequences of tannins themselves. This is a form of feedback on which animals can regulate their intake. Studies by Provenza and colleagues (Provenza 1995a,b; Provenza *et al.* 1996, 1998) have shown that animals can detect differences in the protein or energy contents of feeds via post-ingestive feedback and respond by changing their preferences to the dietary alternative most able to satisfy these short-term deficiencies. The manner in which this occurs is essentially through mild, short-term conditioned food aversions (CFAs) which are dynamic, depending on the nutritional status of the animal (see below). Indeed, Provenza and co-workers have suggested that goats developed CFAs towards condensed tannins (Provenza *et al.* 1990).

Toxicosis/Emetic Stimulation

Once a PSM is absorbed, then it can only act as a herbivore deterrent if it exerts toxic effects. That is, if it causes some disruption of normal physiological functioning which is negatively perceived by the animal. There is an enormous variety of ways in which this can happen which are not within the scope of this review. The salient point is that many of these effects manifest as emetic stimulation (approximately "nausea and vomiting" but note discussion of difficulty with this interpretation in Chapter 3). Emesis arises from stimulation of the area postrema of the brain, which may respond to endogenous emetic agents released from the gut (such as serotonin) in response to a dietary toxin, or to toxic compounds in system circulation, due to an incomplete blood-brain barrier (Andrews *et al.* 1988). Thus emesis provides a strong post-ingestive feedback associated with intake of a PSM and herbivores quickly learn to associate these effects with the taste and/or smell of diets causing them (Provenza *et al.* 1990, 1994a,b). This general concept is integral to the development of conditioned food aversions to PSMs (discussed below) which forms a basis for a more general theory of how herbivores appropriately regulate their diets in spatially and temporally variable environments (Provenza 1996, 1998).

Disruption of Acid-Base Balance

After absorption, detoxification and excretion of most PSMs occurs through biotransformation to less toxic, more water-soluble products. These products are frequently strong organic acids and thus create an acid load against which the herbivore must buffer itself to maintain acid-base homeostasis which is vital for normal metabolic functioning. This may result in reduced levels of urea recycling or increased levels of muscle protein catabolism which are a substantial cost to herbivores. Foley and co-workers (Foley 1992; Foley *et al.* 1995) suggested that this may be a common pathway by which the consequences of ingestion of PSMs could be quantified. However, while they showed quantifiable costs and these have been shown to potentially limit intake (Illius and Jessop 1995), they could not identify an appropriate feedback mechanism that would enable herbivores to regulate food intake on this basis. Nevertheless, sheep have been shown to be able to alter their preferences for diets on the basis of their capacity to attenuate acidosis (Phy and Provenza 1998).

Pre-ingestive Feedback From Plant Secondary Metabolites

Before ingesting a potential food item, an herbivore receives may receive signals, in the form of taste and smell, indicating the palatability of the diet. It may respond to the strength and nature of these signals by deciding to feed or not, or may even regulate its intake on the basis of the strength of the flavour cues alone (Launchbaugh *et al.* 1993). There are essentially two ways in which pre-ingestive feedback may cause an animal to avoid, or reduce its intake of, a food item. These are through an inherent aversive property of the taste stimulus itself (unpleasant stimulus of the trigeminal nerves), or through a conditioned (or learned) association between a taste stimulus (which is not necessarily inherently unpleasant) and negative post-ingestive feedback (a conditioned flavour aversion).

Taste Aversion Via Trigeminal Stimulation

Sensations received through the trigeminal nerves may loosely be defined as the common chemical sense (Silver 1987). Aversion due to trigeminal stimulation is characterised by an immediate response to encountering some irritant or astringent stimulus without measurable ingestion (Nolte *et al.* 1993; Mason *et al.* 1996). Perhaps the best known example of a trigeminal stimulant of mammals is capsaicin, the active constituent in many species of peppers which causes the burning sensation. It is interesting to note also, that the effectiveness of such compounds varies remarkably between vertebrate classes. For example, a study of the effectiveness of capsaicin and its analogues showed a range of effectiveness against mammals that varied in relation to the molecular structure and that this trend was reversed in birds (i.e. compounds repellent to birds were not repellent to mammals and vice versa) (Mason *et al.* 1991).

Much of the research conducted on the effect of trigeminal stimulants on feeding behaviour has focussed on birds, with an emphasis on applied use of the information, such as repelling birds from crops (Rogers 1974; Clark and Shah 1994; Clark 1996). There have been fewer studies of mammals, and these have generally also be aimed at practical applications (Mason *et al.* 1991; Nolte *et al.* 1993; Hoover and Conover 1998). Studies of the role of trigeminal stimulation in natural plant-herbivore systems are even fewer. In the only study I know of to date Jakubas and Mason (1991) showed that coniferyl benzoate on quaking aspen buds had irritant properties that reduced the intake of buds by European starlings. It was argued that trigeminal stimulation may be of general importance in the detection of PSMs by herbivores, due to the irritant or astringent properties of many compounds (Jakubas and Mason 1991).

Trigeminal stimulation may not be enough to deter feeding by herbivores except in situations where palatable alternatives are available in abundance. Where there is no post-ingestive effect the aversive stimulus is often easily overcome by mammals (Hoover and Conover 1998) or by birds (Alcock 1970; Rogers 1974). In fact, the reverse may become true, with the positive stimulus of capsaicin in food familiar to many of us (e.g. in Asian cooking). Therefore, the aversiveness of trigeminal stimulants may need to be reinforced by post-ingestive feedback forming a conditioned flavour aversion (see next section) in order to be more widely effective (Alcock 1970; Rogers 1974). At the least, to determine the importance of trigeminal stimulation requires experiments to separate the effects of taste from post-ingestive effects. Such experiments include bypassing the buccal cavity in administering the PSM such as by gastric lavage (Pass and Foley 1998) or using surgery to severe the trigeminal nerves (Jakubas and Mason 1991).

Conditioned Flavour Aversion

A conditioned flavour aversion arises when an herbivore learns to associate the smell and/or taste of a food with negative post-ingestive feedback (an internal malaise) arising from that food (Provenza 1995a). The flavour cue need not be directly associated with, or derive from, the PSM causing the post-ingestive feedback, but must vary in strength in accordance with the concentration of the active PSM. This cue can then be used by the herbivore to regulate its intake of the diet, based on the strength of the flavour stimulus alone (Launchbaugh and Provenza 1994) potentially enabling it to restrict intake to below the level where post-ingestive effects are felt. Note that the feedback must be internal and of a particular type. External feedback (such as trigeminal stimulation) does not produce conditioned food aversions (Clark 1996). The internal feedback usually arises from stimulation of the emetic system (see above) (Provenza *et al.* 1990) although toxins such as cyanide may generate CFAs (O'Connor and Matthews 1995; O'Connor and Matthews 1997) but do not appear to act on the emetic system (Garcia *et al.* 1985).

The formation of a CFA is shown experimentally by offering the animal both the causative agent and the flavour stimulus together (conditioning) and offering the flavour stimulus alone to determine if it induces the animal to reduce its food intake (e.g. Launchbaugh *et al.* 1993;

Launchbaugh and Provenza 1994). Conditioned flavour aversions are especially likely to be formed with novel foods (Provenza *et al.* 1994a). When a negative post-ingestive feedback is received while the animal receives a novel and a familiar diet together, the CFA develops for the novel food. Thus herbivores need not have an instinctive aversion to any particular food containing a PSM to enable them to avoid intoxication in unfamiliar environments (Provenza 1996; Provenza *et al.* 1998). Conditioned food aversions are also dynamic, with animals constantly resampling diets and "recalibrating" their response to PSMs, enabling them to cope also with changes in foods both temporally and spatially, as well as variations in their own physiological state (Provenza 1995b; Wang and Provenza 1996; Wang and Provenza 1997).

EUCALYPTUS-ARBOREAL MARSUPIAL FOLIVORE INTERACTIONS: STATE OF KNOWLEDGE PRIOR TO THIS STUDY

In this section I describe developments in understanding of the *Eucalyptus*-arboreal marsupial folivore interaction parallel to those listed above. I show that prior to this thesis the most important compounds conferring resistance to marsupial folivory in *Eucalyptus* were unknown. Reasons for this shortcoming are discussed, followed by the discovery of highly deterrent compounds (study of which is the substance of this thesis) and a summary of their chemistry.

Background to *Eucalyptus*-Arboreal Marsupial Folivore Interactions

Eucalyptus *Foliage as a Food Source*

Eucalyptus trees are often found to be growing on poor soils and consequently their foliage contains low amounts of dietary nitrogen and is heavily lignified (Cork and Sanson 1990; Landsberg and Cork 1997) and as such to attain a high degree of folivory on this diet requires specialised digestive adaptations (Cork 1996) (see below). In accordance with the theories of plant allocation of resources to defence (discussed above) *Eucalyptus* foliage also has appreciable amounts of carbon-based PSMs (terpenes and phenolic compounds). Because these two groups of compounds do not have any other known function, occur in high concentrations and are toxic to other organisms (at least in the case of terpenes (McLean and Foley 1997)) both groups of compounds have long been reputed to be deterrent feeding by marsupial folivores (see below). However, while ingestion and metabolism of these compounds has been shown to have metabolic costs (Foley 1987, 1992 Foley and Hume 1987a; Foley *et al.* 1987) the evidence for high concentrations of these compounds limiting food intakes, in nature or captivity, has been inconclusive (see below).

The Marsupial Folivores of Eucalyptus

The genus *Eucalyptus* dominates many Australian forests, and therefore an understanding of the interactions between this genus and its herbivores is fundamental to a broader understanding of Australian ecosystems. Despite the ubiquity of eucalypts in Australia there are few mammals that feed on their foliage to any great extent. In fact, only three arboreal species, the koala (*Phascolarctos cinereus*), the greater glider (*Petauroides volans*) and the common ringtail possum (*Pseudocheirus peregrinus*) are able to subsist of a diet solely of *Eucalyptus* foliage, while a fourth, the brushtail possum (*Trichosurus vulpecula*), may include high proportions of *Eucalyptus* foliage in its diet but supplements the diet with other more nutritious foods. Other terrestrial species, all macropodids (e.g. swamp wallabies, *Wallabia bicolor*, and Tasmanian pademelons, *Thylogale billardierii*), may all also feed less exclusively on *Eucalyptus* foliage.

Perhaps as a consequence of the narrowness of this feeding guild, the interactions between these marsupial species and their eucalypt food have been the subjects of numerous studies over many years. There is a trend in specialisation of these species on a *Eucalyptus* diet, ranging from the brushtail possum to the ringtail possum, the greater glider and finally the koala, perhaps one of the most specialised of mammalian herbivores.

Brushtail possums are an extremely versatile generalist feeder, which is perhaps best exemplified by their success in colonising New Zealand habitats (Rose *et al.* 1993; Owen and Norton 1995). The food items of this species may include grasses, fruits and flowers, insects and foliage from a range of species from shrubs to trees (Fitzgerald 1984; Kerle 1984; Procter-Gray 1984; Statham 1984). However, while in some circumstances a large proportion of the diet may consist of *Eucalyptus* foliage (Freeland and Winter 1975; Kerle 1984; Foley and Hume 1987b) they rarely specialise on *Eucalyptus* foliage in the wild, and this is probably because they lack the necessary digestive adaptations to cope with the high proportions of indigestible material in *Eucalyptus* foliage (Foley and Hume 1987c; Cork and Foley 1991).

The remaining three arboreal marsupial folivores of *Eucalyptus* are able to utilise a foliage diet more exclusively because they share an important digestive adaptation: selective retention of fine particles (Cork and Warner 1983; Chilcott and Hume 1985; Foley and Hume 1987c). Digesta are separated into fine and coarse particles and the former retained in the caecum, while large particles are excreted. Fine particles have a higher surface area to volume ratio that increases the rate of fermentative digestion while larger particles, which are inefficiently fermented, are selectively expelled reducing the constraints of gut fill and passage rate. These retained particles are also those that are higher in nitrogen and other nutrients. This adaptation is particularly important because of the opposing allometric trends of gut size and metabolic rate:

smaller animals have higher mass-specific metabolic rates (Nagy 1987) but their gut sizes are proportionally similar (Demment and Van Soest 1985). Small herbivores must extract more energy and nutrition from a similar sized pool than do large herbivores so must increase the rate of passage and/or the efficiency of fermentative digestion or select a more nutritious diet. Small arboreal herbivores survive at close to the theoretical limits of body size and selective retention appears to be a necessary adaptation for the to attain a high degree of folivory (Cork and Foley 1991).

Ringtail possums occur in a range of forest types throughout much of eastern Australia. They may specialise on *Eucalyptus* foliage (Pahl 1987; McArthur and Sanson 1991) but in other areas or at other times include flowers and buds, and foliage of understorey shrubs in their diets. In addition to selective retention of fine particles they are coprophagic, producing specialised faecal pellets from caecal contents, which are reingested and may help them to cope with low dietary nitrogen (Chilcott and Hume 1985). Ringtail possums are also able to cope with the putative digestion-inhibiting effects of *Eucalyptus* tannins, apparently by breaking down tannin-protein complexes in the caecum (McArthur and Sanson 1991, 1993b).

Greater gliders and koalas are both highly specific *Eucalyptus* folivores, with the koala especially long renowned for its particular eating habits (Fleay 1937; Pratt 1937). Both use selective retention of fine particles in coping with the indigestible component of their diets, but neither employs coprophagy as adults (Cork and Warner 1983; Foley and Hume 1987c). Greater gliders appear to show very strong preferences for young foliage (Kavanagh and Lambert 1990), while koalas appear less constrained. It is difficult to determine why and how these two species are so specialised on *Eucalyptus* foliage, to the extent of being unwilling or unable to feed substantially on the foliage of other species (even avoiding many of the several hundred species of *Eucalyptus*). It is tempting to suggest that it results from differences in their capacity to deal with eucalypt toxins, but as I discuss below, there is little conclusive evidence of the role of any particular toxin in the feeding choices of any of these marsupial species. The detoxification capacity of the koala may be more specifically directed at the particular groups of toxins to be found in *Eucalyptus* foliage, while perhaps brushtail possums spread their capacity over a range of differing classes of PSMs found in widely varying plant species. Thus the generalist may be able to feed on a range of species but have a limited intake of any one, while the specialist may have high intakes of one species but be unable to cope with event small quantities of the qualitatively different PSMs in unrelated plant species. The only data thus far available to support this come from a study of the capacity of these species to metabolise *Eucalyptus* terpenes, showing that the more specialised marsupial folivores were able to oxidise *p*-cymene more completely than the generalist (R. Boyle *personal communication*).

The Common Ringtail Possum as a Study Animal

The common ringtail possum is an ideal species to use to study these interactions, because it not only thrives on a diet of only mature *Eucalyptus* foliage, but is easily kept in captivity and rapidly adjusts to an artificial diet. Thus it is amenable to both the approaches described above, correlative experiments with natural foliage diets and bioassay experiments with artificial diets. In contrast, neither koalas nor greater gliders can be kept for substantial periods on an artificial diet and koalas particularly are difficult to maintain for long periods because of the quantities of foliage required. Therefore this thesis concentrates on the common ringtail possum, as an ideal *Eucalyptus* folivore for captive experiments. Some data of my own for koalas is incorporated into Chapter 2 while data for brushtail possums also is presented in Chapters 3 and 4 resulting from complementary experiments carried out by others in the research group under Dr Foley's supervision (See Declaration on p. i). Appendix 1 also presents complementary data for two macropod species.

Phenolic Compounds in *Eucalyptus* as Marsupial Deterrents

The physiological effects of phenolic compounds in *Eucalyptus* are less well known than those of terpenes (see below) because they are less well characterised. Phenolics in *Eucalyptus* are usually measured as "total" phenolics and condensed tannins, both as extracted in 50% aqeuous acetone (Cork and Krockenberger 1991). An unknown proportion of the lower molecular weight phenolics may be absorbed and exert toxic effects, but these are little known (Cork and Sanson 1990). Condensed tannins have been better studied, but again are considered as a group, rather than individual compounds. Two of the marsupial species on which this thesis focuses (koala, common ringtail possum) and the greater glider are generally not susceptible to the proposed digestibility-reducing effects of condensed tannins (McArthur and Sanson 1991, 1993b). This is thought to be a result of retention of fine particles in the caecum, allowing extended periods for microbial breakdown of tannin complexes (McArthur and Sanson 1991; McArthur and Sanson 1993b). The brushtail possum, however, lacks this digestive specialisation and does show reduced digestibility of the diet when fed *Eucalyptus* foliage (Foley and Hume 1987a), but not when fed a purified tannin in an artificial diet (McArthur and Sanson 1993b).

The effects of total phenolics and condensed tannins on feeding and habitat preferences of the *Eucalyptus* specialists, especially koalas, have been repeatedly studied (Ullrey *et al.* 1981; Cork and Pahl 1984; Geritz 1987; Cork and Sanson 1990; McArthur and Sanson 1991; Hume and Esson 1993; Bednarik 1996) but there has been little effort to explain brushtail possum preferences between or within *Eucalyptus* species on the basis of these compounds (Freeland and Winter 1975). The only consistent trend to arise thus far from such studies is a broad scale effect on habitat quality for arboreal folivores of *Eucalyptus*. A threshold value of the ratio between nitrogen and total phenolics appears to exist, below which viable populations of these animals are not supported, and above which population densities may be high, but are extremely variable (Braithwaite *et al.* 1983; Cork 1992; Cork and Catling 1996). This indicates that other factors are important at the site level, such as habitat complexity or structural features (Lindenmayer *et al.* 1990, 1991, 1994; Pausas *et al.* 1995, 1997), or perhaps an unknown PSM which varies over a small scale (see Chapter 6).

Terpenes in *Eucalyptus* as Marsupial Deterrents

The physiological effects of terpenes, and their effects on feeding, have been more thoroughly studied. This reflects several features of terpenes: they are more easily identified and quantified; they are known to have toxic effects on some species, and; they are a prominent feature of *Eucalyptus* foliage (the smell of each eucalypt species is a characteristic property derived from its terpene profile), which are believed to be used by marsupial folivores as a cue to the leaf's palatability (Betts 1978; Hindell *et al.* 1985; Zoidis and Markowitz 1992). There are, however, some difficulties associated with interpretation of foliar terpene data. Firstly, the method of extraction may affect the terpene profile. Steam distillation is often used to extract terpenes (Foley *et al.* 1987; Hume and Esson 1993) and the high temperatures may cause degradation of some individual compounds (Ammon *et al.* 1985). Secondly, the metabolic fate of terpenes has been investigated with both natural diets (Foley *et al.* 1987; McLean *et al.* 1993) and in bioassay experiments (Southwell *et al.* 1980; Krockenberger 1988; Carman *et al.* 1994; Carman and Rayner 1994; Carman and Garner 1996; Carman and Rayner 1996), but these experiments have failed to test the limits of animals to tolerate high concentrations of terpenes in the diets.

The results of studies attempting to relate marsupial folivore food preferences with terpene concentrations or profiles of foliage have been inconclusive. In correlative studies, relationships have generally been weak (Betts 1978; Zoidis and Markowitz 1992; Hume and Esson 1993) or even contradictory within the one study (Southwell 1978). One major contributing factor to these poor correlations is the failure of previous studies to account for intraspecific variation. Although strong preferences shown by marsupials between trees within *Eucalyptus* species have been identified in both field studies (e.g. Hindell and Lee 1987; Pahl 1987) and experiments with captive animals (Pahl and Hume 1990), intraspecific chemical variation has not been investigated. Thus there is a major source of potential variation not included in the models developed thus far. Investigation of within species variation may also yield the added advantage of minimising qualitative variation between samples, allowing development of more appropriate models focussing on specific terpenes, or other PSMs (note that this is unlikely to be profitable in the case of "total" phenolics because such assays do not allow appropriate distinction between compounds). A final difficulty with assessing the role of terpenes in marsupial folivore-*Eucalyptus* interactions is that, in the few cases where bioassays have been conducted specifically to test the effects of terpenes on food intakes of these animals, they have not used concentrations as high as those found in resistant foliage (Krockenberger 1988; M. Harvey and I. D. Hume *personal communication*).

The general conclusion from the above, is that to date we have been unable to explain the feeding preferences exhibited by either captive or free-ranging arboreal marsupial folivores between or within species of *Eucalyptus*. This is because the identities of the most important PSMs in this interaction were unknown.

Diformylphloroglucinol Compounds (DFPCs) in *Eucalyptus* as Marsupial Deterrents

Prior to the commencement of my research for this thesis, and indeed fundamental to its beginning, was the discovery of a group of compounds to which herbivore resistance could be attributed.

It had been observed in Melbourne, Victoria, that ringtail possums showed very strong preferences between individual *E. ovata* trees (Pahl 1984, 1987; Geritz 1987). Some trees were heavily defoliated by ringtail possums, while others appeared resistant. Using a technique of bioassay-guided fractionation, as described earlier, Pass et al. (1998) investigated the chemical basis for this interaction. They were able to isolate a specific chemical, macrocarpal G (Fig. 1d) to which they could attribute the majority of the feeding deterrence shown by resistant trees. This was the first time that variation in resistance to herbivory within a *Eucalyptus* species could be attributed to a defined chemical factor.

In this thesis I have further investigated the importance of the group of compounds to which macrocarpal G belongs. I refer to them collectively as diformylphloroglucinol compounds (or DFPCs), referring to the two formyl (or aldehyde) groups on each phloroglucinol molecule of compounds so far identified as active against marsupial folivores. Note also that an alternative, more general, nomenclature refers to them as acylphloroglucinol derivatives and includes compounds with fewer, or no, formyl groups (e.g. Ghisalberti 1996). All the relevant compounds also consist of an isoprene or terpene sidechain attached to the molecule. There are a number of sub-groups: simple DFPCs (Figs. 1a,b); euglobals (Fig. 1c); macrocarpals (Fig. 1d), and; sideroxylonals (Fig. 1e). The euglobals do not appear to show high feeding deterrency in marsupials (Pass *et al.* 1998) and thus I have concentrated my research on the other three groups of DFPCs (simple DFPCs, macrocarpals, sideroxylonals). That I have studied the effects of all three groups in different chapters is reflective of the developments through the course of this project in terms of extraction and analytical techniques making the use of certain compounds more feasible than others. It does not necessarily indicate greater ecological importance of one or other of the sub-groups of DFPCs. That must await the accumulation of substantially more data than are available at this time.

As the DFPCs are a relatively newly discovered chemical group, and their significance in this field is certainly only very recently recognised, I now briefly review the chemistry of each of the sub-groups mentioned above. A comprehensive review is given by Ghisalberti (1996).

Simple Diformylphloroglucinol Compounds

Eucalyptus and several other genera of the Myrtaceae contain fully substituted DFPCs, some of which are steam volatile and frequently isolated during phytochemical investigations aimed principally at the volatile essential oils (Boland *et al.* 1991; Ghisalberti 1996). The most widespread compound is torquatone (Figure 1a) which can comprise up to 40% of the steam-volatile constituents of *E. torquata* (Bowyer and Jefferies 1959). A range of other compounds varying in the level of oxidation of the nuclear carbons have been described including jensenone (Figure 1b), which dominates the steam-volatile extract of *Eucalyptus jensenii* leaf (Boland *et al.* 1991; Boland *et al.* 1992). These compounds, together with the diformylphloroglucinol-terpene adducts described below, can have substantial biological activity in a number of different systems (Ghisalberti 1996) which is dependent on the particular molecular structure (Bolte *et al.* 1985). Jensenone is a potent deterrent to feeding by marsupial folivores (Chaps 3, 4 and 5) while closely related compounds such as torquatone show reduced activity (Chap 5).

Diformylphloroglucinol-Terpene Adducts

In the past 10-15 years a number of compounds have been identified which result from terpenes bonded to simple DFPCs (Ghisalberti 1996, Pass *et al.* 1998). These are known as euglobals, so named as they were first extracted from *E. globulus* (e.g. euglobal III, Figure 1c) (Kozuka *et al.* 1982a; Kozuka *et al.* 1982b) and macrocarpals, first identified in *E. macrocarpa* extracts (e.g. macrocarpal G, Figure 1d) (Yamakoshi *et al.* 1992). The two groups are distinguished by the form of the bonds between the terpene and the DFPC structure.

Euglobals

There are two groups of euglobals, adducts with monoterpenes and adducts with sesquiterpenes. Although there are undoubtedly more compounds to be discovered, those already known are formed with only a restricted number of terpenes. The euglobals contain a monoterpene moiety derived most commonly from β-pinene, although compounds with sabinene and β-phellandrene have also been identified. A second group of euglobals are formed with sesquiterpenes, most commonly bicyclogermacrene.

Macrocarpals

Macrocarpals differ from euglobals in that they lack the ether linkage between the aromatic and terpenoid parts of the structure. The fact that euglobals show relatively low activity compared to macrocarpals may indicate the importance of the phenolic group adjacent to the terpene adduct in determining deterrent capacity. All terpene adducts of known macrocarpals are derived from sesquiterpenes. Macrocarpals show high antiherbivore activity (Chapter 2).

Sideroxylonals

A limited number of compounds have been described which can generically be termed dimers of the simple diformylphloroglucinols. These include sideroxylonal A (Figure 1g) and B, robustanol A and grandinal. Of these, only the effect of sideroxylonals on mammal feeding has been investigated here, with strong indications of high deterrency (Chapter 6).

STRUCTURE OF THESIS

As noted in the preface to this thesis, I have used the paper format for each chapter. This is both because this project is amenable to such a structure and because it expedites the publishing of the work in a wider forum. Each chapter is self-contained, and is presented in an approximately chronological order. Later chapters address problems or shortcomings identified in earlier chapters.

I have noted in this introductory review the requirements for identifying and ascribing a role to any particular PSM in a plant-herbivore relationship and also the surprising lack of conclusive evidence for this in the *Eucalyptus*-marsupial folivore interaction. I have attempted in this study to address these issues, in order to develop a convincing set of data for the role of DFPCs, while also further assessing the roles of terpenes and "total" phenolics in diet choice of marsupial folivores. I have overcome qualitative differences between species in their PSM profiles by focussing instead on the wide range of intraspecific variation in herbivore deterrence of individual *Eucalyptus* trees. I believe this is the first study to do so in relation to the *Eucalyptus*-marsupial folivore interaction.

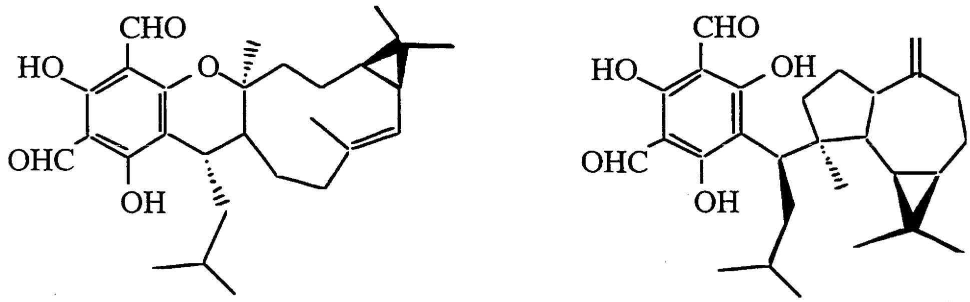
In summary, I have identified DFPCs as the major PSMs responsible for deterring marsupial folivores. I have also provided insights into both the pre- and post-ingestive feedback used by these animals to assess leaf palatability and developed the methods to assess these in a manner appropriate for detailed field studies. More specifically I have found that:

- There is extremely strong intraspecific variation in the resistance of individual *Eucalyptus* trees to browsing by marsupial folivores (Chapters 2 and 6) which occurs at a spatial scale relevant to individual animals (Chapter 6)
    - This correlates extremely strongly with terpenes and DFPCs
    - Only DFPCs can actually cause this effect
    - The role of terpenes is secondary, as a cue to the concentration of deterrent DFPCs in the foliage
    - "total" phenolics and condensed tannins do not appear to play any role in these feeding decisions (Chapters 2 and 6)
- The deterrence of DFPCs to marsupial folivore feeding is at least partly due to causing post-ingestive emesis (Chapter 3)
- The secondary role of terpenes is due to a conditioned food aversion arising from their strong pre-ingestive feedback (taste and smell) being associated with the post-ingestive emesis caused by DFPCs (Chapter 4)
    - This is effective in natural diets because of a strong correlation between foliar concentrations of terpenes and DFPCs (Chapter 6)
- The particular molecular structure of DFPCs is important in determining their deterrent effects on marsupial feeding (Chapter 5)
- Near infrared spectroscopy (NIRS) is an effective technique for assessing the foliar concentrations of DFPCs in *Eucalyptus* (Chapter 6)
    - It can also be used to directly estimate likely food intakes of folivores
    - NIRS enables the data and concepts contained in this thesis to be applied to field study of the effect of variation in foliage chemistry at the level of individual trees, minimising the constraints of sample preparation and laboratory analysis.

I conclude with a general discussion in Chapter 7 to consolidate my findings and address future directions for this research.

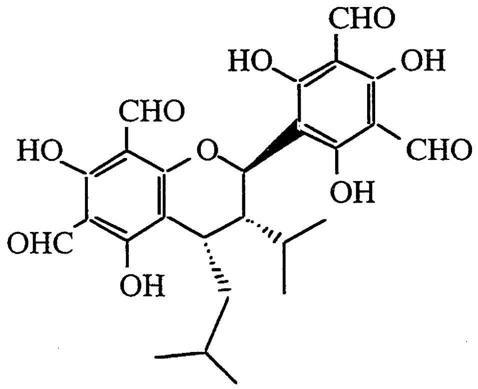
(a) Torquatone

(b) Jensenone



(c) Euglobal III

(d) Macrocarpal G



(e) Sideroxylonal A

Figure 1. Structures of representative diformylphloroglucinol compounds (DFPCs)

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CHAPTER 2. 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 focussed 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 deterrency resulting from secondary leaf chemistry have been inconclusive because assays used have focussed 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 deterrency 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 body mass$^{-0.75}$.d^{-1}. Koala intakes of foliage from the same individual trees ranged from 22.4 to 36.3 g.kg body mass$^{-0.75}$.d^{-1}. When fed foliage from different individual *E. viminalis* trees, common ringtail possums ate between 1.26 and 6.28 g.kg body mass$^{-0.75}$.d^{-1} while koalas ate from 14.3 to 45.9 g.kg body mass$^{-0.75}$.d^{-1}. 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 potential groups of plant secondary metabolites that may cause deterrency; 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*, terpene, phenolic, herbivory, condensed tannin

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 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 varations 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 occassionally 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 seconday 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 antifeedant 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 (*Pseudocheirus 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 0.6m wide x 0.6 m high and 0.75 m long in a room with a 12:12 hour 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 (*Phascolarctos 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 x 3 m long x 2.5 m high and subject to natural light:dark cycles (approximately 12:12). 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.

Deterrency of Foliage of Individual Trees to Feeding by Ringtail Possums

In our initial experiment we aimed to examine the range of individual variation in deterrency in *Eucalyptus 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 deterrency from highly preferred to strongly deterrent. All trees were mature, ranging between 6 m and 15 m and had foliage of similarly high apparent quality, in terms of abundance of young growth, and greenness, softness and lack of damage to leaves.

Deterrency 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 hungy. 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 deterrency, especially if offered a highly palatable alternative.

Eight common ringtail possums were offered leaf from 8 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 27.5g 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: (i) 12M sulphuric acid for 1h at 25 °C then 1M sulphuric acid for 2 h in a boiling water bath (conditions were found optimal for liberating monosaccharides from cellulose); (ii) hydrolysis in the 1M 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 β-glucosidase (Sigma) in sealed apparatus containing a separate well containing 1M 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 (Pass, Eschler, Foley unpublished). Other aldehydes in the extracts contribute only a small proportion of the total aldehyde proton peak (Eschler unpublished). 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.

Biossay 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 preceeding 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 6x6 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 6 concentrations of the suspected deterrent compound and this was removed and replaced by untreated basal diet at 0500h.

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, Lawler unpublished) 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 x 10^{-5}.g dry weight^{-1}: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) dicholoromethane: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}C NMR data (in d4-methanol) with published values (Yamakoshi et al. 1992) confirmed the earlier identification.

RESULTS

Deterrency of Individual Trees to Feeding by Ringtail Possums

Our intial experiment clearly showed that there is great variation in the deterrency 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 body mass$^{-0.75}$.d^{-1}. 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 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).

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 deterrency 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 deterrency 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 Figures 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 (Lawler unpublished). 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 (Lawler unpublished). Only one *E. viminalis* and no *E. ovata* contained trace amounts of cyanide, thus cyanide did not appear to contribute to the deterrency 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 (Lawler unpublished).

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 0.73×10^{-5}moles.g dry weight^{-1} and at 5.87×10^{-5}moles.g dry weight^{-1} 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, #9, was 2.5 g.kg body mass$^{-0.75}$.d^{-1} (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.

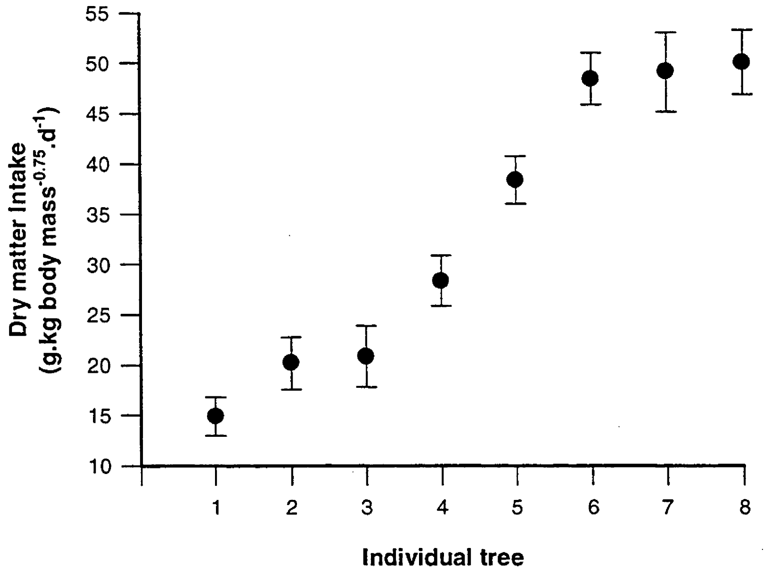

Figure 1. Voluntary intake of *Eucalyptus ovata* foliage by common ringtail possums fed foliage from individual trees in no-choice experiments.

Data are means ± std error for 8 animals (see methods for full protocol)

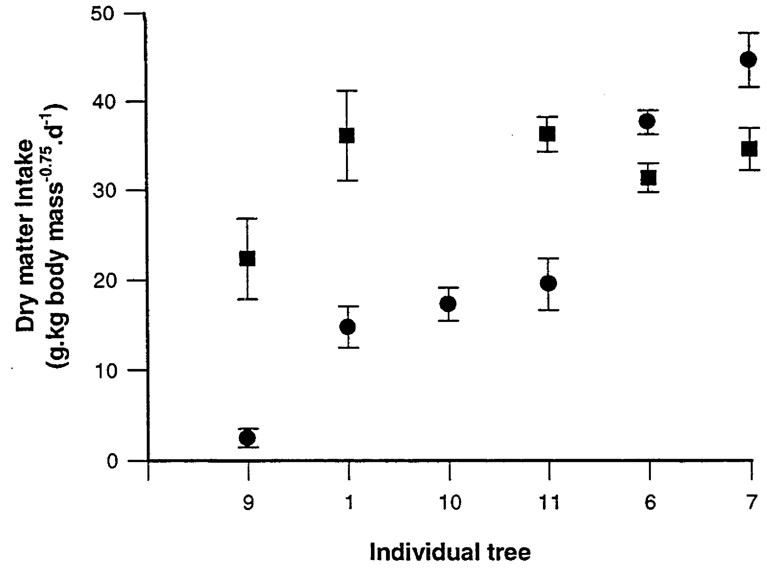

Figure 2. Voluntary intake of *Eucalyptus ovata* foliage by common ringtail possums (●) and koalas (■) fed foliage from individual trees in no-choice experiments.

Data are means ± std error for six animals for ringtail possums and five animals for koalas (see methods for full protocol).

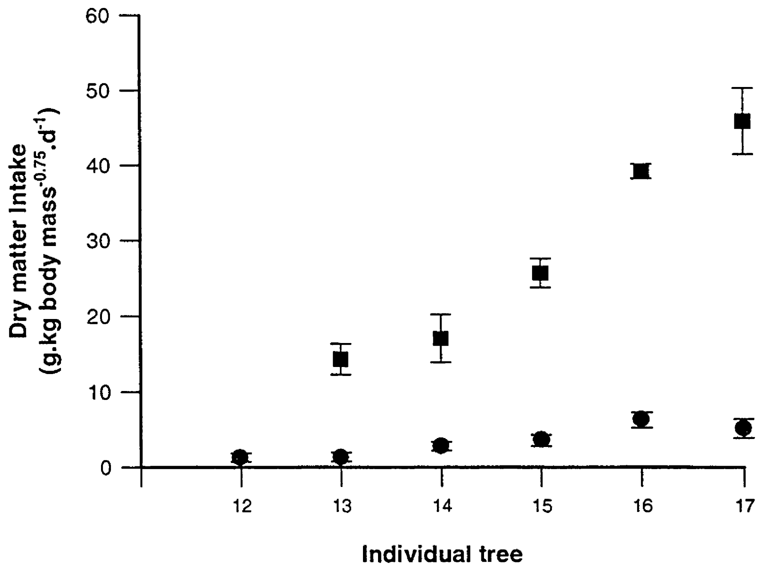

Figure 3. Voluntary intake of *Eucalyptus viminalis* foliage by common ringtail possums (●) and koalas (■) fed foliage from individual trees in no-choice experiments.

Data are means ± std error for six animals for ringtail possums and five animals for koalas (see methods for full protocol)

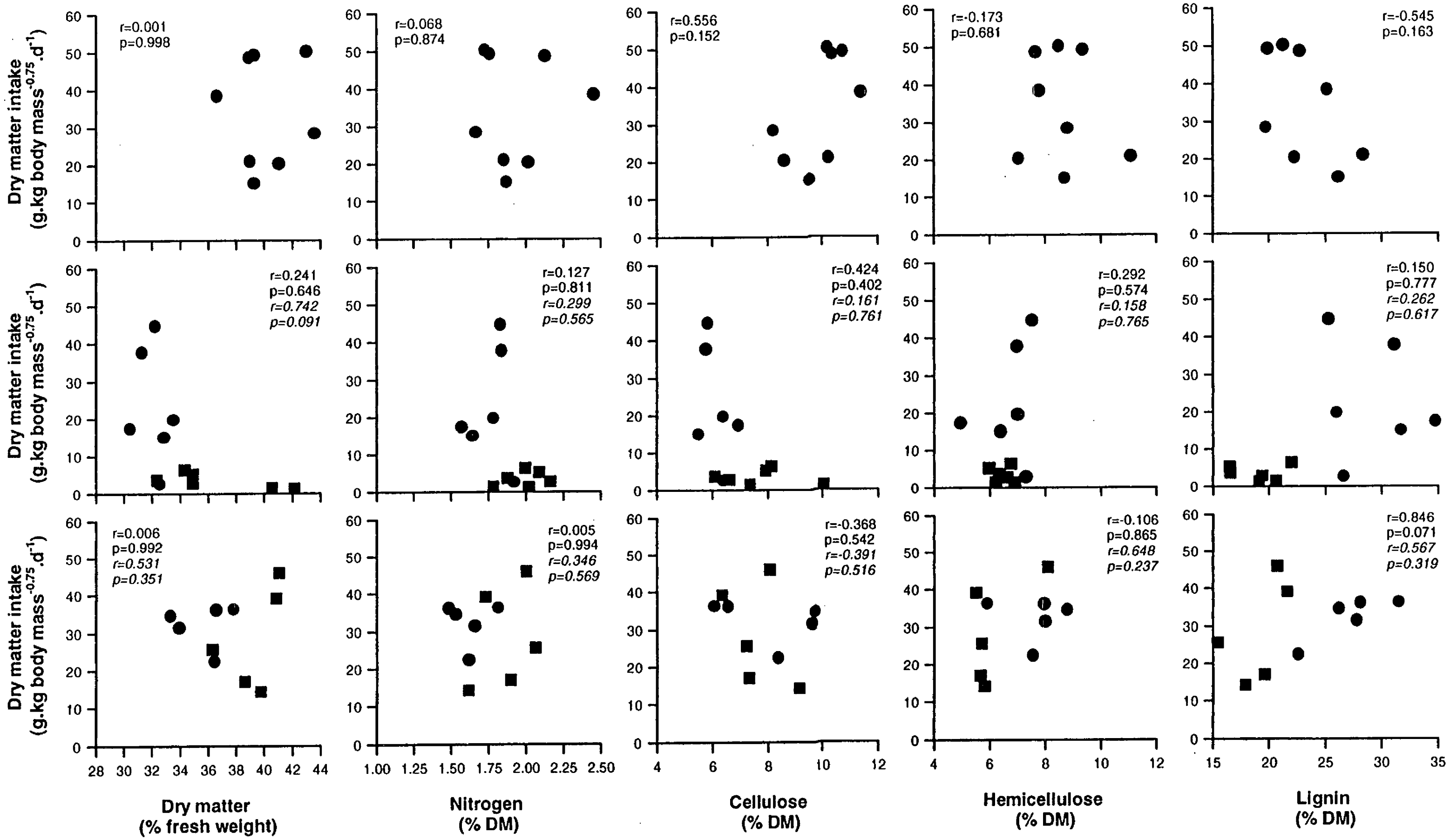

Figure 4. Relationships between mean intakes of foliage from experiments shown in figures 1 to 3 and nutritional characteristics of the leaves. (Top row: ringtail possum data shown in figure 1.
Second row: intakes by ringtail possums of *E. ovata* (●) and *E. viminalis* (■). Bottom row: Intakes by koalas of *E. ovata* (●) and *E. viminalis* (■). Numbers given are values of Pearson correlation coefficients and their corresponding probability values for *E.ovata* (normal font) and *E. viminalis* (italics).

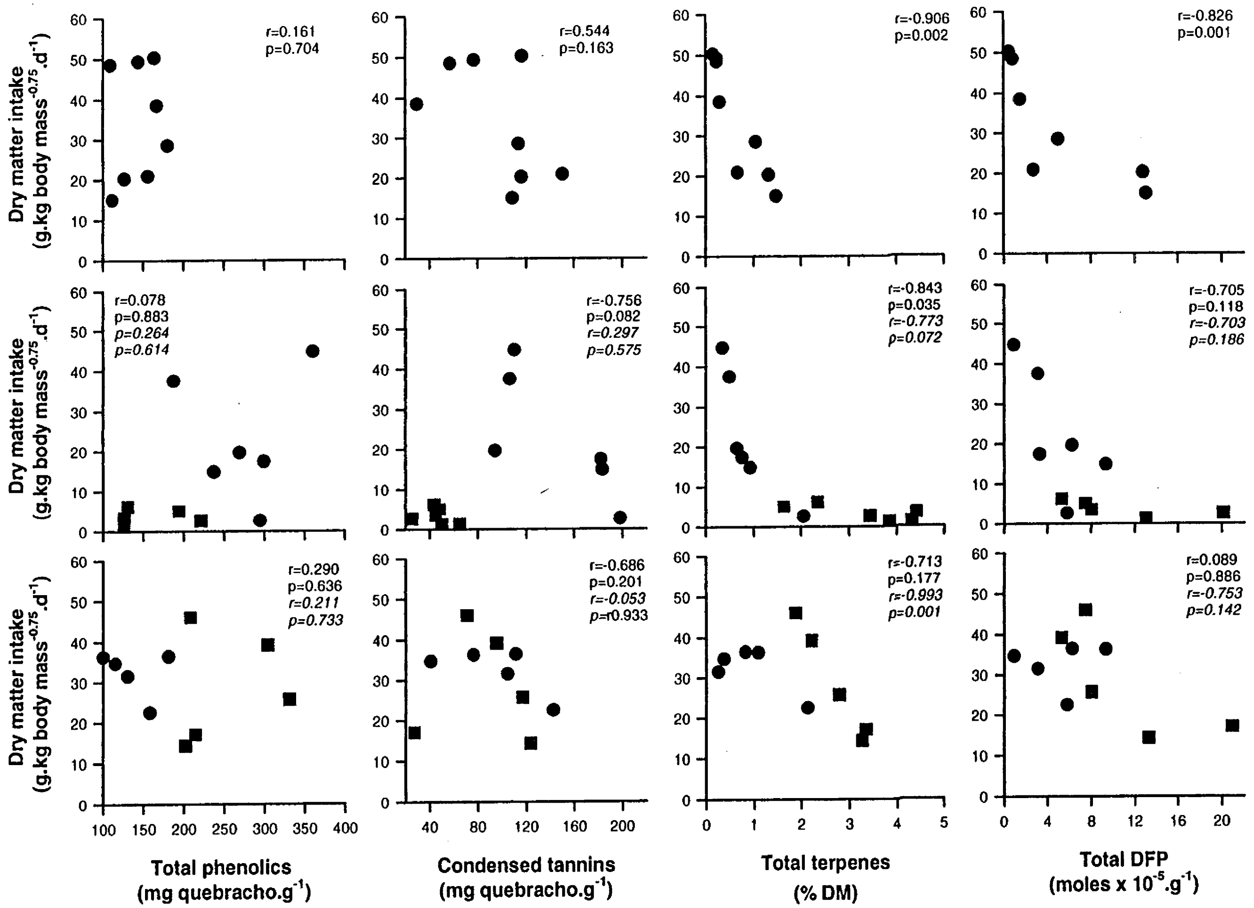

Figure 5. Relationships between mean intakes of foliage from experiments shown in figures 1 to 3 and reputed deterrent compounds of the leaves. Legend as for figure 4.

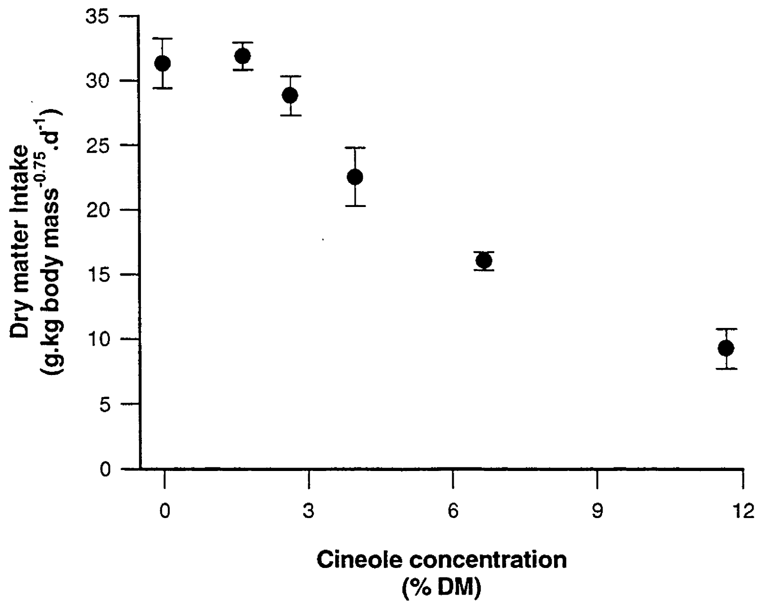

Figure 6. Relationship between voluntary intake of an artificial diet by common ringtail possums in no-choice experiments and the concentration of cineole added to the diet. Data are means ± std error for six animals.

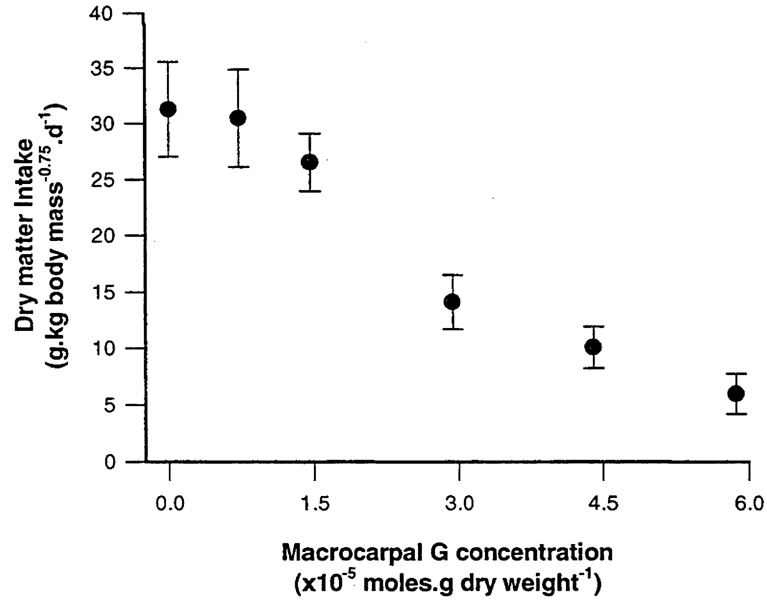

Figure 7. Relationship between voluntary intake of an artificial diet by common ringtail possums in no-choice experiments and the concentration of macrocarpal G added to the diet. Data are means ± std error for six animals.

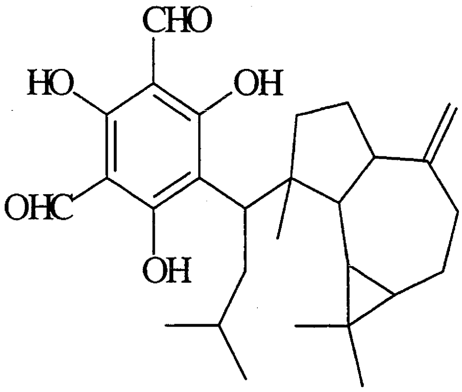

Figure 8. Structure of macrocarpal G.

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 (Lawler and Foley personal observation). Both koalas and ringtail possums appear to smell leaves carefully before ingestion (Zoidis and Markowitz 1992, 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 200mg. 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 (Lawler, Pass, Foley unpublished). Common ringtail possums regulated ingestion to limit jensenone intake to very similar molar quantities as seen here for macrocarpal G. Jensenone is a powerful antifeedant for both brushtail and ringtail possums and its action is mediated through the release of $5HT_3$ (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 Provenza et al.'s (1994) arguments 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 antifeedant. Clearly further experiments exploring the interrelations between volatile terpenes, DFPs and feeding deterrency are needed to evaluate these scenarios.

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 Foley unpublished). 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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CHAPTER 3. ADMINISTRATION OF A $5HT_3$ RECEPTOR ANTAGONIST INCREASES THE INTAKE OF DIETS CONTAINING *EUCALYPTUS* SECONDARY METABOLITES BY MARSUPIALS.

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ABSTRACT

The effect of a naturally occurring plant phenolic constituent (the acylphloroglucinol derivative, jensenone, derived from *Eucalyptus jensenii*) on the food intake of two folivorous marsupials, the common ringtail (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*) was studied. When fed diets containing varying concentrations of jensenone, both species regulated their intake of jensenone so as not to exceed a ceiling intake. This ceiling was about twice as high for common ringtails as for common brushtails from Northern Australia. Southern populations of common ringtails showed greatly reduced capacities to tolerate jensenone. When common brushtails were injected (0.5 $mg.kg^{-0.75}$ body mass) with ondansetron (a selective antagonist of serotonin $5HT_3$ receptors), they ate significantly more jensenone than animals injected with physiological saline. The same pattern was observed when common ringtails were fed diets containing both jensenone and ondansetron (0.035 $mg.g^{-1}$ wet mass of diet). Ondansetron injection had no effect on food intake when the food did not contain jensenone while addition of higher doses of ondansetron to diets of common ringtails very slightly reduced food intakes of a non-jensenone diet. When common brushtails were given 50mg of jensenone by gastric lavage, their average subsequent intake of dietary jensenone matched the difference between the daily threshold and the dose given, though the response of individuals was highly variable. Lavage with water alone had no effect on subsequent jensenone intake compared with the pre-dose period. We interpret these results as evidence that the antifeedant effects of jensenone and related compounds are partly mediated by serotonin acting on $5HT_3$ receptors most likely via "nausea" that conditions a food aversion.

Keywords serotonin, marsupial, *Eucalyptus*, emesis, conditioned food aversion, *Pseudocheirus*, *Trichosurus*, ondansetron

INTRODUCTION

Many vertebrate herbivores feed so as not to exceed a threshold dose of particular plant secondary metabolites (Meyer and Karasov 1989; Jakubas et al. 1993; Pass et al. 1998). In effect, feeding is regulated so that the animal's intake of dry matter is reduced as the concentration of plant secondary metabolite in the diet is increased. In most cases, these patterns are very tightly controlled and there may be no significant increase in the intake of the plant secondary metabolite even when the dietary concentration is increased 5-10 fold (e.g. Jakubas et al. 1993).

How is this impressive regulatory feat achieved? Many authors have speculated on the effect of plant secondary metabolites on mammalian herbivores but there have been few demonstrations of the effects of particular compounds on animal metabolism that could result in specific feedback signals. Foley (1992) and Foley et al. (1995) argued that the effects of many plant secondary metabolites could be ascribed to disturbances in acid-base metabolism but they could find no evidence for specific regulatory mechanisms. Guglielmo et al. (1996) suggested that dilution of useable nutrients by plant secondary metabolite's was responsible for the selective foraging of ruffed grouse (*Bonasa umbellus*) but again it is not clear how this dilution effect could function as a feedback control.

In contrast, Provenza and co-workers (e.g. Provenza 1995; 1996; Provenza et al. 1990; 1992) have argued that animals feeding on diets rich in plant secondary metabolites develop conditioned food aversions mediated by feedback from the "emetic centre" of the medulla. They hypothesized that animals should adjust their feeding so as not to exceed some threshold intake on the basis of this feedback. Provenza et al. (1992) argued that this feedback does not have to result in overt illness and that the animal need not even be conscious of the event that acts as a powerful trigger to provide sufficient signal for the animal to modulate its intake. Thus Provenza and co-workers have identified a possible regulatory mechanism that could explain why animals feeding on food containing plant secondary metabolites do, in fact, limit their food intake.

There are difficulties with interpreting feedback from plant secondary metabolites as emesis or vomiting *per se*. Many herbivores do not vomit and, even in those species that do, the degree to which we can attribute our perception of nausea to animals remains uncertain (Andrews et al. 1988; Veyrat-Follet et al. 1997). Secondly, emesis can result from many different pathways, all mediated by different neuroactive agents (Veyrat-Follet et al. 1997). Finally, previous work in this area has been conducted with rats and sheep given LiCl (Provenza 1995; 1996; Provenza et al. 1992; 1994) rather than naturally occurring plant secondary metabolites. Consequently it is difficult to interpret physiologically Provenza's concept of "emetic stimulation" in the context of herbivores eating natural plant diets.

In this study we address these difficulties by examining the response of the marsupials *Trichosurus vulpecula* (common brushtail possum) and *Pseudocheirus peregrinus* (common ringtail possum) to dietary jensenone. Jensenone (Fig. 1) is an acyl-phloroglucinol derivative found in the foliage of *Eucalyptus jensenii* (Boland et al. 1992; Ghisalberti 1996) and is a simple form of the complex phloroglucinol-terpene adducts (e.g. macrocarpal G; Fig. 1) that are responsible for the selective feeding of these marsupial folivores (Pass et al. 1998; Lawler et al. 1998).

We sought first to establish whether the animals regulated their intake of food according to the concentration of jensenone in the diet and secondly, whether we could increase voluntary food intake by injecting the animals with a potent serotonin ($5HT_3$) receptor antagonist, ondansetron. Serotonin at the $5HT_3$ receptor mediates part of the emetic response in humans and laboratory animals. Ondansetron is widely used to control nausea and vomiting during some cancer therapies (Butler et al. 1988) and aids in restoration of normal food intake of chemotherapy patients (Beck 1992). We expected that if serotonin acting at the $5HT_3$ receptor mediated the antifeedant effects of jensenone, then administration of a drug such as ondansetron would lead to a significant increase in food intake relative to controls.

MATERIAL AND METHODS

Animals and Basal Diets

This research was approved by the Animal Experimentation Ethics Committees of James Cook University and the Australian National University and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twelve common brushtail possums (*Trichosurus vulpecula*) and 12 common ringtail possums (*Pseudocheirus peregrinus*) were caught by hand in woodland near Townsville (northern Australia). A further 12 common ringtail possums were collected near Canberra (southern Australia) for later experiments. Animals were maintained in individual metabolism cages in an air-conditioned room maintained at 21± 2°C. The room was maintained on a 12:12 light:dark regime and the lights were connected to a dimmer that allowed gradual changes in the light intensity to simulate dawn and dusk. Both species are nocturnal feeders in nature but in captivity the common brushtail may feed irregularly during the day.

All animals were fed a palatable basal diet that consisted of (% wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% lucerne hay ground to pass a 2 mm sieve, 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. These analyses followed methods as described in Foley (1992) and Lawler et al. (1997). 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 food refusals was determined by drying all refusals for 24 h at 80°C. Free drinking water was always available *ad libitum.*

Experiment 1 - The Effect of Jensenone on Dry Matter Intake of Common Ringtail and Brushtail Possums.

Common Ringtail Possums

We measured the dry matter intake of six adult (four male, two female) common ringtail possums (mean body mass 0.74 kg) fed six concentrations of jensenone in an basal diet. The protocol used was a no-choice experiment as a 6 x 6 Latin square. The concentrations used were: 0, 0.06, 0.12, 0.24, 0.36 and 0.48% of the wet weight of the diet and the diets were prepared as described above. Treated food was provided *ad libitum* at 1700 h (one hour before the dark period). Urine was collected into plastic bottles set in a slurry of solid CO_2 and kept frozen at -20°C until assayed for glucuronic acid (Blumenkrantz and Asboe-Hansen 1973) as a potential index of biotransformation of jensenone (Foley et al. 1995).

The treated food was replaced with untreated basal diet at 0500 h (1h before the end of the dark period) because animals offered high concentrations of jensenone had eaten very little. Common ringtail possums are very reluctant to feed outside of their usual nocturnal period and this ensured they had some time to feed. However, they typically ate only a small amount during the remaining period of darkness (< 35% of normal intake), so treatment days were alternated with days when only untreated basal diet was offered, to ensure the welfare of the animals and reduce carry-over effects between periods.

Common Brushtail Possums

Experiments carried out with brushtail possums followed the same design as for ringtails with six adult males (mean body mass 2.28 kg) and the same jensenone concentrations used. Food was offered at 1630 h and removed at 0830 h, when *ad libitum* amounts of untreated basal diet were provided. This was removed at 1200 h. As brushtail possums are more inclined to feed outside of their normal nocturnal period, their intakes of the untreated basal diet were consequently higher and it was not considered necessary to alternate treatment days with non-treatment days for these animals.

Experiment 2. - Effect of Ondansetron on Intake of Dry Matter and Jensenone in Common Brushtail and Common Ringtail Possums.

A. Intraperitoneal Injection of Ondansetron

Eight common brushtails (mean body mass 2.20 kg) and, in a separate experiment, eight common ringtails (mean body mass 0.74 kg) were allocated among four treatments in double Latin square designs in which we measured dry matter intake and jensenone intake. Jensenone was added to the diets at a concentration of 0.2% (wet weight) (brushtail) or 0.35% (wet weight) (ringtail) of basal diet. Treatments were: Dietary jensenone plus an injection of 0.5 ml of 0.9% sterile saline; Dietary jensenone plus an intraperitoneal injection of 0.5 $mg.kg^{-0.75}.d^{-1}$ of ondansetron; Basal diet plus an injection of ondansetron, and; Basal diet plus saline. Protocols for feeding the animals were as for experiment 1, except that for the common ringtail possums, the beginning of the dark period was changed to an hour earlier a week prior to the commencement of the experiment. This was to ensure that the ondansetron injection was given as close to the beginning of the feeding period as possible. We purchased ondansetron ("Zoffran": Glaxo) as an aqueous solution (2 $mg.mL^{-1}$) and used the contents of a single 2 mL vial within 30 minutes.

B. Dietary Administration of Ondansetron

In common ringtail possums there was no effect of intraperitoneal ondansetron on jensenone intake, which contrasted with the results for common brushtail possums (see below). We reasoned that this was due to different feeding behaviours after injection which resulted in the common ringtail possums not feeding until much of the injected ondansetron had been metabolized (see Discussion). The experiment was therefore repeated at a later date (with common ringtail possums collected in southern Australia) with ondansetron mixed with the diet to maintain higher plasma levels of ondansetron while feeding on the jensenone-treated diet. Ondansetron was purchased as 8 mg tablets ("Zoffran": Glaxo), crushed in a mortar and pestle and added at 0.035 $mg.g^{-1}$ (wet weight) of the diet. The experimental design was as described above with a jensenone concentration reduced to 0.12% (wet weight) due to an apparently lower threshold for jensenone in common ringtail possums from southern Australia.

Experiment 3. - Effect of Jensenone Given by Gastric Lavage on Intake of Dry Matter and Jensenone in Common Brushtail Possums.

Eight male brushtail possums (mean body mass 2.27 kg) were randomly assigned to two groups, test ands control. All animals were fed the basal diet with 0.1% (wet weight) jensenone *ad libitum.* The four animals in the test group were given 30 mg of jensenone by gastric lavage so they could not taste it. Jensenone was administered dissolved in 1 ml of 2.0% $NaHCO_3$ and the lavage tube then flushed with 2 ml of water to ensure the correct dosage. This was amount approximately 30% of the total amount of jensenone the animals were voluntarily ingesting (see results for experiment 2). Therefore, if jensenone was a metabolic deterrent and caused post-ingestive effects, dry matter intake should be reduced by approximately 30% relative to control values. The four possums in the control group were dosed in the same way with 0.9% saline. This experiment was repeated with a second group of eight male brushtail possums (mean body mass 2.32 kg), because the results of the first experiment were equivocal. In this experiment the animals were dosed with 50 mg of jensenone but all other procedures were the same.

Extraction and Purification of Jensenone.

Eucalyptus jensenii foliage was collected from natural stands at Mount Bundy in the Northern Territory of Australia and from a small number of cultivated trees at Gympie in SE Queensland. The foliage was air-dried and ground to pass a 2 mm screen and 1 kg lots were extracted in 20% acetone:light petroleum for 6 h in a Soxhlet apparatus. The extracts were concentrated and combined with 1 L of di-ethyl ether and then washed 2-3 times with 0.3M NaOH. These washes were acidified (12M HCl) and the precipitate washed with ethanol and re-crystallized from acetone to give jensenone (98% by ^1H NMR) in a yield of about 2.8% (DM).

Statistical Analysis

In all experiments, possible differences in mean dry matter intake and jensenone intake were analysed by analysis of variance. Terms accounting for the main effects of possum, day, treatment and possible carry-over effects of treatments were included in this analysis. Where carryover effects were found to be highly non-significant ($P > 0.35$) they were dropped from the model and the data re-analysed (Ratkowsky et al. 1993). Where jensenone intake was compared between treatments, the treatment with no jensenone was omitted from the analysis. In experiment 2 we were less interested in overall differences between treatments, but rather between specific combinations of treatments. These comparisons have therefore been made using t-tests.

Measurements of intake of plant secondary metabolites and excretion of metabolites have been scaled to $M^{0.75}$ because several studies (Walker 1978; Freeland 1991) have shown that interspecific detoxification capacity scales to this exponent. Body mass in each experiment is provided to allow alternative scaling factors to be calculated.

RESULTS

Dose-Response Experiments in Common Brushtail and Ringtail Possums

Increasing the concentration of jensenone in the diet of both common brushtail and common ringtail possums led to significant reductions in dry matter intake (DMI) ($p < 0.0001$ for both species, Fig. 2a). In both species, at jensenone concentrations above approximately 0.12% (wet weight), DMI tended to decrease with increasing jensenone concentrations. Both species appeared to regulate food intake to remain below a ceiling dose of jensenone (Fig. 2b). Brushtail possums were unwilling to ingest more than approximately 70 $mg.kg^{-0.75}.d^{-1}$ and this was constant across all concentrations of jensenone ($p = 0.108$). The amount of jensenone eaten by common ringtails varied significantly ($p = 0.002$), but this was because the amount eaten was lower at 0.06% jensenone, perhaps due to partial satiation reducing their willingness to take in the compound. If this concentration was omitted, jensenone intake was shown to be regulated at a constant level ($p = 0.124$), much higher than the common brushtails, of approximately 140 mg $.kg^{-0.75}.d^{-1}$.

Only trace amounts of glucuronic acid were excreted by both species and this was independent of the jensenone intake (brushtails $p = 0.349$, ringtails $p = 0.521$). The mean excretion rates were 111 $\mu mol.kg^{-0.75}.d^{-1}$ in the common ringtails and 102 $\mu mol.kg^{-0.75}.d^{-1}$ in the common brushtails.

Effect of Ondansetron on the Intake of Jensenone by Common Ringtail and Brushtail Possums

A. Intraperitoneal Injection of Ondansetron

Brushtail possums that received injections of ondansetron ate significantly more jensenone than those receiving injections of saline solution ($p < 0.001$, Fig. 3). Ondansetron alone had no effect on dry matter intake in the brushtails ($p = 0.652$). Nonetheless, ondansetron did not restore food intake of animals fed jensenone in the diet to the levels seen in the control groups ($p < 0.001$).

In contrast in ringtail possums, injections of ondansetron had no effect on the intake of jensenone ($p = 0.696$, Fig 3). Again, ondansetron itself did not affect dry matter intake ($p = 0.837$) but dry matter intake of the jensenone diet remained at about 30% of that observed in controls ($p < 0.001$).

B. Dietary Administration of Ondansetron

When ondansetron was added to the diet, thereby increasing internal levels of ondansetron simultaneously with jensenone ingestion, intakes of jensenone were substantially increased ($p < 0.001$, Fig 3) but again not fully restored to the level of intakes on control diets ($p = 0.001$). Ringtail possums very slightly reduced intake of diets treated with ondansetron only relative to untreated controls ($p = 0.020$).

Effect of Gastric Lavage of Jensenone on Intake of Jensenone-Containing Diet by Brushtail Possums

When the animals were dosed with 30 mg of jensenone (about 30% of their average voluntary intake of jensenone per unit metabolic body mass), there was a trend towards reduction in subsequent dry matter and jensenone intake, relative to controls (Fig. 4a) but there was no significant difference between either the pre- and post-dose intakes of the treated animals ($p = 0.424$) or the post-dose and expected intakes for the treated animals ($p = 0.295$). This was due to the highly variable responses of the animals: two animals reduced their intakes to close to the expected levels while the other two slightly increased their intakes. The intakes of the control animals in this experiment did not change ($p = 0.853$).

Increasing the oral dose to 50 mg (about 50% of the average voluntary intake per unit metabolic body mass) resulted in a greater reduction in subsequent intake of jensenone but again the data were sufficiently variable that this reduction was not significantly different from pre-dose values ($p = 0.176$). Much of the variability was due to two of the animals vomiting within 3 h of the lavage. Nonetheless, the average intake of jensenone was almost identical to that expected if jensenone acted in a dose dependent fashion ($p = 0.992$) (Fig. 4b). Again, animals lavaged with water as a control did not reduce their intake of jensenone compared with pre-dose values ($p = 0.869$).

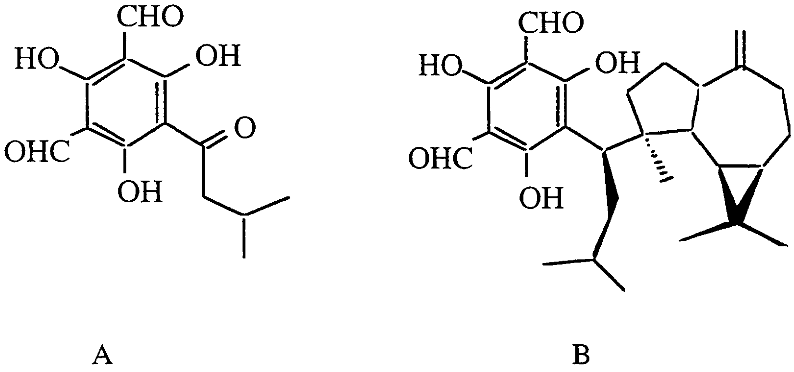

Figure 1. Planar structures of (A) jensenone and (B) macrocarpal G, acylphloroglucinol derivatives of *Eucalyptus*.

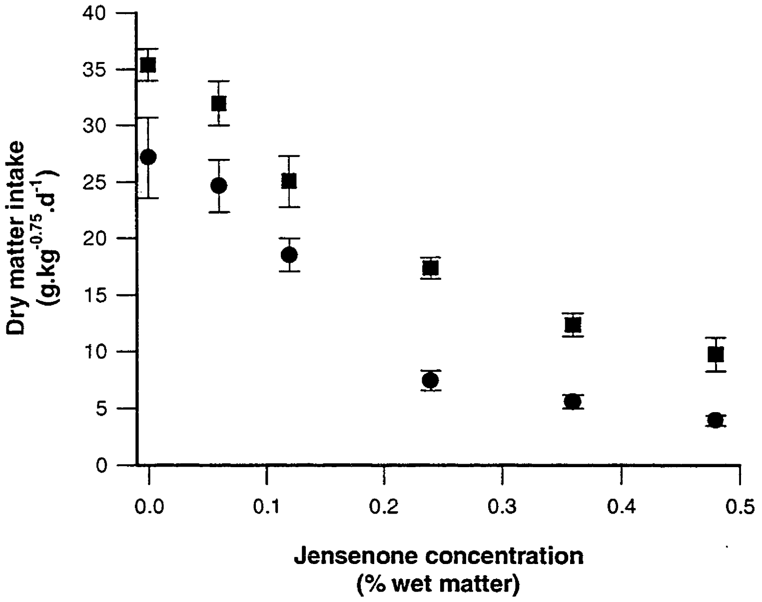

Figure 2 a. The effect of jensenone on the dry matter intake of common ringtail and brushtail possums offered food containing jensenone at one of six concentrations.
Squares are ringtail possums, circles are brushtail possums.

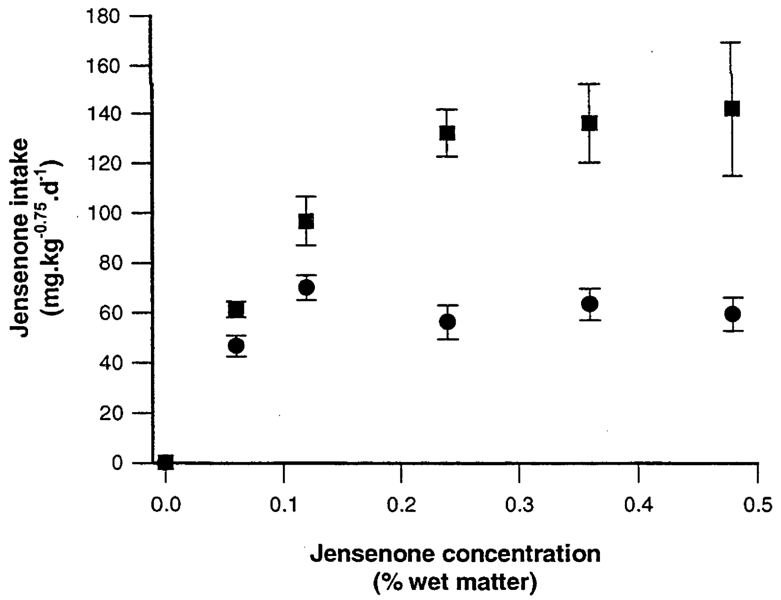

Figure 2 b. The effect of varying concentrations of dietary jensenone on the intake of jensenone in common ringtail and brushtail possums.
Squares are ringtail possums, circles are brushtail possums.

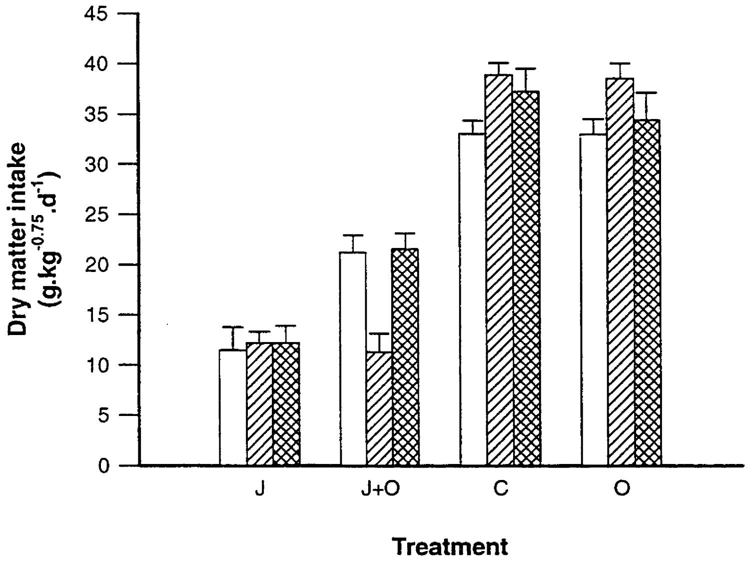

Figure 3. The effect of administration of ondansetron (O) or water (C) on dry matter intake of common ringtail possums and brushtail possums fed dietary jensenone (J).

Unshaded bars are brushtail possums injected intraperitoneally and fed 0.2% (wet weight) jensenone, diagonally shaded bars are northern Australian ringtail possums injected intraperitoneally and fed 0.35% jensenone, crosshatched bars are southern Australian ringtail possums administered ondansetron orally and fed 0.12% jensenone.

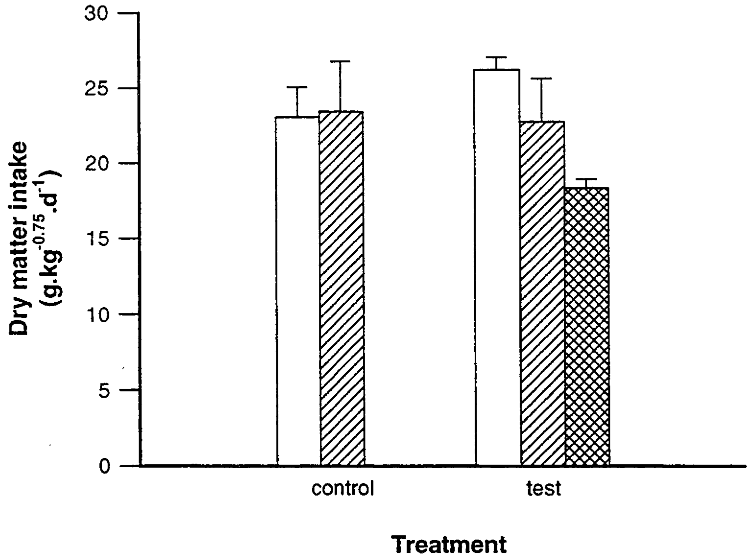

Figure 4a. The effect of 30 mg jensenone given by gastric lavage to common brushtail possums fed 0.1% (wet weight) dietary jensenone.

The expected dry matter intake is calculated assuming that jensenone causes a dose-dependent decrease in food intake as shown in Fig. 2. Unshaded bars are pre-dose intakes, diagonally shaded bars are post-dose intakes, crosshatched bar represents expected post-dose intakes.

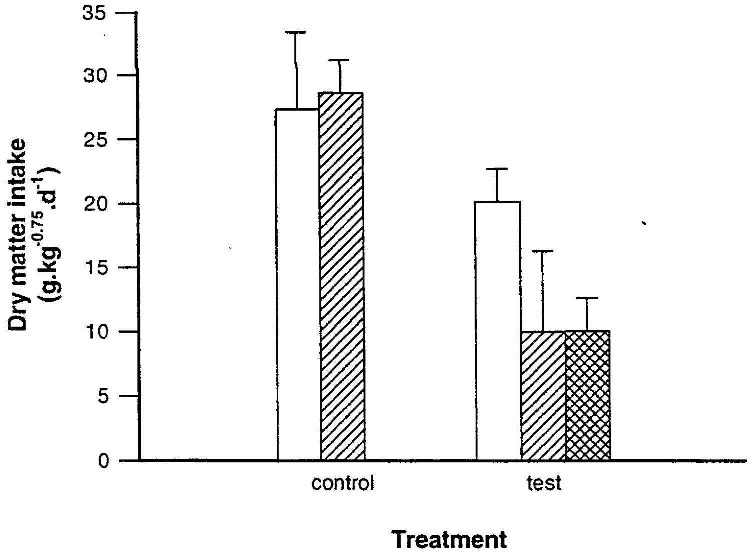

Figure 4b. The effect of 50 mg jensenone given by gastric lavage to common brushtail possums fed 0.1% (wet weight) dietary jensenone.

The expected dry matter intake is calculated assuming that jensenone causes a dose-dependent decrease in food intake. Legend as per Figure 4a.

DISCUSSION

Regulation of Toxin Intake is Mediated by Serotonin

This study has provided clear evidence that both common ringtail (*Pseudocheirus peregrinus*) and common brushtail possums (*Trichosurus vulpecula*) regulated their feeding so as not to exceed a threshold intake of jensenone and that at least part of this regulatory ability is mediated by serotonin. Because ondansetron is such a selective antagonist of serotonin $5HT_3$ receptors (Butler et al. 1988), we can be certain that part of the antifeedant action of jensenone is mediated via serotonin through this receptor site but it remains uncertain exactly where the receptors are located. Jensenone may cause the release of serotonin from enterochromaffin cells in the small intestine (Veyrat-Follet et al. 1997). Serotonin receptors may thus be found in the gut on visceral afferent fibres which in turn carry signals to the brain or, less likely, the toxins may be carried in the systemic circulation and exert a central effect at the chemoreceptor trigger zone.

Other related research has used less selective anti-emetic agents, or mixtures of agents, which make interpretation of results more difficult. Two earlier studies used either high doses of metoclopromide (Aldrich et al. 1993) or else cocktails of metoclopromide, dexamethazone (which enhances the effects of metoclopromide) and diphenhydramine (Provenza et al. 1994) to attenuate effects of either LiCl or alkaloid-infected grass on food intake of sheep (Aldrich et al. 1993). Although both studies showed an elevation of food intake in response to the drugs, the less selective nature of the agents makes it difficult to explain the results in terms of specific physiological pathways. Metoclopromide in particular, is primarily a dopamine (D_2) receptor antagonist but at high doses also acts as an antagonist of $5HT_3$ receptors (Andrews et al. 1988; Veyrat-Follet et al. 1997). High doses of metoclopromide can also have significant effects on gastrointestinal motility (Veyrat-Follet et al. 1997) and although the same is true for ondansetron (Butler et al. 1988), the lower doses of this agent that are necessary to attenuate emesis, means that these side effects are less likely to occur and did not occur in this study. Accordingly, we conclude that ondansetron affected food intake through its action as a $5HT_3$ receptor antagonist rather than through any action on gut motility.

Although it is valuable to be able to specifically identify receptor sites that are involved in modulating feeding, we can still not be certain whether the feedback that is used as a regulatory point is due to nauseous sensations. Nausea remains a subjective sensation (Andrews et al. 1988; Veyrat-Follet et al. 1997) and we do not know whether animals perceive the same sensations that we do. In the case of the brushtail possums, a bolus dose of jensenone given by lavage caused vomiting in two animals, so we could cautiously interpret the effects of ondansetron in terms of a reduction in the nauseous sensations induced by ingestion of jensenone (Veyrat-Follet et al. 1997). However, to an unsatisfactory extent, we can only say with certainty that the feedback which allows marsupials to regulate their intake of jensenone is mediated in part through $5HT_3$ receptors.

Because of these uncertainties, it seems best to discontinue use of the general term "emetic stimulation" when referring to the mechanism by which herbivores regulate their intakes of plant secondary metabolites. Several groups of herbivores appear not to have the ability to vomit and this makes it difficult to attribute to them feelings of nausea. While it may be possible in some circumstances to make objective measurements of physiological traits related to nausea, such as plasma vasopressin (Andrews et al. 1988), difficulty will still be encountered in calibrating these measures against the animals' perceptions. A further problem may be encountered if these measurements are not appropriate for the particular emetic stimulus. For example, while plasma vasopression may increase in response to an emetic stimulus, other stimuli do not increase vasopressin (Andrews et al. 1988). In contrast, describing the feedback processes as envisaged by Provenza and co-workers in terms of specific receptors, draws attention to the many other effects mediated by these pathways and raises the possibility that receptor-based differences between herbivore species and individuals may be consistent with different tolerances for specific toxins, for example those that we have observed between common ringtail and brushtail possums.

Although we have identified a partial control mechanism that explains how jensenone intake is regulated, we still do not know whether jensenone has more widespread effects or what its metabolic fate is in the animals. The increase in jensenone intake when animals were administered ondansetron, together with the effect of gastric lavage on jensenone intake of brushtails, confirms that jensenone exerts its effects, not through taste or smell, but by causing an "internal malaise" (sensu Provenza 1995). Making the link between the regulatory mechanism and toxicological processes is an important goal in understanding the effects of plant secondary metabolites on mammalian foraging.

Jensenone is a diformylphloroglucinol derivative that shares its core phenolic moiety with a range of other compounds that have been identified as natural feeding deterrents in *Eucalyptus* such as Macrocarpal G (Fig. 1; Lawler et al. 1998; Pass et al. 1998). All members of this family of compounds contain a phenolic moiety linked to a terpene but in the case of jensenone, the full mono- or sesqui- terpene found in most of the compounds is replaced by an isoprene unit. This lipid-soluble side chain is presumed to facilitate the passage of the compound across membranes and so carry the reactive phenol and aldehyde groups to sites where they may damage cells (Pass et al. 1998). We have not been able to recover jensenone, or its metabolites, from the faeces or urine and thus assume that the whole dose has been absorbed (S. McLean, S. Brandon and W. Foley unpublished data).

Inter- and Intra- Specific Differences in Response to Jensenone and Ondansetron

In our initial experiments, where results are directly comparable between species, common brushtails consumed only half the amount of jensenone eaten by common ringtails. Common brushtails are perceived as generalist browsers whereas the greater reliance of the smaller sized common ringtails on *Eucalyptus* foliage has led to them being labelled as specialists (e.g. McArthur and Sanson 1993). Although we corrected jensenone intakes for metabolic body size, the difference between the two species persisted. Irrespective of the size of organs such as liver, smaller species with their higher mass-specific metabolic weight should be able to biotransform and clear toxic components from the body more rapidly than larger species (Freeland 1991). Since detoxification capacity scales to about the same exponent as does metabolic rate (Walker 1978; Freeland 1991), we could expect that expressing intake in this way should have eliminated the differences between the species. That it did not, suggests that in the Northern Australian populations of animals used, the common ringtails possess mechanisms that allow them to tolerate and/or excrete jensenone and its metabolites over and above the advantages conferred by body size alone.

It is perplexing then, that in our later experiment with ringtail possums from Southern Australia the threshold jensenone intake was substantially reduced. We propose two hypotheses. The first is that perhaps there is a geographic difference in the ability of this species to tolerate jensenone. The initial experiments were conducted in Northern Australia while the latter experiment (oral dosing of ondansetron) was conducted with possums collected some 2500 km south. Whether there are genetic differences between populations in their capacity to detoxify these compounds, or perhaps phenotypic differences, due to to different levels of exposure to diformylphloroglucinol derivatives is uncertain at this time. The latter seems unlikely as these compounds are found in most *Eucalyptus* species and there is no reason to suspect they are in higher concentrations in northern species (Pass, Foley, Eschler unpublished data).

The second explanation is a component of learned avoidance of jensenone or reduced physiological capacity due to repeated exposure to this and related compounds. The animals used in the second experiment had previously been occasionally fed leaf diets high in sideroxylonals (dimers of jensenone - Ghisalberti 1996) and had also been used in two other experiments using jensenone (Lawler, Foley, Eschler unpublished data). Over these last three experiments there was also a smaller but notable decrease in their jensenone threshold from approximately 80 to 50 $mg.kg^{-0.75}.d^{-1}$ (Lawler unpublished). This suggests some reduction in threshold due to repeated exposure, but the difference between northern animals and southern animals at their first captive exposure to jensenone was nevertheless significant.

We do not believe differences in the purity of jensenone between experiments were important. Our data show that all jensenone samples were highly pure and any differences certainly could not account for a three-fold decrease in the threshold. Experiments with southern populations of brushtail possums conducted concurrently with these later ringtail possum experiments, and using the same batch of jensenone, did not show a corresponding decrease in threshold (threshold approx. 60 $mg.kg^{-0.75}.d^{-1}$; J Stapley personal communication) reinforcing our impression that there are differences between populations of ringtail possums in their capacity to tolerate, or detoxify, jensenone. Controlled experiments with possums of both regions tested simultaneously, to avoid various confounding factors, are required to unequivocally demonstrate interpopulation differences in jensenone tolerance.

Differences in the feeding patterns of the two species are most likely responsible for differences in their response to injected ondansetron. Brushtail possums commenced feeding immediately after the injection of ondansetron and presumably fed when plasma concentrations of the drug were highest. However, the food intake in animals injected with ondansetron was still less than that of controls, suggesting either that there is some other feedback that is limiting the intake or more likely that the drug was cleared from the plasma rapidly enough to render it ineffective in the latter part of the night. In humans, ondansetron must be re-injected every 8 hours or oral supplements taken to maintain effective plasma concentrations (Butler et al. 1988; Rudd and Naylor 1996). Even though the dose we gave both possums was about 10 times the normal human dose, it must have still be cleared rapidly enough to reduce its effectiveness and so may not have been effective for the duration of the 12 h feeding period (F. Mitchelson, personal communication).

We believe that a similar effect explains the lack of effect of injected ondansetron in the common ringtails. In contrast to the brushtails, the ringtails retreated to their nest boxes immediately after the injection and showed little inclination to feed in the first four hours after the injection. This coupled with their smaller size (which should lead to a more rapid clearance of the drug), meant that the dose that we were able to give them was ineffective when their feeding was at its peak. Oral dosing via addition of ondansetron to the diet, which presumably raised plasma ondansetron levels to correspond with jensenone intakes, confirmed that the drug could at least partly ameliorate the effects of jensenone.

CONCLUSION

Understanding how plant secondary metabolites affect animal food choice is an important step in quantifying the metabolic costs for certain diets and foraging patterns. Other mechanisms which might control the ingestion of plant secondary metabolites have been proposed (e.g. acid-base regulation: Foley et al. 1995) but these experiments are the first to demonstrate a specific regulatory mechanism. It remains now to demonstrate what the effects of jensenone are in the animals and to determine whether the intake of other plant secondary metabolites are regulated in a similar fashion but clearly, serotonin acting at the $5HT_3$ receptor is a major factor regulating feeding in these marsupials.

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CHAPTER 4. AN ECOLOGICAL EXAMPLE OF A CONDITIONED FLAVOR AVERSION IN PLANT-HERBIVORE INTERACTIONS: THE EFFECT OF TERPENES OF *EUCALYPTUS* LEAVES ON FEEDING BY COMMON RINGTAIL AND BRUSHTAIL POSSUMS

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ABSTRACT

We investigated the hypothesis that feeding deterrence of common ringtail possums (*Pseudocheirus peregrinus*) and common brushtail possums (*Trichosurus vulpecula*) by *Eucalyptus* terpenes (in this case 1,8-cineole) is a result of a conditioned flavor aversion (CFA), due to the association of terpenes with post-ingestive effects of another group of *Eucalyptus* toxins, the diformylphloroglucinol compounds (DFPCs). Wild-caught common ringtail and common brushtail possums showed a dose-dependent reduction in food intake when 1,8-cineole was added to the diet. However, after continued exposure, over 12 days, to increasing amounts of cineole in the diet, both species substantially increased their intakes of cineole relative to control animals. This indicated that the aversion to cineole was a conditioned response rather than a physiological limitation in their ability to detoxify terpenes. Subsequent exposure to a diet including both cineole and jensenone (a simple DFPC also found in *Eucalyptus* and considered to cause postingestive emesis) in corresponding amounts was able to recondition the dose-dependent aversion. Consequently, animals which had been given jensenone showed an aversion to cineole-rich diets which matched that of animals in the control group. This supported our hypothesis that the effect of terpenes on feeding of these marsupial folivores of *Eucalyptus* is due to a CFA. Possums can cope with levels of terpenes in the diet which far exceed those occurring naturally. The role of terpenes in marsupial folivore-*Eucalyptus* interactions appears to be to act as a cue to the levels of toxic DFPCs in the leaves, rather than acting as toxins in their own right.

Keywords Conditioned flavor aversion, plant toxin, *Eucalyptus*, *Pseudocheirus*, *Trichosurus*, terpene, cineole, jensenone, food intake, emesis

INTRODUCTION

Conditioned flavor aversions are considered to be an important mechanism modulating diet choice in free-ranging herbivores (Provenza, 1996). Animals are thought to regulate their intake of some plant secondary metabolites (PSMs) below toxic levels by learning to associate a distinctive taste of a diet with negative postingestive feedback resulting from the PSM. Studies of this phenomenon in browsing mammals have mostly been conducted using ruminants and have used toxins (principally LiCl: e.g. Wang and Provenza, 1997) and/or artificial flavors such as orange or onion as the taste stimulus (e.g. Ralphs et al., 1995; Wang and Provenza, 1997) which do not occur naturally in the diets studied. Although these studies have shown how CFAs could develop in free-ranging animals, the stimuli used are not ecologically realistic. In contrast, Kyriazakis et al. (1997) used two naturally occurring plant secondary metabolites (PSMs) to condition aversions in sheep but again they used flavor stimuli which the animals are unlikely to encounter in nature. In this study we examine the development of a conditioned flavor aversion in the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*), both arboreal marsupial folivores of *Eucalyptus*. We used both a flavor stimulus (1,8-cineole - a monoterpene) and a toxin (jensenone - a diformylphloroglucinol compound- see below) which occur together in *Eucalyptus* leaves.

It has been suggested that terpenes deter feeding of a wide range of browsing mammals, including the marsupial folivores of *Eucalyptus* (e.g. Southwell, 1978; Hume and Esson, 1993) because small amounts can be toxic to some animals (McLean and Foley, 1997). However, correlations between food intake and the concentrations of foliar terpenes have explained little, if any, of the differences in foliage intake by these animals (Southwell, 1978; Hume and Esson, 1993). In contrast, we showed that intraspecific variation in food intake by common ringtail possums correlates strongly with the total terpene concentration of the leaves (Lawler et al., 1998a). However, in these experiments, food intake also correlated with the concentration of a newly discovered group of compounds, the diformylphloroglucinol compounds (DFPCs). A strong correlation between food intake and foliar DFPC content has now also been found with two other species of *Eucalyptus* (Lawler, Foley, Eschler unpublished data).

In Lawler et al. (1998a) we suggested that foliar terpenes could not cause the variation in intakes at the concentrations observed between individual trees. When added to an artificial diet, the terpene concentration required to reduce food intake was far greater than that found in leaves on which food intakes were reduced to similar levels. In contrast, addition of DFPCs to the diet reduced intakes at levels in close agreement with the trend seen in leaves. This finding was supported also by the more recent studies of two other *Eucalyptus* species (Lawler, Foley, Eschler, Schliebs unpublished data). Consequently, we suggested that foliar terpenes may cue animals to the concentration of the true toxins in the leaves. Terpenes have strong and distinctive odours and tastes and are responsible for the characteristic smell of *Eucalyptus* leaves, while DFPCs are non-volatile and give off very little noticeable odour (Lawler personal observation). All DFPCs contain a side-chain derived from common terpenes (e.g. β-phellandrene, bicyclogermacrene) (Ghisalberti, 1996) and so we suspected that concentrations of the two compounds would be correlated if production of DFPCs is substrate-limited (Lawler et al., 1998a). Such correlations have now been found in three *Eucalyptus* species (Lawler and Foley, unpublished). Upon initial inspection and tasting of a leaf a folivore would first encounter information about the terpene components of the leaf and might use this to, in effect, estimate the DFPC content of the leaves and therefore its likely postingestive effects, based on previous experience. Indeed, we have found that even with our relatively limited olfactory capacity, we could predict the relative amounts of DFPCs between foliage samples of different individual trees within *Eucalyptus* species, simply by crushing and smelling the leaves upon collection (Lawler and Foley unpublished).

In this study we aimed to test the following three hypotheses:

1. Wild-caught folivorous possums held in captivity will show a strong aversion to *Eucalyptus* terpenes, due to their previous experience;

2. This aversion can be reduced or removed by feeding the animals an artificial diet to which increasing amounts of terpene are added, and;

3. Once removed, the aversion can be "reconditioned" by giving the animals a diet consisting of both the terpene and another *Eucalyptus* toxin known to produce negative postingestive effects in corresponding concentrations.

METHODS

This research was approved by the Animal Experimentation Ethics Committee of the Australian National University and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twelve common ringtail (*Pseudocheirus peregrinus*) and 12 common brushtail (*Trichosurus vulpecula*) possums were collected from *Eucalyptus* woodlands and kept individually in metabolism cages (described by Lawler et al, 1998a) and maintained on a basal diet made fresh each day. The diet fed to ringtail possums consisted of (% wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% ground lucerne hay (to pass a 2 mm sieve), 5.5% ground rice hulls, 4.7% ground Weetbix (a wheat- based breakfast cereal) and 1.6% acid casein. The diet for common brushtails was modified for the purposes of other experiments and consisted of 55% apple, 15% carrot, 15% banana, 5.8% sugar, 1% lucerne, 3% Weetbix, 5% rice hulls and 0.2% casein. All animals maintained body mass on this basal diet.

For each experiment, a *Eucalyptus* terpene (1,8-cineole) and a simple DFPC (jensenone (Boland et al., 1992)) were added to the diet in varying concentrations and aversion/deterrence measured as a reduction in food intake relative to control values. No alternative food was offered during experimental periods. For ringtail possums, all experimental periods were separated by a day on which only the basal diet was offered, in order to reduce carryover effects and ensure the welfare of the animals. This was not considered necessary for brushtail possums as they readily feed during the day and so were offered an untreated diet between 0800 h and 1200 h on each experimental day which similarly reduced carryover effects and ensured the health of the animals.

Cineole was chosen as a representative terpene as it is one of the most common terpenes found in *Eucalyptus*, often dominating the total volatile terpene extract (Boland et al., 1991), and has specifically been suggested as a feeding deterrent in studies of both mammals (Southwell, 1978; Reichardt et al., 1990; Zoidis and Markowitz, 1992) and insects (Edwards et al., 1993). Cineole was purchased from Sigma Chemical Company, Australia.

Jensenone was used as a representative DFPC as it is structurally similar to other active DFPCs, is active in similar molar quantities (Lawler et al., 1998a), and is the only DFPC which we have thus far been able to isolate in sufficient quantity for these kinds of experiments (Lawler et al., 1998b). We also have evidence that jensenone acts by stimulation of the emetic system, as much of its effect on intake can be attenuated by administration of ondansetron (Lawler et al., 1998b), an anti-emetic drug which is a $5HT_3$ receptor antagonist (Butler et al., 1988). In this way it is similar to LiCl, the emetic stimulant so frequently used in other studies (e.g. Launchbaugh and Provenza, 1993; Provenza et al., 1994b; Wang and Provenza, 1997). Jensenone was extracted from air-dried foliage of *Eucalyptus jensenii* and ground to pass a 2 mm sieve and extracted in 20% acetone:light petroleum in a soxhlet apparatus. The extracts were concentrated and combined in di-ethyl ether and washed with 0.3 M NaOH. These washes were then acidified with HCl and the precipitate washed with ethanol and re-crystallized from acetone to give jensenone (98% by ^{1}H NMR) (Eschler and Foley unpublished data).

As cineole is volatile it was added to the diet by adsorbing it onto the dry components immediately before adding the fruit. Jensenone is non-volatile and insoluble in water so it was dissolved in acetone and then added to the dry component of the diet, after which the acetone was evaporated before mixing with the fruit component of the diet. Control diets were treated similarly with acetone.

Independence of Effects of Cineole and Jensenone in Common Ringtail Possums

As this experiment relied on our ability to predict the amount of reduction in food intake associated with jensenone and cineole together, it was necessary to first investigate whether there was any interaction between the effects of the two compounds before attempting to remove any aversion to cineole. In this experiment, carried out on ringtail possums, there was no significant interaction (see results), so we considered it unnecessary to also conduct this experiment with brushtail possums as their reactions to both compounds are similar (Lawler et al., 1998b; Stapley unpublished).

Three concentrations of each compound were used. Cineole was added to the diet at concentrations of 0, 6.3 and 9.8 % of total dry matter (DM) and jensenone was added at 0, 0.84 and 1.26 % of the total dry matter of the diet. In an orthogonal design this resulted in nine possible treatment combinations. A truncated Latin square design was used with nine possums over five treatment periods, so that over the experiment each possum received five different randomly allocated treatments.

Removal and Re-conditioning of Aversion to Cineole

The experiments with the ringtail possums and brushtail possums were carried out separately, with those on ringtail possums conducted earlier. Following the ringtail possum experiments, some adjustments to experimental design were made for the brushtail possums and differences in the protocols (principally for stages 1 and 3) are listed below. For both species the animals were divided randomly into two groups of six (a control and a treatment group) and the experiments were carried out in 5 sequential stages. The complete series of experiments took 35 days for ringtail possums and 27 days for brushtail possums.

For clarity the five stages of the basic design, and expectations consistent with the above hypotheses *(in italics)*, are summarized here and full details of each experiment given below:

Stage 1. initial /pre-acclimation aversion to cineole

- The ability of both groups to ingest cineole in no-choice experiments was tested.
- *Both groups should show a similar strong reduction in food intake when cineole is added to the diet.*

Stage 2. acclimation to cineole

- The treatment group was acclimated to increasing levels of dietary cineole over 12 days while the control group was fed only the basal diet.
- *Animals in the treatment group were expected to increase their intake of cineole to levels well beyond the tolerance shown in stage one.*

Stage 3. post-acclimation to cineole

- The tolerance of each group to cineole was again tested.
- *Animals in the treatment group were expected to show significantly less reduction in food intake than the animals in the control group in response to dietary cineole.*

Stage 4. reconditioning aversion to cineole

- The aversion was reconditioned by giving the treatment group a diet including both jensenone and cineole in corresponding concentrations.
- *Jensenone should reduce food intake to levels similar to those expected on the basis of the initial aversion to cineole.*

Stage 5. post-reconditioning aversion to cineole

- The tolerance of both groups to added cineole (without jensenone) was again tested
- *The reduction in food intake due to dietary cineole (in the absence of jensenone) should be similar to that seen in the control group and be dose-dependent*

Stages 1 and 3. Experimental Details

Ringtail possums Tests of the ability of the two groups of animals to tolerate cineole were carried out as cross-over designs involving two treatments and two periods. On the first day, three animals in each group were given diets of 9.8% (on a dry matter (DM) basis) cineole while the remaining three were given the basal diet alone. The following day all animals were fed the basal diet and the next day the treatments were reversed.

Brushtail possums Similar tests were made using three concentrations of cineole (0, 4.9, 9.8% DM). Animals in both the treatment and control groups were divided into two sub-groups and the diets offered in a 3 x 3 Latin square design to each group of three animals over three consecutive nights. A more extensive design with more concentrations was avoided as it was thought that there may be some loss of the aversion in a longer experiment.

Stage 2. Experimental Details

Six animals were acclimated to increasing levels of cineole in their diets while the remaining six were fed only the basal diet alone. The initial concentration of cineole was 1.75% DM; this was increased by approximately 0.7% DM per day to a maximum of 10.5% DM for ringtail possums and by approximately 1.2% DM per day to a maximum of 16.5% DM for brushtail possums. This stage, and stage one, were necessary as the animals were caught from *Eucalyptus* woodland and their intakes of foliage diets had been shown to correlate strongly with leaf terpene concentrations (Lawler unpublished). Hence they were likely to already have a CFA towards cineole. That is, it is possible that the animals had already made associations between cineole or some other foliar terpene and diet choice. This possibility had to be evaluated and its influence (see results), removed in the later stages of the experiment. It is not possible to find possums that we could confidently say had not been exposed to terpenes in their natural diets.

Stage 4. Experimental Details

The treatment group was given the basal diet to which cineole and jensenone were added in corresponding concentrations. That is, concentrations of each compound which, on the basis of previous data (ringtails: first experiment described above; brushtails Stapley unpublished data) would produce the same level of reduction in food intake. Knowing the animals' threshold for jensenone, the amount of jensenone added to the cineole-rich diet was that required to reduce food intake to the same level as seen when cineole alone was incorporated before removal of the aversion. The purpose of this was to allow the animals to learn to associate the concentration of cineole with a given level of postingestive discomfort. Three concentrations of each compound were used. These were (jensenone/cineole as % DM): 0.42/4.39, 0.84/8.78 and 1.26/13.17 for ringtail possums and 0.176/4.5, 0.29/7.5 and 0.43/9.8 for brushtail possums.

A 6 x 6 Latin square design was used to allocate treatments amongst the six animals, so that each animal was given each concentration twice over the course of the experiment.

Stage 5. Experimental details

For both species, three treatment levels were used as described above for brushtail possums in stages 1 and 3. This was adopted at this stage for ringtails (and all stages for brushtails) in order to show that any "reconditioned" aversion was also dose-dependent, rather than a simple avoidance. It was not initially considered necessary to examine dose-dependence for the previous cineole aversion of the ringtail possums as this was shown in the experiment testing for interactive effects of jensenone and cineole (see above).

Statistical Analysis

All experiments were treated as mixed model, row-column designs with possums and days as random effects and cineole concentration, group (treatment or control) and the group by cineole interaction as fixed effect. Parameters of the models were estimated by Restricted Maximum Likelihood theory (REML) (R. Cunningham personal communication). For the key experiments, data presented are for the effect of cineole on dry matter intake and the cineole by group interaction effect on dry matter intake. That is, we expected that cineole would reduce dry matter intake and, where the aversion to cineole was similar between control and treatment groups (Stages 1 and 5), the interaction would be non-significant, while where the treatment group had a reduced aversion, relative to the controls, the interaction would be highly significant (stage 3).

Data Presentation

All data are presented graphically. Where formal statistical tests have been performed (stages 1, 3 and 5) means are presented with a bar showing least significant difference (LSD) derived from the analysis. Where we have not performed formal tests of differences (stages 2 and 4), data are shown as means ± standard errors.

RESULTS

Independence of Effects of Cineole and Jensenone in Common Ringtail Possums.

There was a significant interaction between dietary cineole and jensenone on food intake of common ringtail (ANOVA: $p<0.001$). However, this was mainly attributable to the overriding influence of jensenone on food intake, rather than any strong additive or synergistic effect of the two compounds combined (Fig. 1). Thus, the combination of the two in the reconditioning of any CFA was considered appropriate.

Removal and Re-conditioning an Aversion to Cineole

The salient data are given in Figure 2, with data for ringtail possums listed on the left (Fig. 2a) and those for brushtail possums on the right (Fig. 2b). Statistical tests are summarised in Table 1. In summary, both species showed an initial aversion to cineole (Stage 1: Fig. 2[i]) which was substantially reduced after continued exposure (acclimation) to cineole (Stage 3: Fig. 2[ii]) and was reconditioned after exposure to dietary cineole with corresponding amounts of dietary jensenone (Stage 5: Fig. 2[iii]). Results for each stage (including intermediate acclimation, Stage 2, and reconditioning, Stage 4) are described fully below.

Stage 1. initial/pre-acclimation aversion to cineole

Common ringtail possums in both treatment and control groups showed a clear initial aversion to dietary cineole which was consistent between groups (Table 1, Fig. 2a[i]). Common brushtail possums showed a similar response to both cineole as a main effect and the cineole by group interaction (Table 1, Fig. 2b[i]).

Stage 2. acclimation to cineole

Both common ringtail and common brushtail possums substantially increased their intakes of cineole over the acclimation period (Figs. 3a, 3b).

Stage 3. post-acclimation aversion to cineole

After acclimation to dietary cineole, both species showed substantially reduced aversions relative to animals which were not acclimated. There was still a significant main effect of cineole, due principally to the remaining strong aversion in the control group (Table 1, Figs. 2a[ii], 2b[ii]).

Stage 4. reconditioning of aversion to cineole

During the reconditioning phase of the experiment, animals of both species clearly regulated food intakes in relation to the jensenone (and hence cineole) concentration of the diet (Figs. 4a, 4b).

Stage 5. post-reconditioning aversion to cineole

After reconditioning, the aversions to cineole of animals in the treatment groups for both species matched those of the animals in the control groups which had never had an opportunity to learn that cineole is not always associated with negative postingestive feedbacks (Figs. 2c[i], 2c[ii])

Table 1. Summary of statistical analysis of comparison of aversion to 1,8 cineole between animals in the Control and Treatment groups

Experiment stage	Ringtail possums		Brushtail possums	
	cineole main effect	cineole by treatment interaction	cineole main effect	cineole*treatment interaction
Pre-acclimation	<0.001	0.403	<0.001	0.472
Post-acclimation	<0.001	0.001	<0.001	0.02
Post-reconditioning	<0.001	0.778	<0.001	0.861

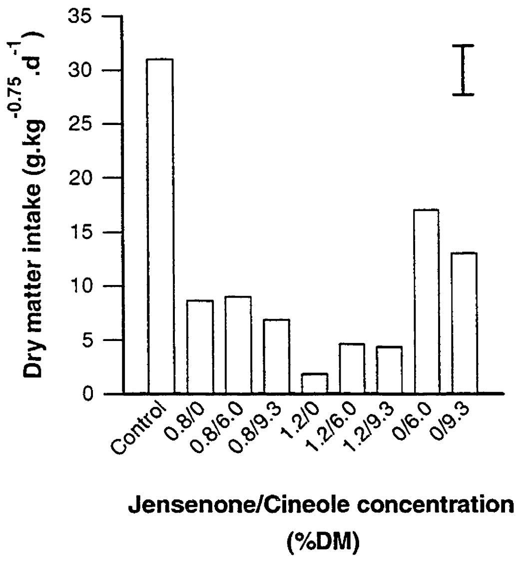

Figure 1. Effects of combinations of 1,8 cineole and jensenone on food intake of common ringtail possums.

Data are means with least significant difference in the analysis represented by the bar in the upper right part of the figure.

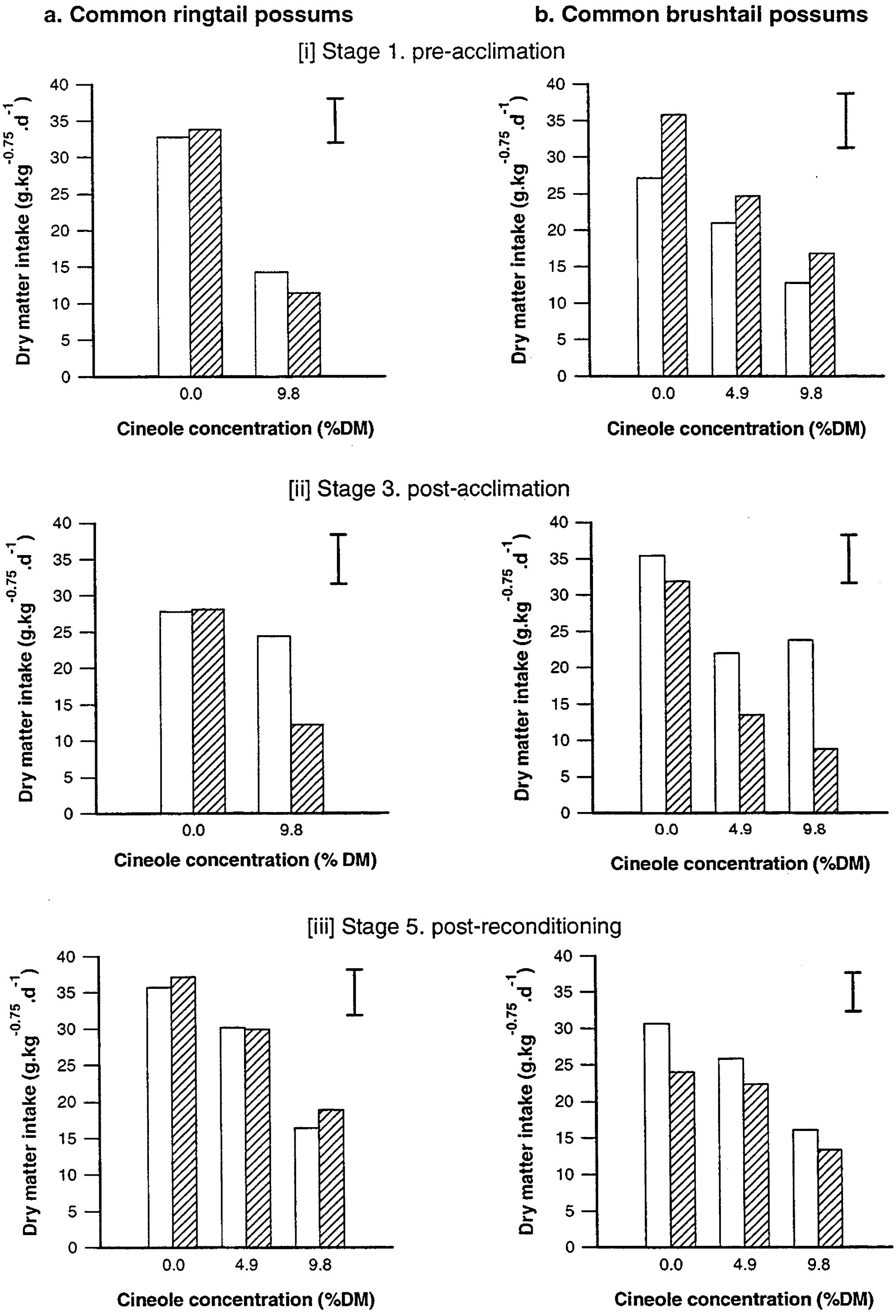

Figure 2. Effects of 1,8 cineole on food intakes of (a) common ringtail possums and (b) common brushtail possums at different stages in the experimental sequence. [i] initial/pre-acclimation aversion after capture from *Eucalyptus* woodland [ii] post-acclimation of animals in the treatment group to increasing dietary cineole [iii] post-reconditioning of the aversion in animals in the treatment group by combining added dietary cineole and jensenone in corresponding amounts.

Shaded bars represent the Control group. Unshaded bars represent the Treatment group. Data are means with least significant difference in the analysis represented by the bar in the upper right part of the figure.

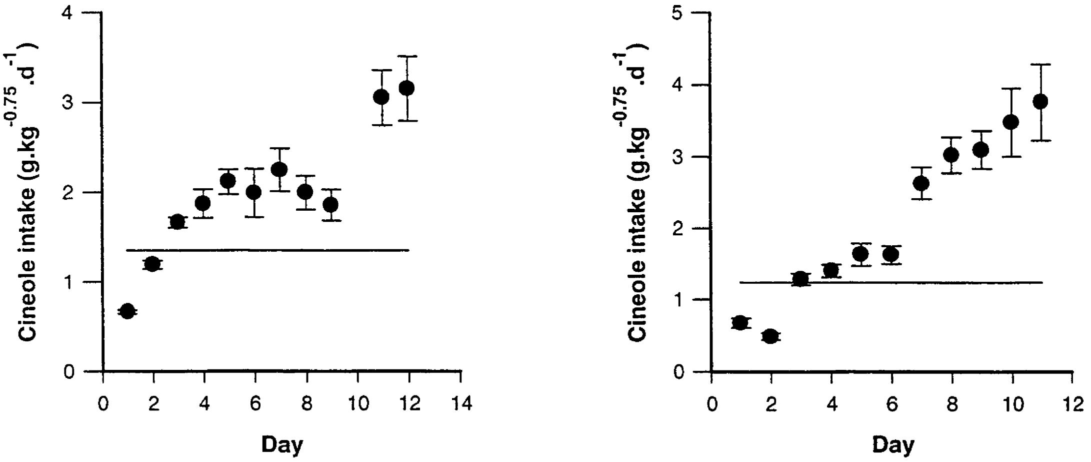

Figure 3. Intake of 1,8 cineole by (a) common ringtail possums and (b) common brushtail possums during acclimation to increasing dietary cineole.

Solid lines represent previous mean maximum intake of cineole by the same animals. Data are means ± standard errors.

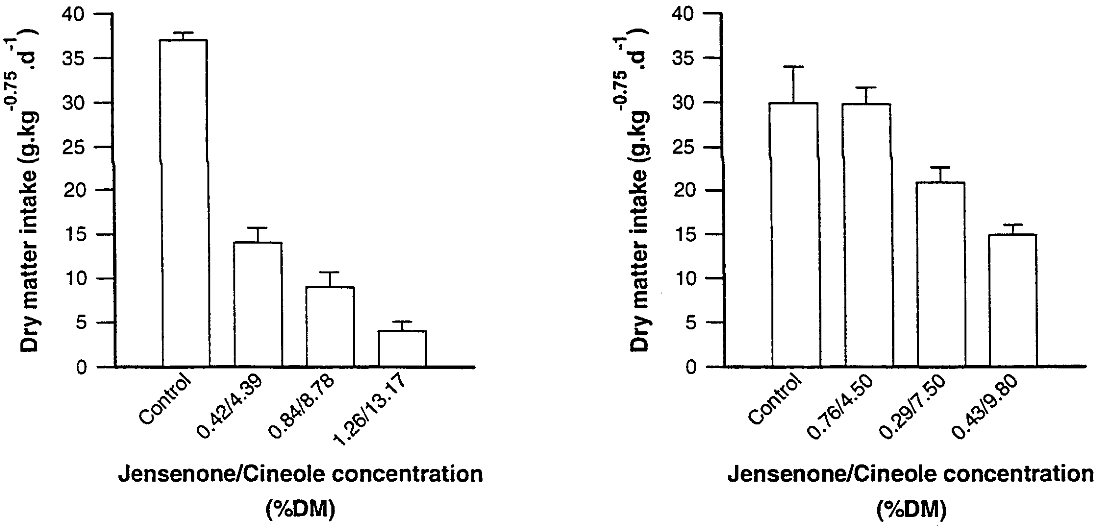

Figure 4. Effect of 1,8 cineole and jensenone in combination on food intake of treatment animals only for (a) common ringtail possums, and (b) common brushtail possums.

First column shows intakes of animals in the Control group concurrent with measured intakes of animals in the Treatment group. Data are means ± standard errors.

DISCUSSION

The data presented here clearly show that feeding deterrence of terpenes in *Eucalyptus* is mediated largely through a conditioned flavor aversion. This aversion can be removed and then reconditioned by disassociating the taste and toxic stimuli and then reinstating the association. Several studies have examined the conditions required to produce a flavor aversion in mammals, however we believe that this is the first study to identify both a salient taste and a toxin which influence a natural plant-herbivore interaction. In studies of browsing ruminants, there have been examples of the aversive compounds being identified (Kronberg et al., 1995; Provenza et al., 1994a) and it has been suggested that the degree of aversion may relate to the concentration of a salient flavor but the compound responsible for the flavor was not identified (Provenza et al., 1994a). It has also been shown that, when flavor and toxins are highly correlated, herbivores learn to regulate food intake based on interactions between postingestive feedback and flavor and then adjust intake on the basis of changes in flavor alone (Launchbaugh et al., 1993).

In *Eucalyptus*, the underlying negative feedback is caused by the DFPCs and this causes an aversion to the taste of terpenes, which occur in concentrations correlating with the DFPCs in the three eucalypt species studied to date (Lawler, Foley, Eschler unpublished data). The aversion to the taste of cineole is dose-dependent: animals eat less of diets with high concentrations of cineole, apparently in the expectation of a greater "internal malaise" (sensu Provenza 1995). It has been shown that one cause of CFAs is stimulation of the emetic system of the body (Provenza et al., 1994b). This appears to be at least part of the mechanism for the conditioning of an aversion due to jensenone (and, we suspect, other DFPCs). Administration of an anti-emetic drug (ondansetron, an antagonist of the $5HT_3$ serotonin receptors) allowed both common ringtail and common brushtail possums to significantly increase their intake of a jensenone-treated diet relative to animals given only the treated diet (Lawler et al., 1998b).

The role of terpenes in deterrence of mammalian folivores of *Eucalyptus* appears to have been overstated (Lawler and Foley in press). They appear not to be the primary cause of the deterrence, but rather their strong, overriding smell and taste serve as a cue to the levels of the true deterrent compounds. Whereas it has been shown that there are physiological costs associated with the detoxification of terpenes by these animals (Foley, 1987; 1992), no study to date has examined whether these costs are indeed limiting to food intake of the animals (Foley and McArthur, 1994). The limiting cost would probably not be reached in the natural diet where concentrations of terpenes rarely exceed 5% (e.g. Hume and Esson, 1993; Lawler et al., 1998a) since this was clearly not deterrent to either the common ringtail possums or the brushtail possums in this study. In contrast, our previous (Lawler et al, 1998a) and ongoing work (Lawler unpublished) shows that the DFPCs can deter marsupials from eating when applied to food in ecologically realistic concentrations.

Nonetheless, assays of terpene content of leaves may still be useful if we use them as do the animals, as a de-facto indicator of leaf toxicity. The DFPCs have been detected in 38/40 *Eucalyptus* species so far examined (Eschler, Pass, Foley unpublished) and, in the three eucalypt species studied in detail to date, the concentrations of the two groups of compounds are closely correlated (Lawler, Foley, Eschler unpublished data). However, the threshold DFPC (and terpene) concentration that results in a reduction in food intake may vary between different species of *Eucalyptus*. Whereas the terpene and DFPC concentration of the foliage within a single species correlate strongly, the slope of this relationship appears to vary significantly between species (Lawler unpublished data). Furthermore structural diversity of DFPCs is such that the biological activities of the different DFPCs may vary as well. The biosynthesis of the DFPCs and the biochemical relationships between the DFPCs and terpenes is an area needing further study.

This begs the question of whether high concentrations of terpenes can confer resistance to herbivores in the absence of DFPCs. That is, can susceptible plants of the same, or different, species as resistant ones, use mimicry to escape herbivory? Launchbaugh and Provenza (1993) concluded that flavor mimicry could reduce the amount of damage that herbivores inflict on plants if those plants had a similar taste and odour to genuinely resistant plants. It is conceivable that plants that are rich in terpenes but poor in DFPCs could gain some measure of protection from marsupial folivores, especially if the different types (low or high DFPC) are individuals of the same species, with very similar terpene profiles. However, CFAs are rapidly extinguished under a range of conditions, and there are restrictions also on the effectiveness of mimicry which are considered below.

While the long-lived nature of CFAs has been emphasized in the past, recent work emphasizes the dynamic nature of foraging in environments where toxins and nutrients are spatially heterogeneous, requiring constant reassessment of the consequences of ingestion of particular foods (Wang and Provenza, 1997). Repeated exposure to the flavor stimulus alone leads to the rapid reduction in the strength of the aversion (O'Connor and Matthews, 1997; this study) and several situations may arise which encourage the animals to re-sample a diet possessing a flavor which previously led to an aversion. Where the amount of non-aversive food is limited, hunger will force the animals to sample aversive foods (Provenza 1996) and in fact, common ringtail possums continued to sample a diet containing toxins even when a toxin-free control was available *ad libitum* (D. Pass personal communication). In free-ranging animals the social context is also important. For example, when associated with animals not possessing the aversion, averted cattle lose an aversion more rapidly (Ralphs, 1997).

Mimicry frequently requires a high proportion of the toxic model before the mimic gains significant advantage (e.g. Lindstrom et al., 1997). It seems likely that flavor mimicry in plants would be an effective strategy to avoid herbivory only when the proportion of mimics in the habitat is very low relative to truly toxic plants of the same flavor. This would be especially true where the herbivore can also incorporate spatial information about the models and the mimics. It is likely also to be necessary to have an abundance of alternative palatable food available to prevent the animals from needing to sample unpalatable items. Terpenes themselves are therefore unlikely to constitute an effective defence against folivorous possums.

The concepts discussed above may prove useful in the management of folivorous possums where they occur as pests of commercial *Eucalyptus* plantations. Commercial operations have suffered significant losses due to predation of trees by marsupials (Montague, 1994) as well as chewing insects. The use of genotypes that are naturally resistant to herbivores may be one means of managing these losses and entomologists have suggested selecting for cineole-rich genotypes which may deter some insect herbivores (Edwards et al, 1993; Farrow, 1993). This may be successful in providing cross resistance against marsupial browsers in the short-term but given our current lack of knowledge of the biosynthesis of the DFPCs (Ghisablerti, 1996) we cannot safely assume that selection for high terpene plants will always result in high DFPC plants. Considerable effort may be expended on producing genotypes that have substantial leaf terpenes but insufficient DFPCs to deter marsupial folivores in the longer term. Folivorous marsupials living within or adjacent to these kinds of trees may quickly lose any aversion based on the taste of terpenes.

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CHAPTER 5. STRUCTURE/ACTIVITY RELATIONSHIPS OF JENSENONE, TORQUATONE AND TWO DERIVATIVES AS FEEDING DETERRENTS OF MARSUPIAL FOLIVORES

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ABSTRACT

It has recently been shown that strong intraspecific variation exists in the resistance of *Eucalyptus* to browsing by marsupial folivores and that this resistance can be attributed to variation in the concentration and identity of diformylphloroglucinol compounds (DFPCs) in the foliage.In this study we address the question of which particular feature(s) of diformylphloroglucinol compounds determine their activity in deterring marsupial feeding. We have used a simple and highly deterrent compound, jensenone, as a model DFPC and compared its activity to structural variants which differ in the types of functional groups on the phloroglucinol molecule. Torquatone, a naturally occurring compound in the steam volatile fraction of *Eucalyptus torquata* foliage has neither the aldehyde nor phenol groups considered likely to confer activity on jensenone. From each of the naturally occurring compounds we have synthesised two intermediates in which we have capped either the phenols (acetyl-jensenone) or the aldehydes (demethyl-torquatone). Addition of jensenone and acetyl-jensenone to diets of common ringtail possums substantially reduced their food intakes. Torquatone showed less activity and there was little reduction in food intake when demethyl-torquatone was added to the diet. We conclude that at least the aldehyde groups attached to the aromatic are important in determing whether these compounds can deter marsupial feeding, whereas the phenol groups may play only a minor role.

INTRODUCTION

There have been many studies of the role of plant secondary metabolites (PSMs) in deterring feeding by mammalian herbivores. In attempting to quantify the relevant compounds they have commonly used broad-scale assays of poorly defined groups of compounds which share perhaps a similar functional group (such as phenolics (Oates *et al.* 1980; Cork 1992; Kool 1992; Hodar and Palo 1997)) and/or the nature of their reaction with certain compounds (e.g. proteins and tannins (Provenza *et al.* 1990; Hume and Esson 1993; McArthur and Sanson 1993; Dearing 1997). However, it is now widely accepted that these methods introduce great variation into the data which can not be accounted for, due to differences in the activity of individual compounds arising from their particular molecular structure (Zucker 1983; Waterman and Kool 1994; Ayres *et al.* 1997). Increased understanding of the importance of PSMs in a plant-herbivore interaction requires that we both identify and quantify, individually, the most active compounds.

Recent work on *Eucalyptus* species and their folivores has shown that a newly discovered group of PSMs, the diformylphloroglucinol compounds (DFPCs) plays an extremely important role in deterring feeding by marsupials (Lawler *et al.* 1998ab,c,d; Pass *et al.* 1998). Where our methods have progressed far enough to quantify individual compounds precisely (currently jensenone, Fig. 1a, and sideroxylonals, dimers of jensenone, Fig. 1e.) we have been able to show that, in *Eucalyptus* species in which these are the predominant DFPCs, they explain the majority of variation in feeding by marsupial folivores between individual trees (Lawler *et al.* 1998a). However, in species where we cannot quantify all individual components, estimates of total DFPCs do not correlate nearly so well with feeding (Lawler *et al.* 1998b), indicating that there is significant variation between different DFPCs in their deterrent activities.

A range of compounds based on acylated phloroglucinol with an isoprene or terpene sidechain have been isolated from *Eucalyptus* foliage (Ghisalberti 1996). The subset of these thus far shown to be active against marsupial feeding have all been DFPCs, characterised by two formyl (aldehyde) groups attached to the phloroglucinol molecule, but differing in the identity of, and nature of bonding to, the isoprene/terpene sidechain (Figs. 1a, e, f, g). Similarities in the levels of activity of macrocarpal G (Fig. 1g), sideroxylonals and jensenone (Lawler *et al.* 1998a,b,c) and the much lower activity of torquatone (Fig. 1b) have led us to suspect that it is the presence of aldehyde and/or phenol groups which determine the deterrent activity of the DFPCs. The lower activity of euglobals, which have an ether linkage to the terpene sidechain (Fig. 1f), relative to macrocarpals (Pass *et al.* 1998), which have free hydroxyls in both positions *ortho* to the terpene, also may indicate that the nature of the linkage between the terpene sidechain and the phloroglucinol molecule, and its effects on H-bonding, is important.

In this study we have compared the activity of four structurally similar simple acylphloroglucinol derivatives in deterring feeding by common ringtail possums (*Pseudocheirus peregrinus*). Two of the compounds ocur naturally in *Eucalyptus* foliage (jensenone and torquatone, Fig. 1b), while the other two have been synthesised, each by modifying one of the natural PSMs. In these modifications we have removed or added functional groups with the intention of creating a series of compounds having the same basic structure and having: both aldehyde and phenol groups; phenol groups only; aldehyde groups only, or; neither aldehyde nor phenol groups. We conclude that the specific molecular structure is an important determinant of deterrent activity, and that certain combinations of functional groups are fundamental to activity, but do not yet have sufficient information on the metabolism of these compounds to identify the underlying mechanism.

METHODS

Animals

This research was approved by the Animal Experimentation Ethics Committee of the Australian National University and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twelve common ringtail (*Pseudocheirus peregrinus*) were collected from *Eucalyptus* woodlands and kept individually in metabolism cages (described by Lawler *et al.* 1998b) and maintained on a basal diet made fresh each day. The diet consisted of (% wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% ground lucerne hay (to pass a 2 mm sieve), 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.

The feeding deterrency of four compounds was tested by offering a diet treated with each of the compounds separately in varying concentrations in a no-choice protocol (see below for experimental design). Treatment diets were offered at 1800 hours and removed at 0600 hours the following morning, when all animals were given the untreated diet. On alternating nights the untreated basal diet only was offered, to prevent carryover effects between experimental nights.

Analytical

Four compounds were tested for their deterrency to feeding by common ringtail possums: jensenone, torquatone, acetyl-jensenone and demethylated torquatone (Figs.1a-d). These represent a series of compounds with the same basic structure, but varying in having: phenol and aldehyde groups (jensenone); either phenol (demethylated torquatone) or aldehyde (acetyl-jensenone) groups, or; neither phenol nor aldehyde groups (torquatone). Note that the structure provided for acetyl-jensenone shows the $(HO)_2HC$ group where the aldehyde group is expected. This is a *gem*-diol, a group which is characterised by the ease which with it undergoes acidic cleavage (such as in the stomach) to revert to the aldehyde.

Jensenone and torquatone both occur naturally in the foliage of *Eucalyptus jensenii* and *E. torquata* respectively (Bowyer and Jefferies 1959; Boland *et al.* 1992) and were thus extracted directly from leaves, while the two other compounds were each synthesised from one of the naturally occurring compounds.

Jensenone

Eucalyptus jensenii foliage, air-dried and ground to pass a 2 mm screen, was extracted in 20% acetone:light petroleum in a Soxhlet apparatus. The extracts were concentrated and combined with 1 L of di-ethyl ether and washed three times with sodium hydroxide solution (0.3 M). These aqueous washes were acidified with hydrochloric acid (12.0 M) and the resultant precipitate collected, washed with ethanol and re-crystallized from acetone to give spectroscopically pure jensenone as a colourless solid (approximately 2.8%) (DM) (see Lawler *et al.* 1998c for details).

Torquatone

Torquatone was extracted from fresh *E. torquata* foliage by steam distillation with cohabation (Foley *et al.* 1987). The resultant oil was loaded onto silica and eluted with 10 % ether: light petroleum. The pure torquatone-bearing fractions were identified by thin layer chromatography and combined. The solvent was then removed *in vacuo* to yield torquatone as a low melting solid. Spectroscopic data was as for the published method (Ghisalberti *et al.* 1995).

Acetyl-jensenone

To a stirred solution of jensenone (20.0g, 0.075mol) and pyridine (22.5ml) in dichloromethane (dry, 200ml) was added acetic anhydride (57ml, 7eq). The reaction was monitored by thin layer chromatography and when all the jensenone was consumed water (200ml) was added. The water layer was removed and the organic layer washed with water (2 x 200ml), sodium bicarbonate solution (2%, 2 x 200ml), water (2 x 200ml), hydrochloric acid (1.0M, 2 x 150ml) and water (2 x 200ml). The organic layer was dried (Na_2SO_4), the solvent removed and the residue further dried under high vacuum to give jensenone triacetate (21.26g, 66%) as a pale oily solid. Spectroscopic data was as for the published method (Boland *et al.* 1992).

Demethylated torquatone

A solution of torquatone (38.20g, 0.136mol) was stirred in neat sulfuric acid (120ml) for 5h., after which thin layer chromatography (10% methanol:dichloromethane) indicated that the reaction had gone to completion. The reaction mixture was poured onto water-ice (200g) and the resultant slurry stirred in an ice bath. The mixture was adjusted to pH 12 by the direct addition of potassium hydroxide pellets (care!), allowed to warm to room temperature and washed with dichloromethane (3 x 300ml). The aqueous layer was then adjusted to pH 4 by the careful addition of sulfuric acid (50%, ca. 160ml) and then extracted with dichloromethane (3 x 400ml). The combined organic layers were then washed with saturated sodium chloride solution (400ml), dried (magnesium sulfate) and the solvent removed *in vacuo* to yield demethylated torquatone as a reddish oil (32.06g, 99%).

Experimental design

In order to test the dose dependency of deterrency of each compound, five concentrations of each plus an untreated control for each compound were offered to the twelve animals over eight nights in a balanced alpha-crossover design, giving four replicates for each treatment (John and Williams 1995). All compounds were tested together to prevent confounding of time with treatments. The extra controls were used to statistically balance the design. Parameters of the model, with compound and concentration as fixed effects were estimated by Restricted Maximum Likelihood theory (REML) (Cunningham personal communication). Data were tested first for linearity across concentrations within each compound and when found not to depart significantly from linearity (χ^2=15.3, df = 12, p= 0.225) the slopes of the dose-dependent linear relationships were compared.

Concentrations of each compound offered were chosen primarily on the basis of preliminary data for deterrency of each of the naturally occurring compounds, as there was insufficient material of each of the synthesised compounds available for substantial preliminary testing. Jensenone is highly deterrent to feeding (Lawler *et al.* 1998c,d) and so was offered at low concentrations, while torquatone is substantially less deterrent (see below; Lawler unpublished data) and was added at higher concentrations. The concentrations of both these compounds in the artificial diet were similar to those found in leaves. We assumed that the alteration in numbers of functional groups on the benzene ring would alter the activity of the synthesised compounds and thus varied the concentrations of the two synthesised compounds relative to their parent compounds. We suspected that acetyl-jensenone would be less active than jensenone and thus used slightly higher concentrations than those for jensenone. We expected that the phenol groups on demethylated torquatone would increase its activity relative to torquatone and thus used slightly lower concentrations. Concentrations of each compound are summarised in Table 1.

RESULTS AND DISCUSSION

Clearly the particular chemical structure of a compound is important in determining its effectiveness as a deterrent to common ringtail possum feeding. There were clear differences in the activity of each of the four groups of compounds (Fig. 2) (χ^2=32.97, df=3, p<0.001). Jensenone was the most effective feeding deterrent, while acetyl-jensenone also was highly deterrent. Torquatone showed substantially less activity and demethylated torquatone the least. The compounds tested here differ only in the nature of the functional groups attached to the benzene ring, yet there is an order of magnitude difference in the amount required to produce the same reduction in food intake. Given such variation in activity between these structurally similar compounds it is clear that conventional "total" phenolic assays are not appropriate to assess the resistance of foliage samples to marsupial folivory. Both jensenone and demethylated torquatone contain the same number of phenolic groups per mole and yet the difference in activity between them is greatest. It should also be noted that jensenone and other DFPCs active against marsupial folivores are not extracted in conventional phenolic assays (Lawler *et al.* 1998b).

A range of similar compounds (including jensenone) have been investigated for other biological activity. Several macrocarpals have been shown to exhibit anti-bacterial activity (Murata *et al.* 1990; Yamakoshi *et al.* 1992; Osawa *et al.* 1996; Sweeney 1997) and sideroxylonal A inhibits attachment of mussel larvae (Singh *et al.* 1996). Grandinol, a simple acylphloroglucinol derivative (similar to jensenone but lacking one of the formyl groups) extracted initially from *E. grandis*, has been shown to be a potent germination inhibitor, while similar compounds showed no such effect (Bolte *et al.* 1984). The euglobals (Fig. 1f) have been investigated as potential anti-tumour agents, with some variation between euglobals of different structure. Detailed structure/activity studies have been carried out with a wide range of variations on the basic acylphloroglucinol structure (including DFPCs) to determine their importance in germination inhibition (Bolte *et al.* 1985) and inhibition of Epstein-Barr virus activation (as a defacto measure of anti-tumour activity) (Takasaki *et al.* 1990). These studies have been more comprehensive (i.e. covered a wider range of structural variation) than has been possible here because of the much lower quantities of compound required for the bioassays. In both cases they have found substantial variation in activity associated with subtle changes in structure and have been able to identify with reasonable precision the requirements for activity. Interestingly, the structural models for both germination and Epstein-Barr virus inhibition have very similar requirements: the acylphloroglucinol structure with a formyl and a ketone group, which may apply also to deterrence of marsupial feeding. However, again there are subtle differences in the requirements for activity: jensenone was an effective Epstein-Barr virus inhibitor (Takasaki *et al.* 1990), but did not inhibit germination (Bolte *et al.* 1985).

A free phenolic group *ortho* to the isoprene unit was identified as important for the germination inhibiting capacity of grandinol. That it may also be important in marsupial folivore deterrence may be indicated by the relative inactivity of torquatone. The reduced activity of euglobals, relative to macrocarpals (Pass *et al.* 1998), may indicate the need for free phenolic groups in both the positions *ortho* to the isoprene/terpene group. Euglobals have an ether linkage at that position masking one of the phenolic groups while this is lacking in the macrocarpals. Grandinol has not been tested against marsupials, but the groups of compounds shown to be active against marsupial feeding (macrocarpals, sideroxylonal, jensenone) all share this feature. Further support for this contention may be given by the close correspondence in the molar thresholds shown by common ringtail possums for jensenone and sideroxylonal. The molar threshold for jensenone in this experiment is very similar to the molar threshold for sideroxylonal of Lawler *et al.* (1998a) (Although it should be noted that the threshold for jensenone may vary significantly between populations (Lawler *et al.* 1998c)). Sideroxylonals are dimers of jensenone and one might therefore expect the molar threshold for sideroxylonal to be half that of jensenone (while the threshold in milligrams would be very similar). However, only one jensenone monomer has all three phenolic groups free while the other has one bound in an ether linkage (similar to that found in euglobals) which may eliminate the deterrent effect of that part of the molecule.

The results obtained in this study were somewhat counter to those expected. As the synthesised compounds were intended to be intermediate in structure between the two naturally occurring compounds, intermediate activities may be expected. This was the case with acetyl-jensenone, although both aldehyde and phenol groups were capped. However, in the methods section we noted that the *gem*-diol groups revert easily back to aldehyde groups in acidic conditions. We suspected also that the high activity shown by this compound was due to its also losing the acetyl groups and reverting back to the parent compound in the stomach. Support for this hypothesis is given by the observation that this occured while attempting to purify acetyl-jensenone after the acetylation reaction using column chromatography. Significant proportions of the resulting product were shown by thin layer chromotography and NMR to be jensenone while no jensenone was evident in the reaction end-product before chromatography (Eschler unpublished data). This is most likely to be due to the acidic nature of the silica gel used in the column. However, only small amounts of jensenone were produced, over several days, when acetyl-jensenone was stirred in pH1 HCl (Eschler unpublished data). It should be noted that our original intention was to produce a more appropriate intermediate by methylating jensenone to produce a compound with the aldehydes present and phenols absent, but our attempts to do so in sufficient quantity failed.

The lower activity of demethylated torquatone relative to its parent compound may be a function of its increased polarity. It contains two of the features of the active compounds (phenol groups, ketone) and therefore may be expected to show greater activity than torquatone. However, the presence of the phenol groups also makes the demethylated torquatone more polar and thus may reduce the likelihood of its being absorbed across lipid membranes. If the action of jensenone (and other DFPCs) is at the surface of the gut wall then lipophillicity may be found to be irrelevant, as may be indicated by the similar deterrency exhibited by jensenone, sideroxylonal and macrocarpal G which vary in polarity. There is currently very little data on the fate of any DFPC post-ingestion. We know that jensenone causes the release of serotonin, apparently from the gut wall, but cannot say whether this is a result of cell damage or stimulation of specific receptors (Lawler *et al.* 1998c). However, when jensenone is incubated in an isolated section of guinea pig gut, it disappears rapidly from the lumen and debris accumulates, indicating cell damage. No jensenone or apparent metabolites can subsequently be found either in the lumen, the gut tissue or the surrounding fluid. It appears that jensenone binds to proteins but it is not yet known if this is specific to certain protein types, such as receptors in the gut wall (S. McLean and S. Brandon *personal communication*). Thus it is not possible at this stage to say whether absorption across the gut wall is a necessary process for these compounds to exert their effects.

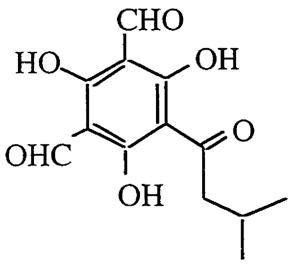
Another feature of the DFPCs not tested here that may be important in determining the antiherbivore activity, and thus interpreting their ecological importance, may be the identity of the terpene moiety. In the only relevant study to date Takasaki et al. (1990) compared the anti-tumour activity of a range of euglobals, that differ only in the terpenoid part of the molecule. While there was some variation between compounds, all were active and they concluded that that part of the molecule was less important than the features of the acylphloroglucinol component described above. We suspect this also to be the case when considering deterrence of marsupial feeding, in light of the similarities in activity between jensenone, sideroxylonal and macrocarpals. However, to date we have not been able to compare directly, for example, two macrocarpals with differing terpene moeities, and believe this is necessary before a firm conclusion is reached.

In conclusion, this structure/activity study has provided information that is useful in an ecological sense, illustrating the importance of identifying the specific molecular structures governing the plant-herbivore interaction. However, the quantities of each compound required for whole animal bioassays with vertebrates severely limit the number of compounds that can be synthesised in appropriate amounts, and this is perhaps reflected by the paucity of such studies in other plant-mammalian herbivore systems. In the only other study that we know of testing structural variants of a PSM against an herbivore that encounters it in the wild, only three compounds were tested (Clausen *et al.* 1986). They identified phenol groups as being important for activity, but noted another co-occurring PSM with a phenol functionality that was inactive. No attempt was made to understand the importance of other parts of the structure or the physiological effects of the compounds. Clearly, for plant-vertebrate herbivore systems, these limitations will in most cases prevent testing an extensive range of variations of the appropriate structure. This approach is unlikely therefore to lead to a complete understanding of the structural requirements. Detailed pharmacological studies of the metabolism of those compounds and the means by which animals can metabolise and tolerate or detoxify them may be a more profitable approach.

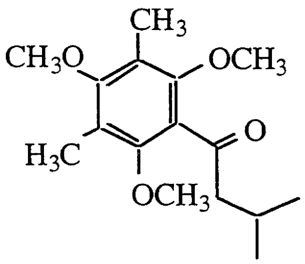
Table 1. Concentrations of each compound used in no-choice bioassay experiment with captive ringtail possums.

Jensenone			Acetyl-jensenone			Demethylated torquatone			Torquatone		
molecular weight	concentration		molecular weight	concentration		molecular weight	concentration		molecular weight	concentration	
	µmoles/g dry matter	% wet matter		µmoles/g dry matter	% wet matter		µmoles/g dry matter	% wet matter		µmoles/g dry matter	% wet matter
266	7.52	0.06	428	13.63	0.18	238	70.03	0.50	280	119.04	1.00
	15.04	0.12		26.48	0.34		140.06	1.00		178.57	1.50
	30.08	0.24		50.62	0.65		210.08	1.50		297.62	2.50
	45.11	0.36		77.88	1.00		280.11	2.00		416.67	3.50
	60.15	0.48		101.25	1.30		350.14	2.50		535.71	4.50

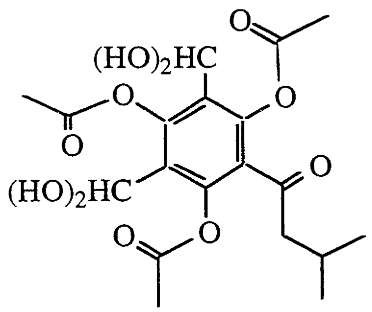
a. Jensenone



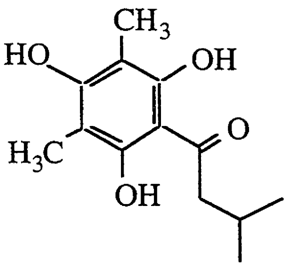
b. Torquatone



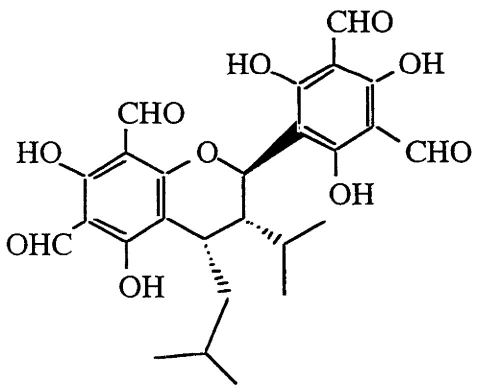
c. Acetyl-jensenone



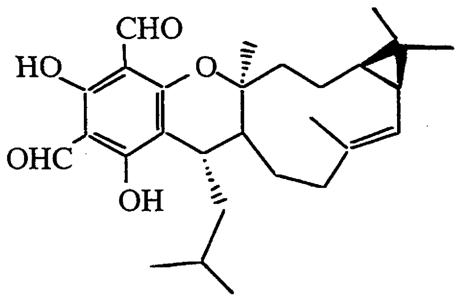
d. Demethyl-torquatone



e. Sideroxylonal A



f. Euglobal III



g. Macrocarpal G

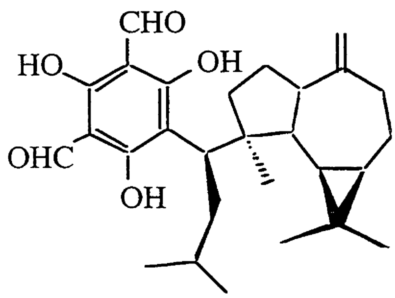


Figure 1. Structures of compounds used in this study and related compounds known to exert deterrent effects on marsupial feeding

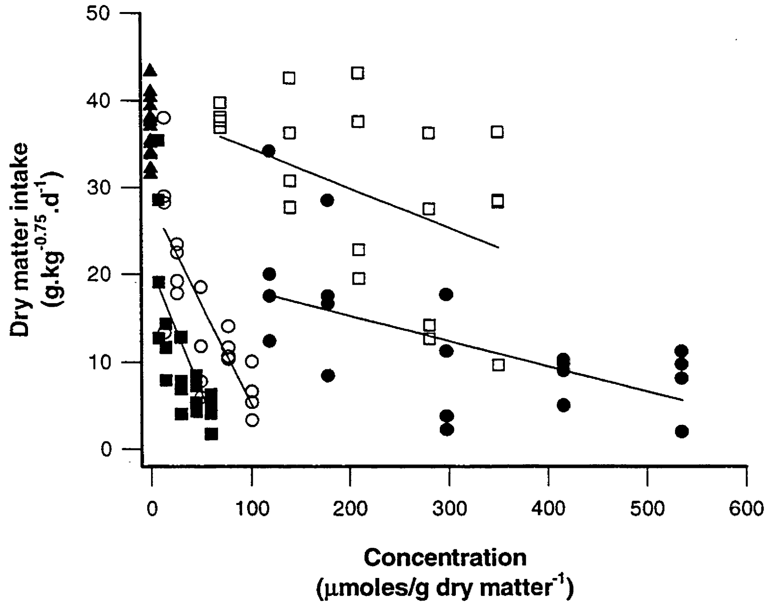


Figure 2. Dose-dependent relationships between food intakes by common ringtail possums and the molar concentrations of potential antifeedant compounds.

Solid squares are jensenone, open circles are acetyl-jensenone, solid circles are torquatone, open squares are demthylated torquatone and solid triangles are controls.

ACKNOWLEDGEMENTS

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CHAPTER 6. FOLIAR CONCENTRATION OF A SINGLE TOXIN CREATES HABITAT PATCHINESS FOR A MARSUPIAL FOLIVORE

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ABSTRACT

We examined intraspecific variation in susceptibility to herbivory by common ringtail possums (*Pseudocheirus peregrinus*) in two species of *Eucalyptus*, *E. polyanthemos* and *E. sideroxylon*, and the chemical basis for that variation. Using a no-choice protocol, we observed dry matter intakes by common ringtail possums ranging from 3.28 to 44 g.kg body mass$^{-0.75}$.d^{-1} for *E. polyanthemos* and 2.4 to 67 g.kg body mass$^{-0.75}$.d^{-1} for *E. sideroxylon*. We investigated, using correlative analyses, the relationships between dry matter intake and a range of foliage chemical characteristics, including measures of nutritional quality (e.g. total nitrogen) and plant secondary chemistry (total phenolics, condensed tannins, terpenes and sideroxylonal, a recently identified *Eucalyptus* toxin). Significant relationships were identified only for terpenes (and 1,8-cineole in particular) and sideroxylonal. Bioassay experiments confirmed that the foliar concentration of sideroxylonal alone was sufficient to explain the variation observed. To enable a field study of variation in foliar sideroxylonal and dry matter intake by possums of *E. polyanthemos*, we developed calibrations of both variables against the Near Infrared spectra of foliage samples. Acceptable calibration equations were developed and we applied these to samples collected from a number of *E. polyanthemos* individuals within an area approximating the home range size of common ringtail possums. We found that foliar sideroxylonal varied from nil to 12.6 mg/g, and predicted dry matter intakes by possums ranged from nil to 42.8 g.kg body mass$^{-0.75}$.d^{-1}. We conclude that significant patchiness in nutritional quality of foliage, resulting from variation in foliar concentrations of a single compound, exists at a scale relevant to the feeding decisions of individual animals. This level of variation must be taken into account if animal foraging is to be understood.

Key words: intraspecific variation, *Eucalyptus*, *Pseudocheirus*, habitat patchiness, terpene, phenolic, herbivory, condensed tannin, sideroxylonal

INTRODUCTION

Patchiness in animal habitats is widely recognised as an important determinant of animal foraging. In studies of vertebrate foraging, patches are most often recognised in terms of the quantity of food available (Laca and Demment 1991). In contrast, the quality of the food resource is often harder to characterise, yet in some environments this may be the cause of the majority of habitat heterogeneity (Astrom *et al.* 1990). It is accepted that in many cases the concentration of plant secondary metabolites (PSMs) may limit the food intake of an herbivore, or deter it from feeding altogether (Cork and Foley 1991, Ganzhorn 1992) and this may contribute to habitat heterogeneity. However, the majority of studies of vertebrate foraging examine only interspecific differences in the amounts and types of PSMs present (Braithwaite *et al.* 1983, Oates *et al.* 1990, Ganzhorn 1992, Cork and Catling 1996) with some notable exceptions (Clausen *et al.* 1986, Reichardt *et al.* 1990, Snyder 1992). This is despite frequent observations of animals showing preferences at the intraspecific level (i.e. between individuals within plant species). Interspecific comparisons may be obscured by qualitative differences in the foliar profiles of PSMs and relationships may be easier to discern within species, where the chemistry is qualitatively similar.

The relationship between *Eucalyptus* species and their marsupial folivores is a case in point. Three species of arboreal marsupials are able to subsist on a diet soley of *Eucalyptus* foliage: the common ringtail possum (*Pseudocheirus peregrinus*), the greater glider (*Petauroides volans*) and the koala (*Phascolarctos cinereus*). As *Eucalyptus* is the dominant tree genus in Australia (Landsberg and Cork 1997), the interactions between *Eucalyptus* species and their marsupial herbivores have been the subject of numerous studies both in the field and with captive animals. While these studies have often identified preferences for individual trees within *Eucalyptus* species (Hindell and Lee 1987, Pahl 1987, Pahl and Hume 1990) the chemical basis for preferences at this level has been little studied. This has occurred for two reasons:

1. Captive animal studies have not specifically addressed intraspecific differences (Pahl and Hume 1990) and/or have relied on crude measures of PSMs such as 'total' phenolics (e.g. Hume and Esson 1993). These measures are insufficient to capture the variation in deterrent activity due to subtle differences in molecular structure between compounds (Clausen *et al.* 1986, Waterman and Kool 1994), and;
2. Whatever our knowledge of relevant chemical constituents, field studies of foliage chemistry are limited to the tree species level by the time and cost constraints of laboratory analysis (Foley *et al.* 1998).

Recent advances in both the detailed chemical identification of *Eucalyptus* PSMs and in rapid methods of assaying foliar chemistry address these limitations. We have now developed a method for the precise quantification of sideroxylonals (Fig. 1), a major subset of the diformylphloroglucinol compounds (DFPC). This is a chemically defined group of compounds known to vary intraspecifically and to affect folivore food intakes (Lawler *et al.* 1998a). This has allowed us to develop far better correlative relationships between feeding and individual PSMs. In this study we report on evidence that the majority of feeding deterrence of herbivore resistant trees of *Eucalyptus polyanthemos* and *E. sideroxylon* can be attributed to variation in foliar sideroxylonal concentration. We discuss also the necessity for the development of analytical tools which allow for the measurement of sufficient numbers of samples to include variation at the level of individual trees in ecological studies of herbivore foraging. We show that near infrared (NIR) spectroscopy can be adopted for this purpose, and that within a population of *E. polyanthemos* variation in leaf quality exists at a scale relevant to the foraging choices of individual folivores.

METHODS

This research was approved by the Animal Experimentation Ethics Committees of Monash University and The Australian National University and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Twelve common ringtail possums (*Pseudocheirus peregrinus*) were caught by hand in woodland dominated by *Eucalyptus* near Canberra and maintained on foliage from *E. polyanthemos* and *E. rossii* collected locally. The possums were housed individually in metabolism cages (0.6m wide x 0.6 m high and 0.75 m long) in a room with a 12:12 hour light:dark cycle at temperatures ranging between 18 °C at night and 22 °C during the day. The room was lit with four 60W incandescent bulbs during the day and by a single 40 W red incandescent bulb at night. Common ringtail possums are strictly nocturnal and so food was offered at 1800 h and removed between 0500 h and 0800 h the next day depending on the particular experimental protocol (see below).

Preliminary Identification of Palatable and Unpalatable Individual *E. Polyanthemos* Trees

A preliminary experiment was conducted to determine whether common ringtail possums ate different amounts of foliage between individual *Eucalyptus polyanthemos*, as we had observed with *E. ovata* and *E. viminalis* (Lawler et al. 1998a). We chose six individual trees ranging from strongly preferred (susceptible) to strongly deterrent (resistant) on the basis of observational and anecdotal data on herbivory levels in the field and on preliminary screening by offering to captive ringtail possums. We then measured the dry matter intake of each foliage by six common ringtail possums using a no-choice protocol.

Each animal was given foliage from an individual tree for an entire night with no alternative food available and the dry matter intake (DMI) calculated using appropriate controls for evaporation. In this experiment food was offered at 1800 h and removed at 0800 h. In order to reduce carryover effects and to ensure the welfare of those animals that fed little, 30 ml of 10% (w/w) aqueous glucose was also provided. Treatments were applied on consecutive nights, using a Latin square design such that each animal received foliage from each tree once and all trees were used on each night of the experiment. Data were analysed by analysis of variance (Ratkowsky *et al.* 1993). Note that in subsequent experiments the protocol differed, as detailed below and in Lawler et al. (1998a).

Foliage offered to animals was always taken from mature trees, ranging between 6 m and 20 m. It was not possible to measure leaf chemistry *a priori* so foliage was chosen on the basis of *apparent* quality. All leaves were fully expanded and mature and in good health (in terms of greenness, softness and lack of damage to leaves). A range of leaf chemistry parameters considered to be of importance to herbivores were measured after the experiments were conducted and their importance in determining feeding rates investigated (see below).

Extended Experiments With *E. polyanthemos* and *E. sideroxylon*

Correlative analyses require adequate degrees of freedom if the results are to be used confidently (Lawler et al. 1998a). Therefore, having shown in the preliminary experiment that there was an eight fold difference in DMI of common ringtail possums between individual *E. polyanthemos* trees, we measured the DMI of a larger number of individual trees to ensure that the correlative analyses had sufficient statistical power. The protocol was similar to that described above except that measurements of dry matter intake of *E. polyanthemos* foliage were only made on alternate nights. On non-experimental nights, only highly palatable *E. rossii* foliage was offered to the animals. Further, experimental foliage was removed at 0500 h and *E. rossii* foliage offered for the remainder of the dark (feeding) period to ensure that all animals had eaten sufficient to maintain their body mass. We continued to use the no-choice protocol throughout the experimental period.

Three experiments were conducted, the first using an alpha row-column design (John and Williams 1995) in which 24 individual trees of *E. polyanthemos* were offered to 12 possums over eight treatment nights, with each tree being fed to four different possums on separate nights. The second and third experiments used 12 different individual trees of *E. polyanthemos* and *E. sideroxylon* respectively with 12 possums, using truncated Latin square designs, such that each tree was fed to five different possums.

These designs were analysed using restricted maximum likelihood (REML) theory. Details of the amount of foliage eaten (DMI) from each tree are presented as best linear unbiased predictors (BLUPs) rather than means, as the former take account of the structure of the model (Ross Cunningham *personal communication*). All intake data are expressed as g dry matter.kg body mass$^{0.75}$.d^{-1} for the following reasons: in analysis log(body mass) was entered as a covariate and found in all cases to have a coefficient range including 0.75; data transformed in this manner satisfied assumptions of normality and homoscedasticity; and it is consistent with the more general pattern described by (Demment and Van Soest 1985).

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. Evaporative loss was always negligible. On the morning following each treatment, these controls were sampled in a manner considered to represent the sampling of those leaves by the animals.

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 freeze-dried and ground to pass a 1 mm sieve.

Dried and ground foliage samples were assayed for the following constituents, as described in Lawler et al. (1998a): nitrogen, cellulose, hemi-cellulose, lignin, cyanogenic glycosides, total polar phenolics and condensed tannins. *In vitro* dry matter digestibility was measured using sequential incubations in pepsin and fungal cellulase as described by (Choo *et al.* 1981).Terpenes were extracted from foliage in sealed vials of hexane at 60°C and analysed by gas-liquid chromatography for identification and quantification of individual terpenes (Edwards *et al.* 1993).

Sideroxylonals were extracted from three grams of freeze-dried and ground leaf for 13 h using a Soxhlet apparatus with 125 ml of 20:80 (v/v) mixture of acetone and light petroleum spirit (40-60 °C boiling point). After extraction the solvent was evaporated off and approximately 10-15 mg of extract dissolved in methanol for HPLC analysis. Sideroxylonal concentrations were estimated by HPLC using a Waters Novapak C18 column (3.9 x 1.5 mm) at a flow rate of 1.0 mL per minute using a solvent of 95% methanol, 4.9% water and 0.1% triflouroacetic acid. Absorbance was measured at 275nm. Although two stereoisomers, sideroxylon A and sideroxylon B were resolved and quantified, sideroxylon B was present at concentrations less than 5% (typically 1-2%) of sideroxylon A. Consequently baseline noise was significant and we only considered sideroxylonal A in our calculations and analyses.

Pure sideroxylonal for standards and bioassays (see below) was extracted in the following manner. Foliage of *Eucalyptus meliodora* was air-dried and ground to pass a 2mm sieve and one kilogram batches extracted in 6 L of 10% ethanol/hexane for 48 h in a large Soxhlet apparatus. The solvent was then removed by evaporation and the residue dissolved in dichloromethane. The residue was then washed through celite, which was then washed successively with hexane, ethyl acetate, 10% methanol (MeOH)/dichloromethane (DCM) and MeOH. The sideroxylonal-rich fractions were combined and the solvent removed. This material was then chromatographed on silica gel and eluted with the following solvents: 50% DCM/40-60 light petroleum, DCM, 5% MeOH/DCM, 25% MeOH/DCM and MeOH. The sideroxylonal-rich fractions were again combined and the solvent removed. The remaining material was dissolved in acetone and chromatographed through Sephadex (LH-20) with acetone. Four fractions were collected and the sideroxylonal-rich fractions combined, the solvent removed and the residue dissolved in diethyl ether. The ether was allowed to evaporate slowly until a precipitate formed. This was filtered and washed with ether to give a white solid with primarily sideroxylonal A and small amounts of sideroxylonal B. Full details of this method and confirmation of the identity of the sideroxylonals will be published elsewhere (Eschler and Foley, unpublished data).

Correlative Analysis of Leaf Experiments

Relationships between DMI for foliage from each tree and foliage chemical characteristics were investigated using stepwise linear regression with a rejection level of $\alpha=0.05$. Comparisons of these data from each separate experiment and each *Eucalyptus* species were made using parameterisation of the model to include different slopes and intercepts. The final model in each case pooled data for the separate experiments where appropriate to produce the simplest model that explained the data.

Bioassay of Effect of Sideroxylonals on Food Intake by Ringtail Possums

The effect of pure sideroxylonal on DMI of common ringtail possums was measured by adding sideroxylonal to a basal diet of fruits and cereal. 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 design was a 6 x 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 h to which was added one of six concentrations of sideroxylonal and this was removed and replaced by untreated basal diet at 0500 h. To determine DMI 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.

Sideroxylonal concentrations were chosen to cover the range measured in *E. polyanthemos* foliage. These were 0, 3.3, 6.7, 13.3, 20.0 and 26.7 (mg/g dry matter). Sideroxylonal was added to the diet by dissolving it in acetone and adding this to the dry components of the diet. The acetone was then evaporated off and fruit added to the diet immediately before offering it to the animals. The control diet was treated with a similar amount of acetone.

Field Survey Of Sideroxylonal Concentrations And Palatability Of *E. polyanthemos* Foliage

In order to assess the relevance of intraspecific variation in foliar chemistry to the choices made by individual animals, we measured the foliar sideroxylonal concentration and the potential DMI of common ringtail possums in a large number of *E. polyanthemos* trees at a field site, using near infrared spectroscopy (see below). This site was located near Queanbeyan in New South Wales, adjacent to the Australian Capital Territory. The area surveyed was 100 x 50 meters and included 87 *E. polyanthemos* trees plus a number of other eucalypt species, including *E. rossii* and *E. nortonii*. Soil type, water availability and aspect were constant across the plot. This size plot was chosen as approximating the home range size of common ringtail possums such that a single possum may potentially encounter all of these individuals while foraging. Samples of healthy adult foliage were taken from all individual *E. polyanthemos* trees, freeze dried, ground to pass a 1 mm sieve and scanned by near infrared spectroscopy (NIRS). We then estimated both sideroxylonal content and ringtail possum intakes for those leaves using calibration equations described below.

Near Infrared Reflectance Spectroscopic Analysis

The principals of near infrared reflectance spectroscopy (NIRS) are summarised in Foley et al. (1998). In brief, the chemical bonds in organic material are represented in the spectrum reflected by a sample irradiated with near infrared light. These spectra can then be calibrated against reference values to develop equations for use in estimating these values for other samples whose spectra fall within the bounds of the calibration population. Effective calibrations can be derived for chemically well-defined attributes such as nitrogen and can also be developed for less well-defined attributes such as fiber, total phenolics, food intake and digestibility (Brooks *et al.* 1984, Redshaw *et al.* 1986, Givens *et al.* 1991, 1992, McIlwee *et al.* 1998).

We used near infrared reflectance spectroscopy to predict the foliar concentration of sideroxylonal A and to predict the potential DMI of foliage collected from a large number of *E. polyanthemos* trees. Samples of dried and ground foliage, prepared as described above, were placed in an oven at 40 °C overnight to minimise the interference of residual moisture with the NIR spectra of the samples. After cooling to room temperature (approx 22 °C) in a desiccator, the spectrum of each sample between 400 and 2500 nm was collected using an NIR Systems 6500 Scanning Spectrophotometer with spinning cup attachment.

Calibration equations were developed to estimate sideroxylonal concentration and DMI by common ringtail possums using reference values derived from the HPLC assays and animal experiments respectively (as described above). Calibrations were made by correlating spectra against reference values using modified partial least squares (MPLS) regression with cross validation to prevent overfitting of the model (Shenk and Westerhaus 1991). Raw spectra (stored as log(1/reflectance)) were treated using standard normal variate and detrend transformations to reduce the influence of particle size (Barnes 1989). Optimal calibration was achieved by using the second derivative of the spectra with a gap size for its calculation of 10nm. The range of wavelengths used was 1108 - 2492 nm. Both equations were highly accurate, returning r^2 values for the relationship between observed and NIR-predicted values of 0.911 for sideroxylonal A and 0.917 for possum intake. These were considered suitable for application to the field survey of *E. polyanthemos*.

RESULTS

The preliminary experiment clearly showed substantial variation in dry matter intake (DMI) of common ringtail possums between individual *E. polyanthemos* trees ($p<0.001$) (Fig. 2) and gave us some insight into the choice of individuals for later experiments. The extended experiments also showed great variation in DMI, covering a range from 3.28 to 44 $g.kg^{-0.75}.d^{-1}$ for *E. polyanthemos* and 2.4 to 67 $g.kg^{-0.75}.d^{-1}$ for *E. sideroxylon* (Fig. 2). Note that the maximum value for *E. sideroxylon* tree #54 exceeds, by almost 50%, all other DMI measurements we have made for common ringtail possums on these and other *Eucalyptus* species. The foliage of this tree was unexceptional in all leaf characteristics measured (including fiber and *in vitro* digestibility measures) and we can offer no explanation for the level intakes recorded. However, due to its extreme value we have treated it as an outlier and performed regression analyses with and without this individual tree in the data set. Its omission does not alter our results, though we consider it to be unrepresentative of our data generally.

Regression analyses showed that only two components of the foliage, sideroxylonal and cineole, significantly explained the decline in DMI seen across trees within species (Table 1, Figs. 3 and 4). When the relationship between cineole and DMI was investigated without sideroxylonal, the final model pooled data from all experiments. In the case of the relationship between sideroxylonal and DMI the slope was the same, but the intercept differed, between the two *Eucalyptus* species. When both sideroxylonal and cineole were included in the initial model, cineole was dropped from the model (Table 1). Note also that total terpenes correlated with food intake (data not shown), but the relationship was weaker than for cineole alone. None of the other measured leaf chemical components (nitrogen, cellulose, hemi-cellulose, lignin, cyanogenic glycosides, total polar phenolics, condensed tannins or *in vitro* digestibility) showed any relationship with DMI that approached significance. There was also a strong relationship between foliar concentrations of cineole and sideroxylonal (Table 1, Fig 5). The foliar concentrations of sideroxylonal and total terpenes were also correlated, but the relationship was weaker than for cineole alone.

The addition of pure sideroxylonal to the artificial diet substantially reduced DMI by common ringtail possums ($p<0.001$, Fig. 4). At 3.3 mg/g sideroxylonal, DMI was decreased by 44% relative to the DMI of the control diet and at 26.7 mg/g sideroxylonal DMI was reduced by 88%. Comparison of these data with data for the same animals feeding on foliage diets shows that sideroxylonal can cause feeding deterrence at ecologically realistic levels.

Improvement on Linear Model of Sideroxylonal Effect on Dry Matter Intake

Following the conclusion that increased foliar sideroxylonal was the cause of reduced DMI, we further investigated the relationship between food intake and sideroxylonal concentration due to apparent curvature in the relationship (Fig. 4). In this we have used only the *E. polyanthemos* data due to the greater number of degrees of freedom available. We began with the assumption that food intake (DMI) is regulated so as not to exceed some threshold dose of sideroxylonal, or simply:

$$\text{sideroxylonal threshold} = \text{DMI} * [\text{sideroxylonal}]$$

such that the relationship between the inverse of DMI and sideroxylonal concentration should be linear if the threshold is constant:

$$\text{DMI} = \frac{\text{sideroxylonal threshold}}{[\text{sideroxylonal}]}$$

The data from the foliage diet did not fit this simple model, implying poor regulation of sideroxylonal intake by possums. This is shown to be the case by plotting sideroxylonal intake against sideroxylonal concentration (Fig. 6) where there was wide variation in sideroxylonal intakes at higher foliar concentrations. However, the data from the bioassay experiment did fit the basic model, with the equation:

$$\text{DMI} = \frac{1}{0.0094(7.1\text{x}10^{-4}) * [\text{sideroxylonal}]}$$

or

$$\text{DMI} = \frac{106.38}{[\text{sideroxylonal}]}$$

describing the data with an r^2 value of 0.89. This indicates that common ringtail possums are indeed able to regulate their intake of sideroxylonal but that perhaps other factors interfere with this when feeding on a foliage diet (see discussion).

We therefore tried a range of models, including linear, quadratic and exponential decay forms, and found that a simple quadratic function gave the best explanation of the whole *E. polyanthemos* data set, with an r^2 value of 0.86 (Table 2, Fig. 4). We also tested the fit of the model derived from the bioassay data when applied to the foliage diet. The model was inappropriate at low sideroxylonal concentrations, giving unrealistic estimates of DMI. However, at concentrations of sideroxylonal above 3 mg/g DM the model provided as good a fit to the foliage data as did quadratic and exponential decay models fit to the same subset of the data (Table 2, fig. 7).

Field Survey of Sideroxylonal Concentrations and Palatability of *E. polyanthemos* Foliage

The concentration of sideroxylonal varied substantially between individual trees within the study area (Fig. 8a). Similarly, the potential DMI estimated by NIRS also varied markedly between individual trees (Fig. 8b). Foliar sideroxylonal concentrations ranged from zero to 12.6 mg/g, corresponding to a range of predicted dry matter intakes of 42.8 to zero g.kg body mass$^{-0.75}$.d^{-1}. The frequency distributions of both variables were approximately normal (sideroxylonal - χ^2 =7.96, 7 df, p=0.34; DMI - χ^2=7.26, 7 df, p=0.40). In several cases, trees with high foliar sideroxylonal concentrations were the nearest neighbours of individuals with very low concentrations.

Table 1. Summary of linear regressions of the relationships between food intake by common ringtail possums fed *E. polyanthemos* or *E. sideroxylon* foliage and chemical composition of the foliage and the relationship between chemical components.

Data for both species were included in the analyses, with appropriate parameterisation to allow for, and test, differences in slopes and intercepts between species. Parameters are presented with standard errors in parentheses.

(n.b. The model for sideroxylonal only also incorporates the model where both sideroxylonal and cineole were added, as cineole was dropped from the model in stepwise regression. The variables *Es* and *Ep* are categorical variables with the value 1 if the species is *E. sideroxylon* or *E. polyanthemos* respectively, and zero if not that species. This allows for differences in slope or intercept in these models which combine data for boht species).

Independent variables	Dependent variable	Regression equation	r^2
sideroxylonal only	food intake	food intake = 37.60(1.64) - 6.55(2.47)**Es* - 2.42(0.21)*[sideroxylonal]	0.75
cineole only	food intake	food intake = 35.86(1.69) - 21.12(2.18)*[cineole]	0.68
cineole	sideroxylonal	[sideroxylonal] = 9.29(0.46)*cineole**Ep* + 6.63(0.75)*cineole**Es*	0.92

Table 2. Summary of regression equations describing the relationship between food intake by common ringtail possums fed *E. polyanthemos* foliage and sideroxylonal content of the foliage.

The latter part of the table compares the fit of a theoretical model based on regulation of toxin intake about a threshold (based on bioassay data) with other models incorporating curvature in the relationship. Parameters are presented with standard errors in parentheses.

Model type	Concentrations of sideroxylonal (mg/g)	Model	r^2
Linear	0-18.24	food intake = 37.60(1.64) - 2.42(0.21)*[sideroxylonal]	0.81
Quadratic	0-18.24	food intake = 40.66(1.50) - 4.19(0.51)*[sideroxylonal] + 0.12(0.03)*$[\text{sideroxylonal}]^2$	0.86
Exponential	0-18.24	food intake = 41.20(3.60)*$e^{-0.14(0.01)*[\text{sideroxylonal}]}$	0.80
Quadratic	3-18.24	food intake = 38.69(8.92) - 3.96(1.82)*[sideroxylonal] + 0.12(0.08)*$[\text{sideroxylonal}]^2$	0.52
Exponential	3-18.24	food intake = 38.25(11.08)*$e^{-0.13(0.03)*[\text{sideroxylonal}]}$	0.53
Regulation	3-18.24	food intake = 1/(0.0094(7.1*10^{-4})*[sideroxylonal])	0.50

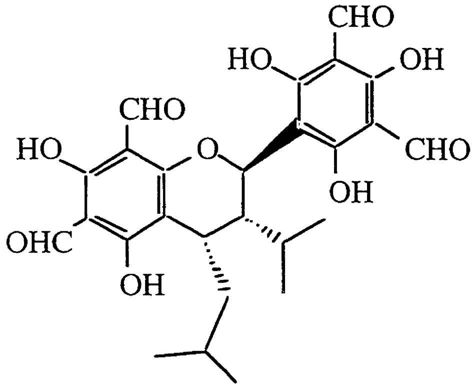

Figure 1. Molecular structure of sideroxylonal A.

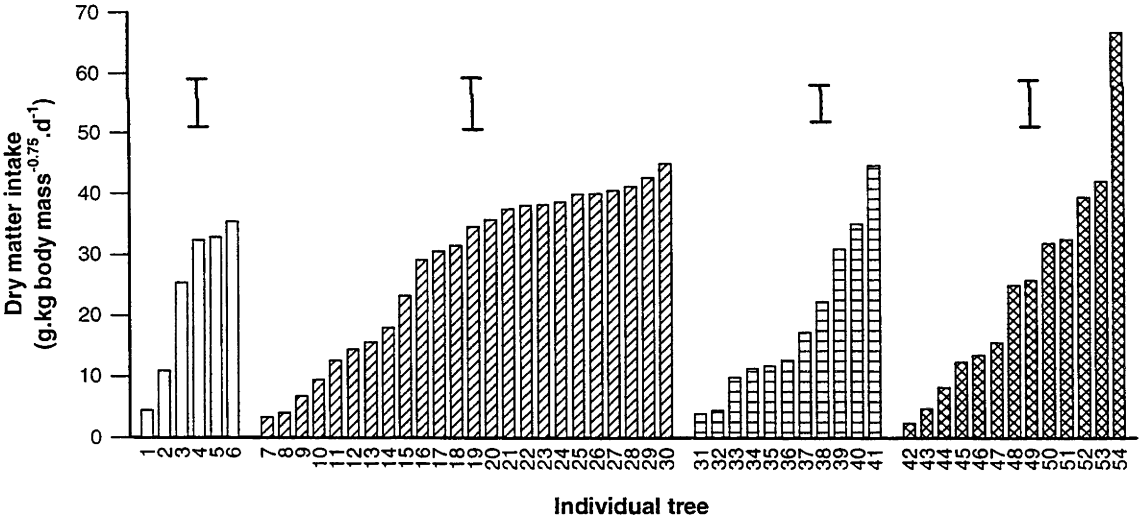

Figure 2. Variation in dry matter intake by common ringtail possums fed *Eucalyptus polyanthemos* or *E. sideroxylon* foliage in no-choice experiments.

Unshaded bars are *E. polyanthemos* from the preliminary experiment, diagonally shaded bars are *E. polyanthemos* from alpha design with 24 trees, horizontally shaded bars are *E. polyanthemos* from Latin square design with 12 trees and heavily shaded bars are *E. sideroxylon*. Vertical bars above each group of data are least significant differences from ANOVA or REML analysis.

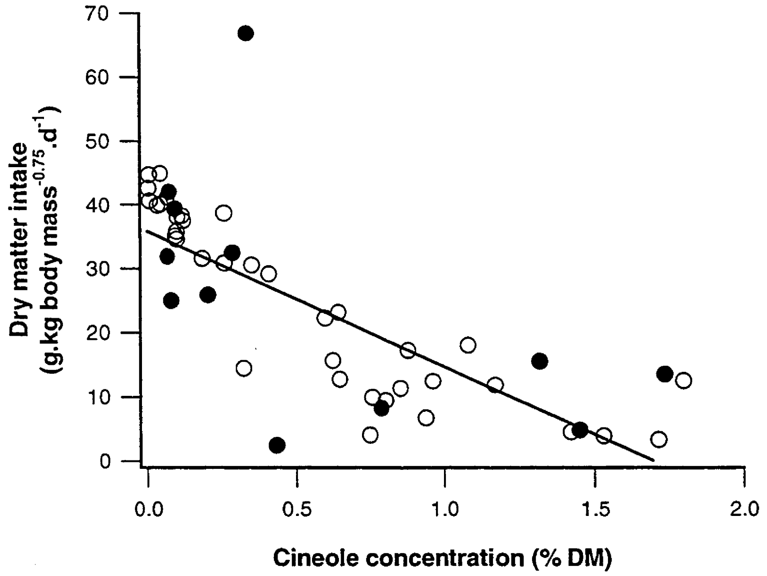

Figure 3. Relationship between dry matter intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage (open circles) or *E. sideroxylon* foliage (closed circles) and cineole concentration of foliage.
The regression equation (solid line) was not statistically different for the two species. Equations summarised in Table 1. Note that tree #54 has been omitted from calculation of the regression equation (see results).

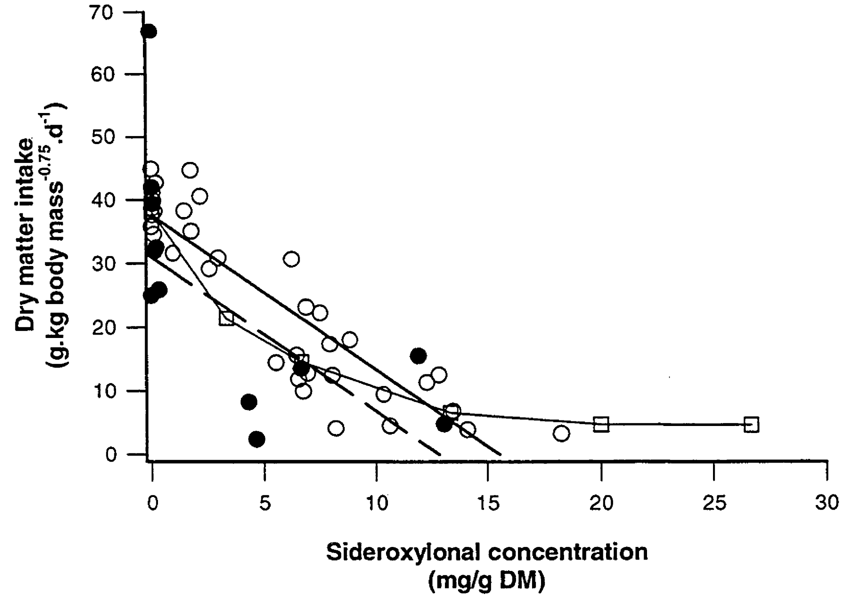

Figure 4. Relationship between dry matter intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage (open circles), *E. sideroxylon* foliage (closed circles) or artificial diet with sideroxylonal added (open squares) and sideroxylonal concentration of foliage.
The regression equations had the same slope, but different intercepts for *E. polyanthemos* (solid line) and *E. sideroxylon* (dashed line). Equations summarised in Table 1. Again tree #54 was omitted from these calculations. Values for artificial diet are joined with lines to identify their positions more readily.

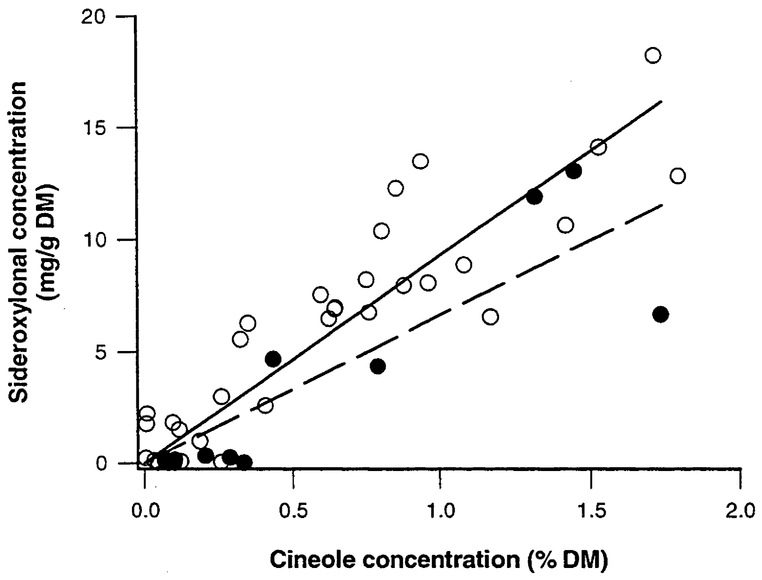

Figure 5. Relationship between cineole and sideroxylonal concentration of *Eucalyptus polyanthemos* foliage (open circles - regression solid line) or *E. sideroxylon* foliage (closed circles - regression dashed line).
Equations summarised in Table 1.

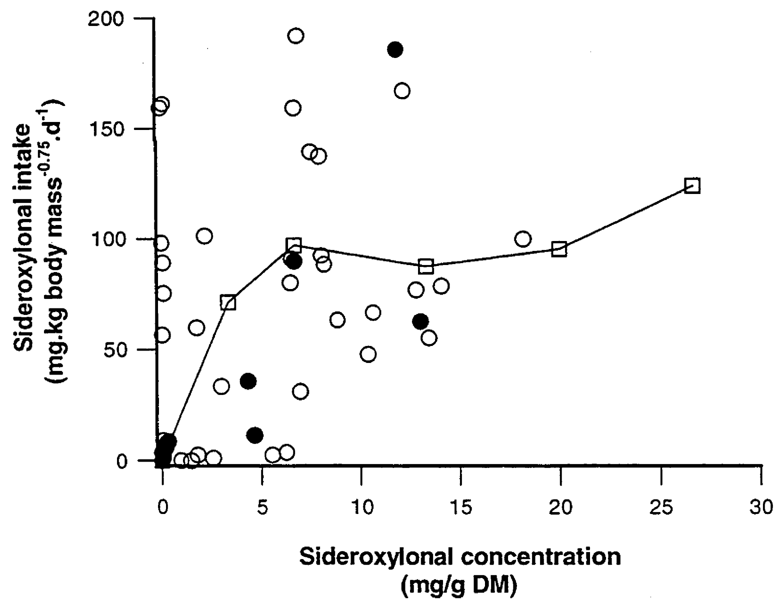

Figure 6. Relationship between sideroxylonal intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage (open circles), *E. sideroxylon* foliage (closed circles) or an artificial diet with sideroxylonal added (open squares) and sideroxylonal concentration of foliage.

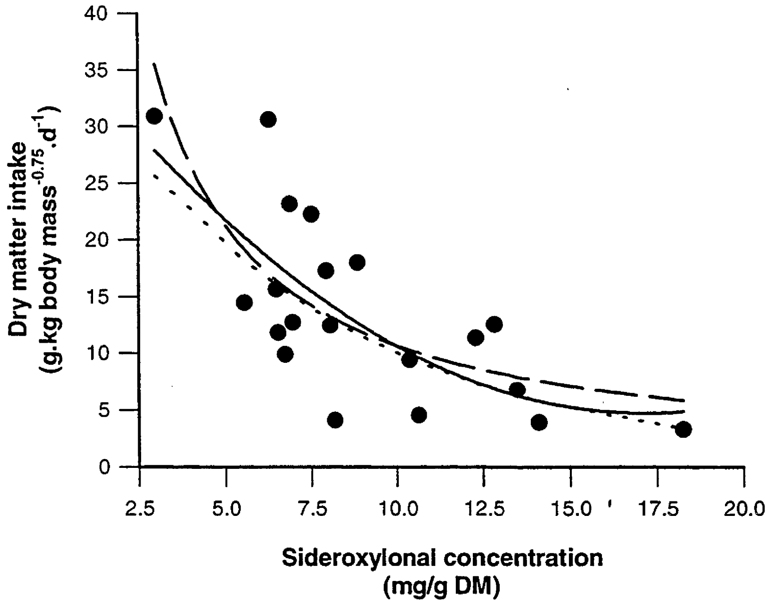

Figure 7. Comparison of different models of relationship between dry matter intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage at limiting foliar sideroxylonal concentrations.

Solid line is quadratic, dotted line is exponential decay and dashed line is toxin intake regulation model. Equations summarised in Table 2.

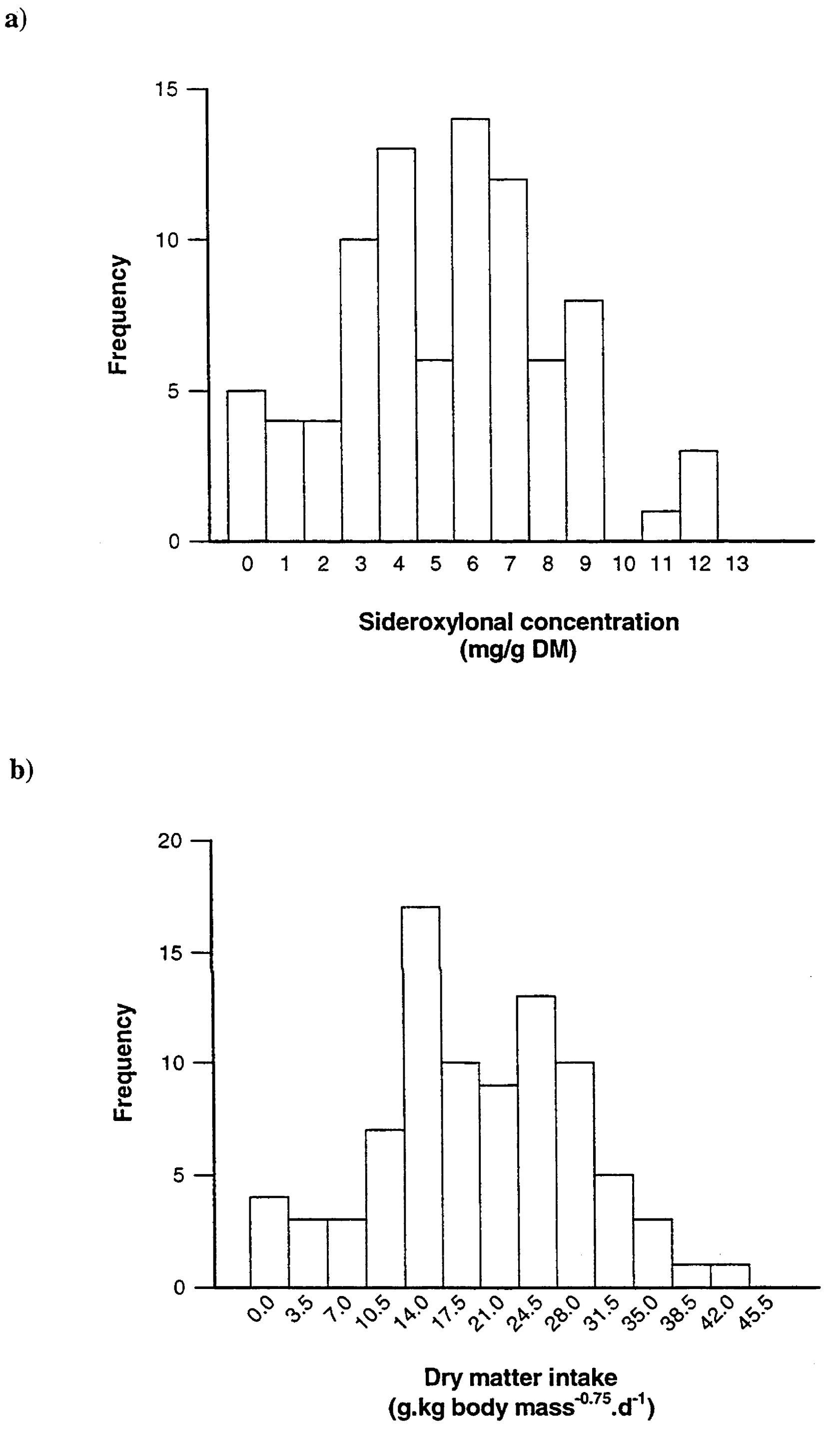

Figure 8. Frequency distributions of varying leaf chemistry between individual *E. polyanthemos* trees within one population. a) foliage sideroxylonal concentrations, b) near infrared spectroscopic prediction of dry matter intake of common ringtail possums.

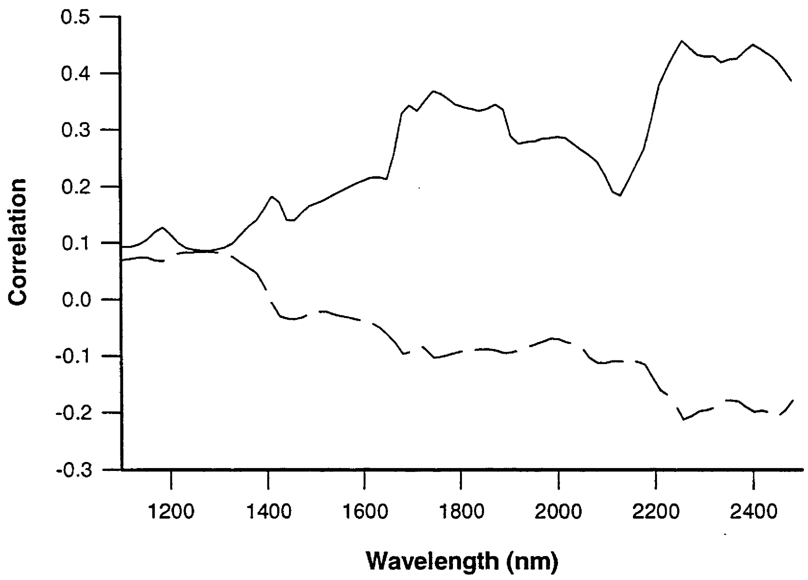

Figure 9. Correlation between individual wavelengths of near infrared spectra of *E. polyanthemos* foliage and sideroxylonal concentration (solid line) and dry matter intake of common ringtail possums (dashed line).

DISCUSSION

Plant Secondary Metabolites Causing Herbivore Deterrence in *Eucalyptus*

We have shown clearly that a wide spectrum of herbivore deterrency can be found between individuals within both *E. polyanthemos* and *E. sideroxylon*. Despite the measurement of a range of other chemical characteristics, including those conventionally made on *Eucalyptus* foliage, only two compounds showed substantial (negative) correlations with food intake. We have shown previously that terpenes could not reproduce the deterrent effect at the concentrations seen in leaves when used in bioassay experiments, while the DFP compounds were active at foliar concentrations (Lawler et al. 1998a,b,c). The data shown here reinforce that finding, with foliar sideroxylonal concentrations alone describing 86% of the variation in food intake between trees, and being shown in bioassay experiments to be the likely causative agent. We have not repeated bioassays with terpenes as the experiments reported previously used cineole (Lawler et al. 1998a,c) which is the major terpene found in the species used here (Boland *et al.* 1991, Lawler, Foley, Matsuki *unpublished data*). In these *Eucalyptus* species, high foliar concentrations of sideroxylonal confer resistance to herbivory by common ringtail possums.

With the attention given in the literature to the role of terpenes in herbivore resistance (e.g. Reichardt *et al.* 1990, Zoidis and Markowitz 1992, Edwards *et al.* 1993, Hume and Esson 1993, Duncan *et al.* 1994) discussion of their role in this interaction is warranted. In Lawler et al. (1998a) we hypothesised that the role of foliar terpenes was not to deter feeding via toxicity but rather that they acted as a cue to the concentration of the true deterrent compounds, the DFPCs. Data reported here and elsewhere by us (Lawler et al. 1998c) strengthens the case for this hypothesis. We suggested that molecular similarities between the two groups of compounds may give rise to a correlation in the concentrations of the compounds to be found in the leaves. All known DFPs incorporate at least an isoprene unit, and often whole terpenes, bonded to the phloroglucinol molecule (Ghisalberti 1996) and thus if production is substrate-limited the amount of each group produced should correspond. This is borne out by the strong correlations found in this study, made possible by the development of a precise assay for the sideroxylonals. We have also shown (Lawler et al. 1998c) that common ringtail possums and common brushtail possums (*Trichosurus vulpecula*) can develop a conditioned flavor aversion to terpenes via an association between terpenes and post-ingestive effects of a simple DFP compound. We conclude that role of terpenes in *Eucalyptus*-marsupial folivore interactions is secondary, as a deterrence cue, rather than acting as primary deterrents in their own right.

Regulation of Sideroxylonal Intake by Common Ringtail Possums

It has been suggested that herbivores are able to regulate their intakes of PSMs over a wide range of concentrations (Jakubas *et al.* 1993, Launchbaugh *et al.* 1993, Pfister *et al.* 1997, Wang and Provenza 1997). In this study common ringtail possums appear to show poor regulatory ability when feeding on foliage diets. We believe that they do possess regulatory mechanisms, that they are mediated at least in part by feedback from $5HT_3$ receptors (Lawler *et al.* 1998b) and are enhanced by the conditioned aversion to terpenes, giving them pre-ingestive feedback (Lawler *et al.* 1998c). The data for the same animals feeding on the artificial diet with sideroxylonal added support this contention and suggest a sideroxylonal threshold of approximately 106 mg.kg body mass $^{-0.75}$.d^{-1}. Consequently, we offer two explanations for this discrepancy between foliage and artificial diets, both stemming from the fact that foliar concentrations of sideroxylonal are high relative to any apparent threshold.

Firstly, the amount that an animal eats in any given feeding bout may be significant relative to the putative threshold. A decision to feed, or not, may cause wide variation. For example, if an animal eats five grams at a time on a foliage with a moderate sideroxylonal concentration (say 10 mg/g), then a decision to include another feeding bout in a period would increase its sideroxylonal intake by 50 mg which may take it well past the threshold before an appropriate feedback signal is received. Similarly, the reverse may happen if the animal overingests sideroxylonal early in the feeding period, receiving a stronger than usual feedback signal and making a decision not to feed further at all. However, as noted above, the role attributed here to cineole should serve to enhance any regulatory ability the possums may have. Animals feeding on trees with high foliar sideroxylonal concentrations should be induced to sample the diet more cautiously due to the high foliar cineole giving them prior knowledge of the likely post-ingestive feedback from the diet.

Another contributor to the high variation in sideroxylonal intake may be methodological. While we are confident that our estimates of food intake by these animals are accurate, some imprecision is introduced by the altered behaviour of the animals feeding on foliage with high sideroxylonal concentrations. Where food intakes were substantially reduced, the animals became very agitated and would chew off branches, strip leaves and eat bark and we had to correct for these behaviours. Small errors in correcting for these behaviours would have little effect on estimates of intake, but combined with the high foliar sideroxylonal concentrations, may have introduced significant variation into estimates of sideroxylonal intake. An error in the estimate of food intake of only three grams on the highest sideroxylonal foliage would lead to an error in the sideroxylonal intake of over 54 mg, or approximately 50% of the threshold seen in the bioassay experiment.

NIR Spectroscopic Measurement of Leaf Toxins and Other Attributes

The identification of the major deterrent compounds found in foliage is but the first step to understanding their role in the plant-herbivore interaction. We have shown here that intraspecific variation in these *Eucalyptus* species is so strong that the foliage chemistry of individual trees must be taken into account if such measurements are to have relevance to the foraging of individual animals. Further study at this scale requires the development first of a precise quantitative laboratory-based assay and then modification of that technique (via reduction in sample preparation, reagent volumes and/or process times) to allow for the numbers of samples required in an ecological study. However, many conventional analytical techniques cannot be streamlined sufficiently to allow for the necessary scale of sample collection and remain one of the major factors limiting experimental designs and sampling schemes (Foley et al. 1998). Such is the case with sideroxylonal analysis, which requires drying and grinding of the sample before exhaustive extraction in organic solvents, evaporation of those solvents then preparation and analysis by HPLC. Where these inflexible limitations exist, other methods must be investigated to relieve these constraints.

NIR spectroscopy is one such means whose utility is only now becoming recognised by ecologists, although a widely accepted technique in agriculture for many years (Foley *et al.* 1998). NIR spectroscopy cannot be used in isolation as an analytical tool, but in the many cases where an acceptable conventional assay is available, and the compound of interest has organic bonds (C-H, N-H, C-O) acceptable calibrations can be developed between NIR spectra of samples and laboratory values. Thus the limiting laboratory stage is used only in the development of a calibration set (and occasional validation of samples) and further samples can be analysed using only NIR spectra. This requires minimal preparation (in this case only drying and grinding of samples), does not damage sample, uses no reagents and takes little time for multiple analyses. Once calibration equations are developed, over 150 samples a day can be scanned and analysed for a large number of components in a single day (Foley et al. 1998).

In this study we have shown that NIR analysis is an effective method for assaying the foliar concentration of sideroxylonals. Acceptable NIR calibration equations can also be developed for estimation of a range of other *Eucalyptus* leaf characteristics, including total nitrogen, neutral detergent fiber and condensed tannins (McIlwee *et al.* 1998). A number of studies have shown that poorly chemically-defined features, such as food intake and digestibility can be reliably estimated with NIRS (Brooks *et al.* 1984, Redshaw *et al.* 1986, Givens *et al.* 1991, 1992) even when the underlying mechanistic basis for the calibration is unknown (McIlwee et al. 1998). In this study, we know the primary determinant of variation in DMI (sideroxylonal), and have a corresponding calibration for that compound. We can therefore illustrate that not only is the calibration for DMI a functional tool, but that it is underlaid by a sound mechanistic base. A graph of the correlation of each wavelength with sideroxylonal concentration and possum intake illustrates the relationship, one being approximately opposite in shape to the other (Fig. 9). Moreover, comparison of coefficients corresponding to each wavelength in the calibration equations for sideroxylonal content and possum intake showed that six of the ten most important wavelengths for each are identical but with opposing signs. That is, an increase in sideroxylonal content equates to a decrease in food intake according to the calibration equations.

Determinants of Nutritional Patchiness in *Eucalyptus* Forests.

Previous studies have identified either total nitrogen or the ratio of nitrogen to phenols (Cork and Catling 1996) as important determinants of nutritional patchiness for arboreal marsupials in *Eucalyptus* forests of south-east Australia. The major difference between these previous studies and ours is the scale of the observations. For example, studies described by Cork and Catling (1996) focussed on variation in the ratio of nitrogen to total phenols (N:TP) in patches from 5 to 30 ha over an area over 400 000 ha and found a negative correlation with the abundance of greater gliders. Landscape scale studies in the past have, of necessity, ignored the variation in herbivore deterrence at the scale of the individual tree but importantly, studies by (Braithwaite 1983) and Cork and Catling (1996) showed that for greater gliders at least, there was a threshold ratio of N or N:TP below which viable populations could apparently not be maintained. However, it is also apparent that above these threshold values there is great variation in population densities, with some forests of apparently high nutritional value supporting very few folivorous mammals. The high level of resistance of some individuals of favoured species, and particularly the proportion of resistant individuals in a particular population of trees, may be the cause of much of this variation.

It should be noted that even though DFPCs contain phenolic groups, they are not detected in standard assays of 'total' phenolics. Assays of total phenolics use polar solvents (usually 50% aqueous acetone (Cork and Krockenberger 1991) that do not extract non-polar phenols such as the DFPCs (Lawler *unpublished data*). Hence, although N:TP ratios might give an indication of broad scale differences in foliage quality, they are not sufficient to identify the high level of variation in herbivore resistance identified in this study. This is further supported by the lack of any relationship between TP and food intake in our data.

We have shown here that resistance of individual *Eucalyptus* trees to herbivory, conferred by a single compound, does occur at a scale small enough to influence the foraging decisions of individual animals. Further, such intraspecific variation is as strong as that found between species. This variation is not captured by conventional assays of leaf chemistry and it is certainly not appropriate to assay foliage of only a small number of individuals of each species if the aim is to describe observed variation in herbivore activity. There are clear examples of one or a few trees suffering much greater herbivore damage than surrounding conspecifics where the herbivores are ringtail possums (Geritz 1987, Pahl 1987, Lawler *personal observation*) or other marsupial folivores (Hindell *et al.* 1985, Martin 1985). In one case we have observed a severe decline in a population of ringtail possums after defoliation resulted in the deaths of suitable individual trees even though healthy (and strongly herbivore deterrent) conspecifics remained (Foley unpublished). There is significant patchiness in nutritional quality of *Eucalyptus* forests for marsupial folivores that may affect foraging of individual animals, with potential flow-on effects for home range sizes and population densities.

We do not of course contend that sideroxylonal is the only possible cause of patchiness. Clearly variation in other factors, such as nitrogen or digestibility may also cause similar effects. However, we suggest that for two reasons, variation in such factors at a scale relevant to individual animals, is unlikely to be as important as that shown in foliar concentrations of sideroxylonal. Firstly, the nutritional quality of a food for a herbivore is the product of how much food it can eat and the digestibility or metabolizability of that food. Previous studies of the nutritional ecology of common ringtails have shown that differences in digestibility may affect animal performance (e.g. Hume *et al.* 1996). However, our results show that variation in intake is the major determinant of nutritional quality in this instance. Across the range of *E polyanthemos* trees, *in vitro* dry matter digestibility varied from 30-75% (30 of 36 trees were between 65 and 75%) whereas DMI varied more than 10 fold. Similarly, digestibility of *E. sideroxylon* foliage varied by a factor of only 1.3 (27-36%) while DMI also varied by more than an order of magnitude. Small changes in digestibility are inconsequential compared to the differences in intake (and irrelevant where DMI is minimal). Consequently, the best measure of nutritional quality of *Eucalyptus* foliage is the voluntary DMI of animals. The second reason that we believe nutritional factors are likely to be less important, at the scale measured in our field study, is that they are strongly influenced by environmental factors (Lawler *et al.* 1997). Within a small, environmentally uniform area nutritional factors are unlikely to vary substantially within *Eucalyptus* species. In contrast, sideroxylonal concentrations were extremely variable on a scale at which environmental conditions are likely to be almost identical. This is indicative of a strong genetic basis to the determination of foliar sideroxylonal concentrations, and the normal frequency distribution amongst individuals points to a multi-allele system (Falconer 1981).

Variation at the level of individual trees is also an important component of habitat variability for boreal herbivores such as grouse (Guglielmo *et al.* 1996) and snowshoe hares (Clausen *et al.*

1986) and other species of arboreal folivores (Glander 1978, Milton 1978). The challenge in these systems, and in *Eucalyptus* forests, is to develop methods which allow this important source of variation to be measured. Chemical analyses quickly become prohibitively expensive if many individual plants have to be assayed to sample the nutritional quality of a forest patch. We have shown that near-infrared spectroscopy (NIRS) can be used to develop rapid and cheap methods of measuring PSMs and other nutritional factors, and perhaps more usefully, potential dry matter intake by folivores (Foley et al 1998; McIlwee et al. 1998; this study). This technique should now be applied to intensive field studies of animal foraging.

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CHAPTER 7. GENERAL DISCUSSION

Our capacity to understand and to further study the effects of PSMs, and leaf chemistry in general, on the feeding of marsupial folivores, has increased markedly since I began the research for this thesis. This has come both through the identification and illustration of the importance of the DFPCs in determining food intakes of marsupial folivores. Another major advance is the realisation that leaf spectral characters (as captured by near infrared spectroscopy (NIRS)) can be calibrated against food intake (and other measures of performance (Brooks *et al.* 1984; Redshaw *et al.* 1986; Givens *et al.* 1991, 1992)) without requiring knowledge of the mechanistic basis for the relationship. We are thus now in a position to extend consideration of the effects of intraspecific variation in foliar chemistry into detailed studies of foraging of marsupial folivores in the field, using NIRS to escape the constraints of conventional laboratory analysis of foliage chemistry. Of course, a better understanding of the relationship between *Eucalyptus* species and their marsupial folivores will be gained as our knowledge of the metabolism and physiological effects of the DFPCs increases, as does our understanding of the effect of detailed molecular structure of particular DFPCs. I discuss these possibilities in the following section. I discuss also the implications of this work for understanding allocation of resources by *Eucalyptus* to defence and the likely factors governing that allocation. More practical applications of the work, including conservation implications and uses for commercial forestry are also discussed. Finally, I emphasise that the ability to develop empirical models of the effects of variation in foliage chemistry on animal performance should not be put aside until our understanding of the physiological effects of DFPCs is complete. We now have the capability to develop models of marsupial foraging at a range of spatial scales with a power never before possible, using NIRS techniques, and I explore these possibilities at the end of this discussion.

DIFORMYLPHLOROGLUCINOL COMPOUNDS AS MAJOR DETERRENTS TO MARSUPIAL FEEDING

Assigning a Role in Deterring Herbivores to a Plant Secondary Metabolite

In the introduction to this thesis I identified the necessary requirements for attributing, and understanding, a role of any PSM in a plant-herbivore interaction. In summary, these were:

1. Herbivores should reduce the amount or likelihood of feeding on a plant in manner that correlates with the concentration of the PSM in the foliage;
2. Addition of the purified PSM to an artificial diet should reproduce the deterrent effect at concentrations similar to those seen in the natural diet;

3. There should be some form of post-ingestive feedback that allows the animal to regulate its intake of the PSM to prevent or limit intoxication;
4. If some pre-ingestive taste cue is available, the intensity of which correlates with the concentration of the active PSM, a conditioned food aversion should arise that further enhances the ability of the animal to regulate its intake of the PSM, and;
5. Ideally, some understanding of the metabolism of the PSM should be gained.

In this study, I have addressed all but the last of these points and can say with confidence that DFPCs are the major deterrents to feeding of marsupial folivores on *Eucalyptus* (Chapters 2 and 6). The ability to quantify sideroxylonals precisely has allowed explanation of most of the variation in feeding between individual *E. polyanthemos* and *E. sideroxylon* trees and addition of sideroxylonal to an artificial diet does reduce feeding at similar concentrations (Chapter 6). Jensenone, and presumably other DFPCs, causes serotonin-mediated post-ingestive feedback which allows the animals to regulate their intake of the compound (Chapter 3) (For simplicity, hereafter I use the term "emesis" to refer to this post-ingestive feedback, but note the difficulty in attributing feelings of nausea to animals, discussed in Chapter 3). The concentrations of DFPCs and terpenes are correlated (Chapter 6) and this allows the formation of a conditioned food aversion to terpenes, whereby common ringtail and common brushtail possums can determine the likely consequences of ingestion before feeding (Chapter 4). It is still unknown quite how jensenone causes post-ingestive emesis as there is little information available on its fate once ingested. The metabolism of DFPCs, and jensenone in particular, has recently begun to be investigated by Stuart McLean and co-workers in the School of Pharmacy, University of Tasmania. I briefly summarise their very preliminary data in a later section.

The identification of DFPCs as major deterrents to marsupial feeding only very recently, despite intensive research effort in the area (see Introduction), highlights the advantages of using an approach (bioassay-guided fractionation) to the problem that avoids preconceptions. The evidence for the role of these compounds is quite conclusive but, as with all developing research fields, there remain significant gaps in our understanding. Most notable is the lack of a detailed field study which addresses both foliar variation in DFPCs (and other leaf chemistry), at the level of individual trees, and animal foraging and performance together. Unfortunately, the resources (especially time) available in this project prevented such a study. However, it remains an important research goal.

The requirements outlined above for assigning an anti-herbivore role to a PSM apply also to other plant-mammalian herbivore systems. There are studies where the data cited to support a putative anti-herbivore role for a PSM are purely correlative (e.g. Duncan *et al.* 1994), or preference bioassays only are performed (e.g. Sinclair *et al.* 1988). In these cases, I suggest that the evidence is inconclusive, especially where the PSM considered to be deterrent to feeding is a terpene. For example, the correlative evidence in Chapters 2 and 6 between marsupial feeding and foliar terpene concetrations may have be taken to indicate that they were the major deterrent compound. But this is refuted by the comparison of active concentrations in foliage and artificial diets, and the evidence for a conditioned aversion to terpenes in Chapter 4. Choice bioassays are indicative of at least an aversion, but do not test the limits to the animal's capacity to tolerate or detoxify a PSM: only part of the role of that PSM is elucidated.

Testing the strength of post-ingestive effects lends stronger support to a more fundamental role in herbivore deterrence. Despite a multitude of pathways for toxic effects and metabolism, the perception of the effects of many toxic PSMs are seen as emesis (Provenza 1995a) (discussed in more detail below) and thus experiments with anti-emetic drugs, as described in Chapter 3, may provide a useful first test of post-ingestive feedback. Alternatively, lavage experiments, as described in Chapter 3 and Pass and Foley (1998) may at least indicate post-ingestive feedback, but give little indication of the mechanism. For example, captive brushtail possums showed an aversion to salicin in the diet, but did not further reduce their intakes of a diet treated with salicin, even when lavaged with a substantial bolus dose. This was taken to indicate that the primary effect of salicin was pre-ingestive (Pass and Foley 1998). In some systems the complexity of the interactions, in terms of numbers of plant species (and hence variety of PSMs) may prohibit such extensive research to illustrate the mechanism underlying the interaction between the herbivore and every plant species it utilises. In these cases, a functional tool able to discriminate between favoured and non-favoured food items may be more valuable. The value of empirical versus mechanistic models, and the application of NIRS analysis to these situations, is addressed in the penultimate section of this discussion.

Further Identification of Important Diformylphloroglucinol Compounds

Clearly an important goal of future research must be the identification of other important DFPCs and the development of methods to quantify them. The structure/activity study (Chapter 5) illustrated the difficulty in assigning importance to any particular part of the molecule. However, some insights were available, and it appears that at least one aldehyde group and the ketone group on the side chain are necessary for antifeedant activity against marsupial folivores. Macrocarpals, sideroxylonals and jensenone show similar activity at equivalent molar concentrations, whereas euglobals show substantially less. Future research should therefore focus on methods to at least identify and quantify macrocarpals as a group, as well as sideroxylonals, and this is currently being pursued (WJ Foley and BM Eschler *personal communication*). Ideally, direct bioassay comparisons should also be made between the activity against marsupial feeding of a variety of macrocarpals (i.e. with differing terpene side chains), to determine if a general macrocarpal assay is sufficient to capture variation in herbivore deterrency between foliage samples. This will be necessary as there are usually several different macrocarpals to be found in a single foliage sample (Yamakoshi *et al.* 1992; Osawa *et al.* 1996).

The difficulties in actually achieving these goals should be noted. During the course of the broader study of which this thesis has been a part (3.5 years), there have been a full-time post-doctoral chemist, a PhD student in chemistry and a number of other staff with chemical backgrounds who have worked on the isolation, identification and development of assay techniques for DFPCs. Thus far three compounds (macrocarpal G, sideroxylonal A and jensenone) have been isolated in sufficient quantity for bioassay experiments (Pass *et al.* 1998; Chapters 2, 3 and 6) and quantitative assays have been developed for only two (sideroxylonal, jensenone) (BM Eschler *personal communication*; Chapter 6), while new DFPCs continue to be identified by other workers (Kokumai *et al.* 1991; Takasaki *et al.* 1994a,b ; Singh and Etoh 1995; Osawa *et al.* 1996). Therefore, although it is tempting to suggest a wide array of avenues for further experimentation these will be limited by the constraints on development of chemical methods. Synthesis of these compounds in the laboratory may be an alternative to attempts to purify them from leaf extracts (Chiba *et al.* 1996a, b; Tanaka *et al.* 1997) but will likely be limited also by the quantity required for whole animal bioassays. This also highlights the value of using empirical models of food intake (and other measures of performance such as digestibility) as can be derived from NIRS. If acceptable calibrations can be developed for these measures in other *Eucalyptus* (and non-*Eucalyptus*) species, before detailed knowledge of foliage chemistry is available, then they should be put to use in field studies as soon as possible.

Metabolism and Physiological Effects of Diformylphloroglucinol Compounds

At the time of writing there are few data on the fate of DFPCs when they enter the body of a marsupial folivore. It is possible to say with confidence that they are absorbed and metabolised because no trace of jensenone, sideroxylonal or macrocarpals, or related compounds, have been found in the urine or faeces of either common ringtail or common brushtail possums fed any of these compounds (S McLean *personal communication*). Research in this area has commenced and is primarily being undertaken by Dr WJ Foley of the Division of Botany and Zoology, Australian National University and Dr S McLean, S Brandon and G Pass of the School of Pharmacy, University of Tasmania. Their preliminary findings are summarised below.

As stated above, no metabolites of DFPCs have been found. When jensenone was incubated in loops of rat ileum, it disappeared rapidly and was apparently metabolised. HPLC analysis showed multiple peaks but these were unstable and eventually consolidated into two peaks at the polar end of the chromatogram. Mass spectroscopy showed only jensenone, perhaps indicating that some jensenone adduct is formed that breaks down under those conditions. A notable observation of the gut incubation was that the gut contents became very thick and mucus-like, indicating significant cell damage.

The next stage of investigation showed that jensenone does indeed form adducts with proteins. Jensenone was incubated *in vitro* firstly with a gut enzyme (trypsin) and then albumin to test if it bound to proteins generally. In both cases jensenone disappeared but no product could be isolated or identified. Glutathione was then tested, as it reacts readily with reactive electrophillic substances, protecting cells from toxicity (Cheeke 1998). Once again jensenone disappeared rapidly but no product could be identified. Finally, acetylcysteine was reacted with jensenone (as it is a simpler compound with same thiol functionality as glutathione). Jensenone disappeared more slowly, but a new, stable peak appeared on HPLC which increased as jensenone decreased. Several milligrams were isolated, MS showing a molecular weight of 238, indicating the loss of CO from jensenone, but NMR analysis has to date been hindered by the lack of sufficient sample. This may give some insights into the fate of jensenone post-ingestion. The rapid *in vivo* disappearance may be due to reaction with glutathione, which is abundant in cells. This would explain why no urinary metabolites have thus far been found. The antifeedant activity may thus result from non-specific toxic effects on cells and/or specific binding to particular gut receptors (S McLean and S Brandon *personal communication*). That the activity is due to non-specific toxic effects is also supported by the wide range of activity shown by DFPC compounds generally (discussed in Chapter 5). Such an effect would also be consistent with cellular damage causing release of serotonin from enterochromaffin cells as one form of post-ingestive feedback arising from jensenone (as suggested in Chapter 3).

However, other toxic PSMs may act at specific receptor sites and this possibility also may be consistent with results of McLean and co-workers. For example, ingenol esters in leafy spurge (*Euphorbia esula*) activate the phosphorylating enzyme protein kinase C which leads to activation of the hypothalmic-pituitary-adrenal axis, leading to the development of conditioned food aversions in sheep and rats (Kronberg *et al.* 1995). These authors also suggest that this action may be similar to that of LiCl, the commonly used “emetic stimulant” in flavour aversion studies, though they do not explicitly state that emesis results from ingestions of ingenol esters. Diterpene alkaloids in larkspur (*Delphinium* spp.) also function by binding to specific receptors, in this case for acetylcholine, and result in neurological disturbance and respiratory failure (Pfister *et al.* 1997). Again it is noteworthy that the authors suggest that part of the effect of toxic larkspur ingestion is perceived by the animals as nausea, or emesis. Therefore, while the specific action of jensenone has yet to be elucidated, the herbivore may perceive its effects similarly, whether it causes general cell toxicity or damage or whether it acts by competitively binding to specific receptor sites. This is consistent with the data presented in Chapter 3 indicating the post-ingestive emesis is one of the major forms of feedback on which animals may regulate their intake of jensenone.

Feedback Effects of Diformylphloroglucinol Compounds

Chapters 3 and 4 showed that at least part of the post-ingestive feedback used by common ringtail and brushtail possums to regulate their intakes of jensenone was mediated by serotonin acting at the $5HT_3$ receptors, and that this feedback was enough to condition an aversion to the flavour of terpenes. However, it was also apparent that, as administered, the ondansetron was not sufficient to completely abolish the effects of jensenone on intakes. There are two possibilities that may explain this observation, the first methodological and the second physiological.

It is quite possible that the route of administration of the drug did not maintain plasma concentrations at sufficiently high levels to prevent feedback for the course of the feeding period. Therefore other methods of administration that allow prolonged release of ondansetron at active levels may further reduce the antifeedant effect of jensenone. This was partly achieved in Chapter 3 by adding ondansetron to the diet of common ringtail possums rather than administering it via inter-peritoneal injection. An attempt to further prolong the release of ondansetron has also been made using osmotic pumps, which are inserted into the animal sub-cutaneously or inter-peritoneally, and provide release of a compound at continuous levels for up to 28 days. Unfortunately, in this instance the pumps were inserted into brushtail possums sub-cutaneously and several of the animals subsequently lost their pumps during the course of the experiment, reducing the statistical power of the experiment to unsatisfactory levels (Stapley 1998). The experiment will soon be repeated with inter-peritoneal insertion of osmotic pumps (J Stapley and WJ Foley *personal communication*).

The alternative possibility is that the post-ingestive feedback of jensenone is derived from several different pathways and that blocking $5HT_3$ receptors can only partially reduce its antifeedant effects. For example. even if the effects of jensenone are manifest only in release of serotonin, there are at least 14 serotonin receptors, of which we have blocked only one. Serotonin may act as a neurotransmitter, neuromodulator or neurohormone, and it affects a variety of behaviours including feeding, sex and aggression (Weiger 1997). Jensenone may also be subsequently found to exert effects other than through serotonin release, such as the release of other neurotransmitters involved in emetic responses (e.g. dopamine (Grant 1987)).

The understanding of the general effects of DFPCs is further hindered by the lack of complementary experiments with other deterrent DFPCs (macrocarpals, sideroxylonals).

Jensenone has been used for logistical reasons as a "model" DFPC due to the relative ease with which it can be isolated in sufficient quantity for bioassay experiments. While it is reasonable to suppose that the effects it exerts are similar to those likely to result from macrocarpal or sideroxylonal ingestion, given the close similarity in molecular structure and activity between these compounds, these experiments should be repeated with these compounds before general conclusions are drawn.

Despite the wide array of specific effects of PSMs, a general rule appears to be that ability of animals to regulate their intakes of PSMs relies proximally on a feeling an "internal malaise" (*sensu* Provenza (1995a)). This applies to PSMs as diverse as jensenone, condensed tannins, PSMs of leafy spurge and larkspur described above (Provenza *et al.* 1990, 1994; Kronberg *et al.* 1995; Pfister *et al.* 1997; Chapter 3). Whether this can generally be attributed to an emetic response *per se* remains uncertain due to poor definition of the make-up of the emetic centre (Grant 1987) and the difficulty inherent in attributing perceptions to animals (Chapter 3). Nevertheless, the emetic response is considered to be centred in the area postrema of the brain, which also has a role in governing food intake, sleep and blood pressure (Andrews *et al.* 1988). As well as providing the negative feedback on which PSM intakes can be regulated, it may apply also to a nutrient-deficient diet (Provenza 1995a; Provenza *et al.* 1995, 1996). A clear example of the similarities in the mechanisms of regulation of nutrient and toxin intake is that ondansetron (the drug used in Chapter 3 to increase possums' intakes of jensenone) completely ameliorated the reduced food intake by rats of a diet deficient in amino acids (Terrynathan *et al.* 1995). This "internal malaise" provides a general mechanism by which animals can regulate their food intakes in response to variation in PSM and nutrient content of diets and their own changing requirements. This idea is fully developed in the reviews of Provenza and colleagues (Provenza 1995a,b; 1996a,b; Provenza *et al.* 1998).

Inter-Animal Differences in Physiological Capacity to Cope with Diformylphloroglucinol compounds

Chapter 2 showed that there is significant variation between koalas and ringtail possums in their capacity to ingest DFPCs in foliage diets. This also extends to apparent variation between ringtail and brushtail possums fed jensenone in an artificial diet and perhaps even to populations of common ringtail possums (though this was heavily confounded with time and exposure to the jensenone in the diet) (Chapter 3). The question must therefore be asked: why and how do different species or individuals cope with different levels of PSM intake?

The answer to the question why there are interspecific differences most likely lies in the level of dietary specialisation shown by these species. As discussed in the introduction, there is a trend from generalist to specialist in the tendency of brushtail possums, ringtail possums and koalas to feed on *Eucalyptus* foliage. Therefore it is appears intuitively likely that the most specialised feeder (the koala) should divert proportionally more of its detoxification capacity towards coping with specifically *Eucalyptus* toxins, while the generalist brushtail would benefit more from a broad-based ability at the expense of coping with any particular toxin. Evidence for this capacity is seen in the corresponding trend between these species in their ability to oxidise *Eucalyptus* terpenes, with brushtail possums < ringtail possums < greater gliders < koalas (R Boyle *personal communication*). Why different populations of the same species (i.e. northern versus southern populations of ringtail possums) differ in their capacity to deal with jensenone is less clear. It may be that different selection pressures operate in each region, with concentrations of DFPCs encountered in nature lower in the north than in the south. There are currently no data on this situation and any discussion must therefore remain speculative at this stage.

How different species or populations within species are able to more effectively tolerate or detoxify DFPCs is also an important issue. As discussed in Chapter 2 body size is unlikely to be important, as the mass-specific capacity to detoxify PSMs should be higher in small animals (Freeland 1991), the reverse of what we see between koalas and ringtail possums. Similarly, the overall liver clearance rate does not differ between brushtail possums and koalas (Pass and Brown 1990) but this does not necessarily reflect the capacity to detoxify individual PSMs. There are numerous examples of herbivore species that can tolerate intakes of PSMs that would be toxic, or even fatal, to other species, of which the ability of black colobus monkeys (*Colobus satanas*) to detoxify alkaloids is perhaps best known (McKey *et al.* 1981). There are also recorded instances of variations within species (goats - (Pritz *et al.* 1997); desert woodrats - (MD Dearing *personal communication*)). In fact, the intake of terpenes by ringtail possums recorded in Chapter 4 is an order of magnitude higher than the fatal dose for humans (McLean and Foley 1997). Again the lack of data on the metabolic fate of DFPCs limits further discussion of the mechanisms by which they are tolerated or detoxified by even one species, much less interspecific or inter-population comparisons.

ECOLOGICAL AND PRACTICAL IMPLICATIONS OF THE ROLE OF DIFORMYLPHLOROGLUCINOL COMPOUNDS

Clearly there is significant intra- and inter-specific variation in foliar concentrations of DFPCs with accompanying variation in their resistance to herbivory by marsupial folivores. Given the apparently clear benefit to be gained from reduced herbivory, one might well ask the question, “why are there any susceptible trees”? In light of the fact that there certainly are susceptible trees, a significant cost must accompany investment of resources in DFPC production. An understanding of the factors governing this investment will yield advantages both in further understanding of the natural system and in practical applications.

Costs and Benefits for Plants of Investment in Diformylphloroglucinol Production

There have been many studies of cost and benefits of plant defence (e.g. Gulmon and Mooney 1986; Bazzazz *et al.* 1987; Gershenzon 1994). Those that have been most successful have clearly defined the defensive measure, be it chemical (Ohnmeiss and Baldwin 1994; Euler and Baldwin 1996; Iwasa *et al.* 1996; Mauricio and Rausher 1997; Baldwin 1998) or mechanical (Gowda 1996; Rohner and Ward 1997). There has also been some success in showing costs and benefits of broad classes of PSMs, such as tannins, as defences (Coley 1986; Sagers and Coley 1995). However, in these studies the evidence for a defensive role of tannins *per se* was weak and correlative only, and Coley (1986) noted the likelihood of varying activity between the different individual tannins grouped together in the total tannin assay. As I noted in the introduction, there have been few instances where the specific chemical defending plants from browsing mammals have been identified. Consequently, there are correspondingly few studies of the costs and benefits for plants of investment in PSMs as defences against mammals specifically. Again, the use of measures of chemically defined PSMs (Bryant and Julkunen-Tiitto 1995) has met with more success than the use of crude measures, such as total phenols (Rohner and Ward 1997). In the *Eucalyptus*-marsupial folivore system we are now beginning to develop sufficient understanding of the interaction to more precisely identify the costs incurred.

In this case, the most significant cost must be an investment in carbon. It appears that increased production of DFPCs is most often accompanied by an increase also in terpenes, and the two have complementary roles in defence. Sideroxylonals and terpenes may constitute over 5% of the dry weight of the leaf in the species measured in this study (and higher in other species, such as *E. melliodora*: B Moore *personal communication*). It should be noted that this is substantially lower than the levels of "total" phenolics frequently used in such studies, but is more reflective of the true investment in defence. The two types of compounds must be produced by common metabolic processes which may reduce the costs of producing the appropriate biochemical machinery. It is not yet known if DFPCs are stable in leaves, or whether they are constantly turned over, and this must be determined before costs can be appropriately quantified (Coley *et al.* 1985). A preliminary indication that they may be mobile is that a field study recorded a decrease in sideroxylonal of mature leaves coincident with flushing of new growth in spring (Watson 1998) indicating a reallocation of those resources.

The benefits to the plants are perhaps less clear. While there is no doubt that high foliar concentrations of DFPCs reduce herbivory, this can only be evaluated in the context of herbivore pressure. There may be circumstances where selection pressure due to herbivory is particularly low, and this may explain the persistence of susceptible individuals in populations. For example, the studies of Braithwaite and others (Braithwaite *et al.* 1983; Cork 1992; Cork and Catling 1996) have suggested that, for large areas of *Eucalyptus* forest, foliage is probably too low in nutrients (especially N) to support viable populations of folivorous marsupials. Thus herbivore pressure would be extremely low, regardless of allocation to defensive PSMs, and this may create a reservoir where susceptible genotypes could persist. It is even feasible that low herbivore pressure is more the rule than the exception in *Eucalyptus* forests, and that the extreme examples of high herbivore pressure seen in recent times (e.g. Martin 1985a,b) may be an artefact of anthropogenic changes in the ability of animals to disperse or other effects. A long-term study of changes in the proportions of resistant and susceptible individuals in populations under varying herbivore pressure may yield some valuable insights.

Factors Affecting Allocation of Resources by *Eucalyptus* to Foliar Diformylphloroglucinols

As discussed above, increased production of DFPCs represents a carbon cost to the plant, particularly as it appears compounded by corresponding increases if foliar terpenes. Concentrations of DFPCs in leaves may therefore be expected to vary with resource availability, as described in the introduction, which may be indicated by the seasonal differences noted above (Watson 1998). There are, however, reasons to believe that resource availability may not be the major determinant of foliar sideroxylonal concentrations.

Foliar terpene concentrations in *Eucalyptus* do not respond according to the resource availability/C:N balance hypotheses (Lawler *et al.* 1997). Therefore, if as appears to be the case, DFPC production is closely linked to terpene production (Chapter 6), DFPC concentrations also are unlikely to vary in accordance with resource availability. Here I return to the point made in the introduction: analyses of variation in total phenolics are perhaps more likely an indication of carbon excess and not a direct measure of allocation to defence. Identification of the particular important phenolic compounds show that this is unlikely, despite the fact that "total" phenolics in *Eucalyptus* do vary with resource availability (Lawler *et al.* 1997). This is further supported by the field data. The extreme ends of the spectrum of foliar sideroxylonal values have been recorded in trees only a few metres apart (Chapter 6). A large-scale field study of foliar sideroxylonal concentrations also showed that over a regional scale (>200 km) 40% of the variation in 108 trees from 18 populations was explained by individual trees within populations (environmental conditions were similar within populations) (Watson 1998).

These data may also be used to support the contention that there is a strong genetic basis to allocation of resources to DFPC production. The approximately normal distribution of foliar sideroxylonal concentrations amongst individual trees in a single site was considered indicative of a multi-locus genetic system (Chapter 6; Watson 1998). Once again, this must be confirmed with further experimental work. However, it does raise interesting practical possibilities for both conservation and forestry. For both purposes, selective breeding programmes may be able to produce plants with different levels of resistance to browsing by marsupials. For conservation purposes it may be desirable to select for less resistant individuals for rehabilitation of damaged habitat (ANZECC 1997), or for feeding of captive animals. Foresters may select instead for increasing resistance to browsing marsupials, which may increase the viability of plantations without the need for fencing or pest baiting programmes which are not economically viable or are publicly unacceptable (Montague 1993, 1994, 1996).

A genetic basis to DFPC production may be further exploited by foresters if the genes can be activated by environmental stimuli. For example, phenolic production may increase in response to UV light (Lavola *et al.* 1997; Schnitzler *et al.* 1997; Lavola 1998) or ozone (Lavola *et al.* 1994; Booker *et al.* 1996; Biagioni *et al.* 1997; Paakkonen *et al.* 1998). Some investigation has already been made of the potential for UV-B activation of sideroxylonal production by *E. sideroxylon* (Watson 1998). There was no effect of UV-B on foliar sideroxylonal concentrations. The potential for ozone induction of DFPC production is untested.

An interesting point to come out of the study of Watson (1998) was the differences in foliar sideroxylonal concentrations between seedlings and adult trees. Seedlings were grown from each of six parent trees and had substantially higher foliar sideroxylonal concentrations than did the parent trees in the field. Despite the fact that differences in growth conditions may have been substantial (and were not taken into account) this may be significant. As noted above, environmental conditions may not be significant in this process. The differences between parents and offspring were such that parents would be susceptible to browsing by common ringtail possums whereas their offspring would be resistant. This is consistent with the proposition of Bryant and Julkunen-Tiitto (1995) that the relatively higher loss of biomass to herbivory by juveniles, and higher probability of mortality, should result in a greater allocation of resources to defence in juveniles.

Importance of Diformylphloroglucinols for Foraging of Marsupial Folivores

A recurring theme of this thesis has been the fact that marsupial folivores of *Eucalyptus* have been repeatedly shown to exhibit preferences between individual trees within species in the field (Pahl 1984, 1987; Geritz 1987; Hindell and Lee 1987, 1988). No study has yet shown a chemical basis underlying these preferences. It is now possible to do so, using both an improved knowledge of the important PSMs, and the ability to make an assessment of quality based solely on the NIR spectrum of a sample.

Data presented in this thesis show the importance of measuring the leaf chemistry of every individual tree within an animal's home range. Some southern Australian woodlands are dominated by the species investigated in this thesis and studies by others (Watson 1998; B Moore *personal communication*) and in which foliar sideroxylonal concentrations vary at a scale relevant to the foraging of individual animals (Chapter 6). Studies of individual animals foraging can therefore be made with at least the preferences for the dominant species (and others not yet examined which are subsequently shown to have sideroxylonals as the dominant DFPC) explained mechanistically. For other food species where DFPCs may be absent or as yet unidentified, NIR calibrations of intake (and other measures such as digestibility (Brooks *et al.* 1984; Redshaw *et al.* 1986; Givens *et al.* 1991, 1992)) can be used to provide further understanding into the importance of leaf chemistry in foraging decisions (see discussion in following section).

A field study would require detailed observation of feeding behaviour of individuals within their home ranges, to determine how data from captive animals presented here translate to preferences in nature. I would anticipate at least a threshold of foliar DFPC above which foliage is never fed on. An absolute limit should exist but a realised threshold would most likely be habitat-dependent, decreasing with increasing availability of palatable foliage (and hence greater opportunity to choose to avoid foliage high in DFPCs). Where there are high proportions of trees with low concentrations of DFPCs then tree use may also vary in response to factors such as digestibility, or metabolisable energy content of foliage, or non-nutritional factors such as habitat structure (Lindenmayer *et al.* 1994; Pausas *et al.* 1995). With detailed information on foliage chemistry of individual trees, and hence food availability, a better understanding may also be gained of determinants of home range size and habitat use (Benhamou 1996; Ransome and Sullivan 1997; Fortier and Tamarin 1998; Hubbs and Boonstra 1998) and allocation of energy to reproduction (Munks and Green 1995, 1997; Krockenberger 1996; Krockenberger *et al.* 1998).

Empirical Versus Mechanistic Models of Food Choice - The Value of Models Based on Near Infrared Spectra of Foliage

In a pure sense, if understanding of a plant-herbivore system is the objective, then models of herbivore food choice should try to identify and quantify the specific chemical components, and physiological processes governing these decisions. That is, they should define the mechanisms underlying the interaction. However, as I have shown in this thesis, to do so for even a single herbivore and a single PSM requires extensive experimentation and is heavily limited by the logistics of purifying, identifying and quantifying the PSM of interest, particularly if it is a compound not well studied. This is especially so when studying mammalian folivores, which generally consume material from a variety of species and in amounts that place severe restrictions on bioassay studies.

In contrast, if our goal is only to be able to better describe the observed feeding behaviour, or perhaps produce information and tools for habitat management for conservation, then perhaps an empirical model is more appropriate. This is only possible provided that such a model can both reliably make the distinction between preferred and avoided food items, and the means are available to develop this rapidly and inexpensively (relative to mechanistic models). NIRS analysis provides such a means and the benefits are substantial.

I have outlined earlier in this discussion the substantial effort required to advance our understanding of the *Eucalyptus*-marsupial folivore system to the point that we can now explain slightly over >80% of the variation in feeding of ringtail possums between individual trees of *E. polyanthemos*. This should also be considered in the context of research efforts spanning some 20 years (e.g. Betts 1978; Southwell 1978; Cork and Pahl 1984; Pahl and Hume 1990; Hume and Esson 1993) which have not identified clear trends. The rapidity and precision of the development of NIRS models stands in stark contrast against this background: the models described in Chapter 6 took less than a day to develop and explain more variation (91%) than does any mechanistic model. Similar success has been achieved with models of food intakes in koalas (B Moore *personal communication*). The NIR spectrum of a sample is a composite of the entire biochemistry of the leaf and, combined with modern computing power and statistical techniques, can identify the important features (i.e. wavelengths in the spectrum) providing a powerful functional tool for investigating foraging behaviour of herbivores (Foley et al. 1998 - see Appendix 5). Modelling intake (or other measures of performance) directly against the NIR spectrum is also efficient because there is no middle stage, of assaying a leaf chemical component in the laboratory (where there is potential loss of precision introduced), which is then modelled against food intake.

A further advantage is the lack of preconceptions about important characteristics of foliage chemistry. The number of studies examining the effect of "total" phenolics on feeding of marsupial folivores without identifying clear trends illustrates the value of this approach. In fact, this may confer another benefit, in that determining the wavelengths contributing to an NIR calibration can indicate the nature of the compounds involved. For example, Clarke and Lamb (1991) surveyed the use of different NIR wavelengths as predictors of dry matter digestibility in ruminants. They found that regardless of forage type, wavelengths associated with C-H groups typical of plant fibre were important. Similarly, in a study of resistance of sugar cane to stem borer attack, Rutherford and Van Staden (1996) used important wavelengths in calibration equations to implicate alcohols and carbonyl components in resistance. An initial empirical approach became an analytical tool that substantially increased progress towards identifying resistance factors through bioassays.

The development of comprehensive calibration equations, such as described in Chapter 6, may also be hindered by the complexity, or lack of necessary knowledge, of the system. Low tree species diversity in some systems, such as some *Eucalyptus* woodlands or boreal forests, simplifies studies of mammalian feeding, and inter- and intra- specific differences in plant deterrence of herbivores may be more apparent. In contrast, in tropical rainforests where other folivorous marsupials, monkeys or lemurs may feed on an extensive array of species (and not feed on even more) it may be difficult, or impossible, to identify such continuous trends in deterrency of individual trees within all food species. However, field studies in these systems frequently note at least interspecific preferences (Oates *et al.* 1980; McKey *et al.* 1981; Procter-Gray 1984; Oates 1988; Ganzhorn 1989; Kool 1992), and also may record intraspecific preferences (Glander 1978; Waterman and Kool 1994). While the development of continuous calibration equations may not be an option, combining NIRS with discriminant functions may yield functional tools (with the potential for analytical insights as described above). The capacity to determine at least the proportion of individual trees of a "food" species likely to be avoided within an area may yield valuable information on food availability and habitat quality.

Application of Near Infrared Spectroscopy to Landscape-Scale Assessment of Habitat Quality

If one of the primary aims of conservation is the management of forest areas to retain habitat suitability for animals, then landscape-scale maps of those features of the habitat important for those animals are required (Coops and Catling 1997). Indeed this is cited as a primary objective for conservation of koalas (ANZECC 1997). As described throughout this thesis, arguably the greatest limiting factor for koalas, and marsupial folivores of *Eucalyptus* generally, is the quality of foliage. There have been to date two major obstacles to the development of habitat maps at scales suitable to management: the inability to discriminate between foliage of acceptable and poor quality for marsupial food, particularly within *Eucalyptus* species, and; the enormous logistical difficulty inherent in assaying foliage quality over large areas of forest. While addressing the first issue, this thesis has perhaps made the second of these obstacles appear even greater, due to the need to sample individual trees within species (or at the very least a representative sample of individuals within each species), increasing the sampling intensity used in previous species-level approaches. However, I conclude this thesis with discussion of how NIR spectroscopy may be performed remotely, using field or airborne scanners, to both incorporate the resolution that the data presented her stipulates is necessary for adequate description of habitat variability, and to escape the limitations of manual sampling and laboratory analysis.

I have shown that the NIR spectrum incorporates much of the information about the biochemical composition of a leaf, and that this can be used to estimate defined chemical characteristics of leaves, such as nitrogen, as well as functional measures such as animal intake. The NIR spectrum is simply that light in the appropriate wavelengths reflected from a sample after being irradiated with light. The same principle can therefore be applied to leaves irradiated with sunlight, or whole canopies, in the field and indeed leaf biochemical information is transmitted almost unaltered from the leaf to the canopy (Kupiec and Curran 1995). It is possible then to record leaf biochemical information remotely, provided the technology is available to capture this information with sufficient precision.

Traditional aerial or satellite telemetry has had poor wavelength resolution. For example, Landsat satellites and other scanners measure NIR light only as broad bands, and thus are unable to capture the available biochemical information, though estimates of leaf area index and habitat complexity could be reasonably estimated (Coops *et al.* 1997; Coops and Catling 1997). New generation scanners have substantially improved in this area. AVARIS, CASI and other scanners (collectively termed hyperspectral (Held 1997)) can measure multiple bands with a band width as narrow as 1.9nm (Held 1997). These have already been adopted for the measurement of leaf chemistry in a variety of Northern Hemisphere situations with promising results. Strong correlations have been developed between NIR spectral data from aerial spectrometry and leaf nitrogen ($r^2 = 0.87$), cellulose ($r^2 = 0.79$) and lignin ($r^2 =0.77$) (Gastelluetchegorry *et al.* 1995; Curran *et al.* 1997; Martin and Aber 1997).

Similar scanners have begun to be adopted for use in *Eucalyptus* forests. Hand-held instruments have been shown to be able to capture sufficient spectral variation to discriminate between a wide range of *Eucalyptus* species (L Kumar and A Skidmore *personal communication*). Attempts are now underway also to develop calibrations of field-spectra of *Eucalyptus* foliage against leaf chemistry (BTurner and WJ Foley *personal communication*). While there will no doubt be substantial logistical hurdles to overcome, the knowledge of the factors determining the resistance or otherwise of *Eucalyptus* foliage to marsupial folivores, combined with the ability also to measure other important nutritional characteristics such as nitrogen, and even directly estimate likely intake, will provide a vital management tool. Given the numerous pressures being exerted on *Eucalyptus* forest ecosystems by anthropogenic activities, research into this area should proceed as a matter of urgency.

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APPENDIX 1. SWAMP WALLABIES AND TASMANIAN PADEMELONS SHOW INTRASPECIFIC PREFERENCES FOR FOLIAGE OF *EUCALYPTUS OVATA*

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SUMMARY

Using cafeteria-style experiments with captive animals, we investigated preferences shown by swamp wallabies (*Wallabia bicolor*) and Tasmanian pademelons (*Thylogale billardierii*) for the foliage of different indvidual trees of *Eucalyptus ovata*. Strong and consistent preferences were shown by both macropod species. These preferences were not correlated with any of the conventional measures of nutritional quality or putative defense compounds often made on *Eucalyptus* foliage. However, the preferences shown by these macropods correlate strongly with preferences shown by common ringtail possums (*Pseudocheirus peregrinus*), an interaction which is far better understood. We interpret this correspondence as being the result of the same chemical factors being important for all three marsupial species.

INTRODUCTION

Browsing of seedlings by marsupials is a major obstacle to the establishment of viable *Eucalyptus* plantations (Wilkinson and Neilsen 1995). Even non-fatal browsing by an animal is sufficient to reduce the future timber value of a seedling through its effect on the growth form of the tree (Montague 1996). This damage, inflicted in many cases principally by macropods, causes losses estimated at up to $400/ha in Victorian plantations (Montague 1996) and the failure of up to 63% of unprotected areas in Tasmania (Neilsen and Wilkinson 1995). Consequently, methods to reduce or eliminate marsupial browsing have been a primary focus of research. Methods such as fencing or poisoning are considered less than ideal due to their cost and negative public perceptions respectively (Montague 1993; Wilkinson and Neilsen 1995; Montague 1996).

One approach often suggested for dealing with this problem, for both vertebrate and insect herbivores, is to exploit natural resistance to herbivory (Floyd and Farrow 1994; Montague 1994). Field studies of wild vertebrates have shown that some *Eucalyptus* species display very strong intraspecific (i.e. between individuals) variation in their susceptibility to browsing by marsupials (e.g. Hindell and Lee 1987; Pahl 1987). If the factors causing natural resistance can be identified, then breeding programmes may select for these traits, conferring a resistance which costs little in dollar terms and minimises the impact on the environment (Floyd and Farrow 1994).

A significant problem in the use of this approach is our poor understanding of what chemical factors in *Eucalyptus* foliage confer resistance to marsupial herbivory. The foliage of eucalypts has long been recognised to be rich in terpenes and phenolics and research into mammalian herbivore resistance has focussed on these compounds. However, few data have accumulated to support a significant role for these compounds (e.g. Cork and Sanson 1990; Hume and Esson 1993). Recently, however, it has been shown that the there is a well-defined group of chemical compounds which can determine the rate at which arboreal marsupials can ingest foliage, and which, in some individual trees, occur in concentrations sufficient to completely deter feeding (Lawler *et al.* 1998a; Pass et al. 1998; Lawler, Foley, Eschler unpublished data).

In this paper, we report on the high degree of variation in palatability of individual trees of *E. ovata* to swamp wallabies (*Wallabia bicolor*) and Tasmanian pademelons (*Thylogale billardierii*). We show that ranking of palatabilites of individual trees is highly consistent between macropod species, and also between the macropods and common ringtail possums. We discuss factors likely determining palatability of *Eucalyptus* foliage to macropods, in relation to the detailed knowledge of such factors affecting the arboreal folivores which is now accumulating.

METHODS

Animals

This experiment was approved by the Animal Experimentation Ethics Committee of Monash University and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Four adult swamp wallabies and six Tasmanian pademelons were housed in separate outdoor enclosures at Monash University in Melbourne. Enclosures measured approximately15m x 20m and had a thick covering of grass. Pelleted foods and water were supplied *ad libitum.*

Eucalyptus ovata was chosen as the study species due to prior knowledge of the variation in food intakes between individual trees shown by ringtail possums (Lawler et al. 1998a). Although *E. ovata* is not a commercial species, we considered that it was more useful to begin these investigations with a species that was known to exhibit significant variation in deterrence, to identify a chemical principle and then to look for that chemical in other eucalypts of commercial value (see Discussion). Six different individual trees of *E. ovata* were collected from areas within 20km of Monash University. Trees were chosen on the basis of an abundance of young, fully expanded foliage and on data from feeding experiments with common ringtail possums (reported in Lawler et al. 1998a).

A cafeteria-style experimental design was used in which six feeding stations were established at even intervals around the perimeter of the pens. Each station was comprised of a bucket of water into which branches approximately one metre long were placed and fastened to the fence to prevent their ends coming out of the water. A sample of foliage from one tree was placed at each feeding station, such that foliage from all trees was offered each night. The experiment was repeated over 6 nights, with positions of foliage re-randomised each night, such that all trees were positioned at all stations by the end of the experiment. Foliage was put in the pens at 1700h and removed the following morning at 0800h. The amount of foliage offered was determined by preliminary feeding in which an abundance of highly palatable foliage was offered and the amount eaten recorded. Branches were weighed before placing in cages and after removal. Leaves dropped on the ground around each station were collected and dried at 80°C for 24 hours. Separate buckets in unoccupied pens were used with foliage samples to corrective for evaporative loss. A sample of this foliage was collected and frozen for later chemical analysis and determination of appropriate dry matter conversion by drying a subsample of foliage. Differences in food intake between trees were analysed by analysis of variance.

Chemical Analysis

All samples were freeze-dried and ground to pass a 1 mm sieve. Samples were analysed for nitrogen, cyanogenic glycosides, total phenolics, condensed tannins, neutral detergent fibre, cellulose, hemicellulose and lignin as described in Lawler et al. (1998a). Relationships between food intake and leaf chemistry were compared by Pearson's correlations.

Comparison of Macropod and Ringtail Possum Intakes

These experiments were run in conjunction with similar, but more comprehensive experiments using common ringtail possums, as described in Lawler et al. (1998a). Hence we were interested to know if those trees which showed resistance to browsing by the macropods were also resistant to browsing by ringtail possums. The experiments with ringtail possums employed a slightly differenct protocol, in which foliage from only a single tree was offered to the animals each night (Lawler et al. 1998a). The small size and low energy reserves of ringtail possums meant that we were unable to offer them the three worst trees offered to macropods because they would not eat enough to maintain themselves. A direct comparison is hence not possible. However, we have recently developed a technique for predicting food intake of common ringtail possums using Near infrared reflectance (NIR) spectroscopy, calibrating food intakes against leaf chemistry as reflected by differences in sample spectra (McIlwee et al. 1998). This model is highly accurate, producing an r^2 value of the relationship between observed and predicted values of 0.94. We have used this equation to estimate the ringtail possum intakes of samples of this foliage in order to compare preferences across taxonomic groups.

RESULTS

Both swamp wallabies and Tasmanian pademelons showed very strong preferences between trees (Fig. 1; $p<0.001$ for both species) which were consistent between the two macropod species ($r=0.93$). Similarly, the preferences of both macropods were also highly consistent with the preferences predicted to be shown by common ringtail possums (swamp wallabies $r=0.97$; Tasmanian pademelons $r=0.91$). No relationships were found between food intake by macropods and any of the leaf chemical measures made.

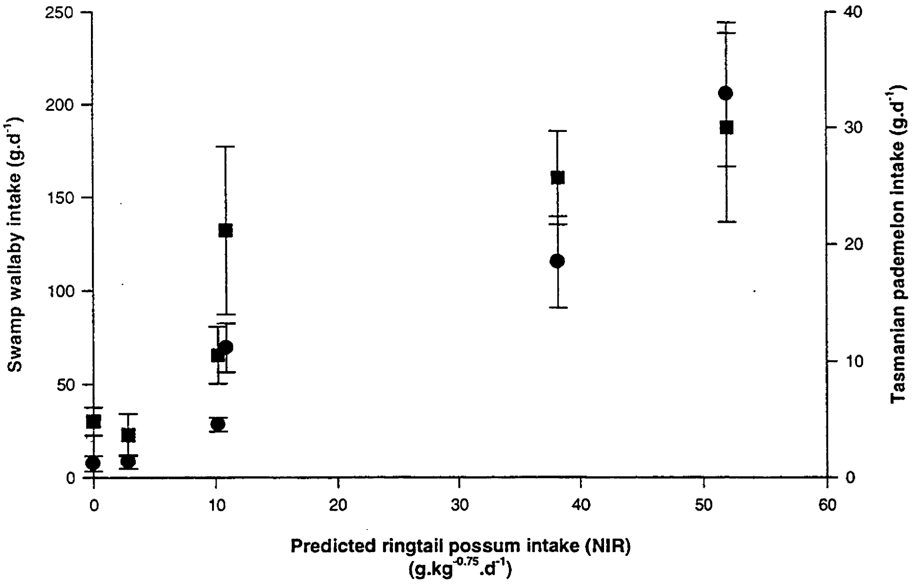

Figure 1. Intake of foliage of six individual *E. ovata* trees by swamp wallabies and Tasmanian pademelons in cafeteria-style experiments.

Data are plotted using intakes by ringtail possums (as predicted by NIR spectroscopy) as the X axis to show the correlation between these measures. Data are means ± standard errors.

DISCUSSION

Variation in the palatability of different species of *Eucalyptus* to swamp wallabies, one of the major pests of eucalypt plantations, has previously been investigated and found to be significant (Montague 1994). We have now shown that there is significant variation between individual *E. ovata* trees which appears stronger than the interspecific differences reported by Montague (1994) and are likely to be even greater in a field situation (Montague 1994). If such variation can be found within commercial species (e.g. *E. regnans*, *E. nitens, E. globulus*) then there is the potential for it to be exploited in breeding programmes to confer natural resistance to macropod browsing of eucalypt seedlings in plantations. Further, it would appear that selection of trees resistant to macropods will also have the added benefit of conferring resistance to arboreal marsupials. However, to do this effectively requires that such variation be found, and ideally, that the chemical attributes causing it be identified.

In this study, none of the chemical characteristics measured showed any relationship with palatability. However, the trend in palatability was strongly consistent with levels of resistance to herbivory by common ringtail possums, an interaction which is now far better understood (Lawler et al. 1998a,b; Pass et al. 1998; Lawler, Foley, Eschler unpublished data). Given such consistency, it is reasonable to surmise that the same chemical features are responsible for a reduction in palatability of *E. ovata* foliage to macropods. In experiments in which *E. ovata* foliage was fed to ringtail possums, two groups of chemical compounds correlated negatively with possum herbivory (Lawler et al. 1998a). These were the terpenes and a recently discovered group of compounds known as diformylphloroglucinol (DFP) compounds. It was further shown that only the DFP compounds could reproduce the resistant effect in bioassay experiments. In addition, concentrations of the two groups of compounds are correlated (Lawler et al. 1998b; Lawler, Foley, Eschler unpublished; Matsuki unpublished data) and ringtail possums appear to use the smell and taste cues of the foliar terpene concentration as a proximal cue to the concentration of the real deterrent, the DFPs (Lawler et al. 1998b). We were unable to measure the specific DFP likely to cause the effect seen (macrocarpal G: Lawler et al. 1998a) in this experiment due to insufficient sample for gravimetric isolation (Pass et al. 1998) and because a suitable quantitative method has not yet been developed. We consider that variation in the concentration of DFPs, and macrocarpal G in particular, in foliage of the individual *E. ovata* trees used here is the most likely explanation for the attendant variation in feeding rates of macropod browsers.

Further investigation of the effect of DFPs on macropod feeding, and variation in their concentrations in commercial eucalypt species, would potentially yield significant advantages in plantation pest management. Other commercial species, *E. globulus*, *E. delegatensis* and *E.*

nitens, contain DFP compounds, the latter two having predominantly sideroxylonals (Pass, Eschler, Foley unpublished data) a group of DFPs which is highly effective in deterring common ringtail possums from feeding (Lawler, Foley, Eschler unpublished data). Further, in all DFP-containing species which we have investigated, we have found significant intraspecific variation in concentrations, covering a range that we consider to confer resistance.

The identification of resistant individuals within a species may prove one of the greater obstacles to progress in this area. However, there are two alternatives which may prove practical. The first is the selection of high-terpene genotypes. As described above, browsing by ringtail possums correlates negatively with terpene concentrations while DFP concentrations are positively correlated with terpenes. As a defacto measure of marsupial resistance, the foliar terpene concentration is useful. However, the biochemical relationship between the two groups of compounds is unknown (Ghisalberti 1996). Thus there may be the potential for selection of high-terpene genotypes to cause some uncoupling of the relationship, perhaps resulting in selection for high-terpene, low-DFP genotypes which are ultimately not resistant to browsing (Lawler and Foley *in press*). A more rigorous technique for identifying and quantifying resistance is NIR spectroscopy. The technique is fully described in McIlwee et al. (1998), but in essence provides a means for the rapid and accurate analysis of large numbers of samples for multiple chemical components. These include "traditional" measures often made such as those listed in this study, but extends also to measurement of relevant DFP compounds, such as sideroxylonals (Lawler, Foley, Eschler unpublished data) and also to less well-defined measures such as animal intake (see above; McIlwee et al. 1998; Lawler unpublished data).

In conclusion, there exists significant variation in palatability of individual trees of *E. ovata* to macropod browsers which is likely to occur in other, commercial *Eucalyptus* species. Further work is required to identify this variation, and to define its chemical basis, however the concentration of DFP compounds at this stage offers the most likely explanation.

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APPENDIX 2. PROBLEMS ENCOUNTERED IN FEEDING MICROENCAPSULATED OILS TO A FOLIVOROUS MARSUPIAL, THE COMMON RINGTAIL POSSUM (*PSEUDOCHEIRUS PEREGRINUS*)

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ABSTRACT

In our study of interactions between marsupial folivores and *Eucalyptus*, we have examined the role of terpenes, including the use of bioassay experiments where terpenes are added to an artificial diet. In pursuit of greater ecological realism in these experiments we have sought means of packaging terpenes more realistically, using microencapsulation to simulate leaf terpene glands. We report here on a preliminary experiment with microencapsulated olive oil (intended for use as an experimental control) in which the food intake of the animals was substantially reduced, to the point that starvation appeared imminent and we aborted the experiment. We discuss why this occurred and recommend caution to others intending to use microencapsulated terpenes (or other oils) in herbivore diets.

Keywords: microencapsulation, terpene, plant-herbivore interaction, possum, *Eucalyptus*

INTRODUCTION

Terpenes are volatile plant secondary metabolites which have been the subject of a number of studies of plant-herbivore interactions (Foley *et al.* 1987; Edwards *et al.* 1993; Hume & Esson 1993; Duncan *et al.* 1994; Lawler & Foley *in press*). Because they are found in varying concentrations between and within species, have distinct tastes and odours, and are toxic to some animals in small doses (McLean & Foley 1997), they are often suspected to be deterrents to feeding by both mammalian and insect folivores. Several studies have investigated their effect on mammalian feeding using correlative analysis of the relationship between food intake and concentration of terpenes in the foliage (Hume & Esson 1993; Lawler *et al.* 1998a). However, such correlative studies do not show cause and effect and bioassay studies, in which isolated terpenes are added to artificial diets, are required to ascribe a definite role to the terpenes (Lawler *et al.* 1998a).

The presentation of terpenes in artificial diets in an ecologically realistic manner is problematic. In plants, terpenes in the foliage are typically contained in microscopic glands. In contrast, most bioassay experiments with terpenes are conducted with the liquid terpenes added freely to the diet or adsorbed onto a dry component of the diet (Reichardt *et al.* 1990; Lawler *et al.* 1998a,b). Because terpenes are highly volatile and odoriferous this results in an artificial diet which, while the total terpene concentration may be similar to that found in foliage, presents much stronger taste and olfactory cues to the animal. If these aspects of the compounds are important (Lawler et al. 1998b), then results obtained with artificial diets may not be comparable to those obtained with foliage diets. The stronger signals would likely exaggerate the importance of the terpenes in artificial diets. As a consequence, we have sought means of packaging terpenes for addition to diets in a realistic manner, one of which has been microencapsulation (Clancy *et al.* 1992).

The microencapsulation technique used was coacervation of gelatin and acacia resin in an emulsion of the target oil in water. Manipulation of the pH of the emulsion causes the gelatin and acacia to bind at the oil-water interface of each oil droplet to form the capsule. The technique has been widely used in a variety of applications, such as feeding of marine larvae (Southgate & Lou 1995), as well as a recent use similar ours, in testing the effects of terpenes on insect feeding (Clancy *et al.* 1992). The size of the capsules can be controlled by the speed at which the emulsion is blended, producing capsules of sizes similar to the terpene glands found in leaves.

METHODS

Preparation of Capsules

Several attempts were made to produce the capsules in our laboratory using the technique described in Southgate and Lou (1995). We were unsuccessful, as terpenes are much less viscous than the oils commonly used in this procedure. Further, we required the capsules to be in powder form for addition to the diet, which required hardening of the capsules with glutaraldehyde (Clancy et al. 1992) before drying. Finally, we contracted a commercial manufacturer to produce capsules containing a terpene (1,8 cineole) and a separate batch containing olive oil, intended for use as experimental controls. Capsules were approximately 12.5% oil by weight.

Diet Composition

In Lawler et al. (1998a) we established that a threshold concentration of cineole of approximately 2.6% of the dry matter (DM) of the diet was required to produce any reduction in food intake by common ringtail possums. We decided to begin experiments with a concentration (approximately 8% DM) which would normally decrease intake to approximately 40% of normal intake, in order for differences between intakes of free and encapsulated diets to be statistically discernible. A preliminary experiment was conducted to test the control capsules (with olive oil) first before commencing a full experiment comparing the effects of free and encapsulated cineole. However, we found that the diet had to be substantially modified to make it palatable to the animals. The capsules were hydrophobic and did not mix well with the diet, requiring the addition of substantially more fruit to make it cohesive and palatable to the animals. The final diet contained approximately 60% capsules (DM) in order to reach an olive oil concentration of 8% (DM) (equivalent to that desired for terpenes in the subsequent experiments). Due to the failure of this initial experiment (see results) it was considered unsafe to continue with further experiments.

RESULTS

All of the twelve adult ringtail possums offered the diet with microcapsules containing olive oil on the first night showed an unexpected reduction in food intake (Fig. 1). Only two of the possums were given the capsule-containing diet again on the second night while the remainder were returned to the normal basal diet. Again the intakes were low. The two animals given capsules for a second night had extremely low intakes and, surprisingly, those not given capsules (which had eaten them the previous night) reduced intakes even further than they had when given the capsules. At this point the experiment was discontinued and after another night of similarly low intakes we began to offer the animals several alternative foods to induce them to feed. These included the basal diet, highly palatable foliage from two species of *Eucalyptus* and honeyed water. Intakes gradually recovered and the animals were again weaned off honey water and foliage and back onto the artificial diet. It was not until 12 days after first giving the animals the microcapsules that intakes of all individuals had reached similar levels to those recorded previously.

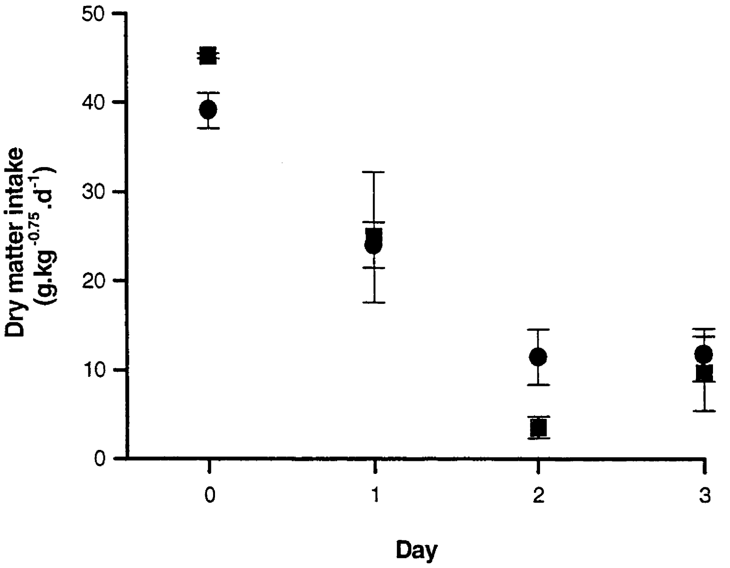

Figure 1. The effect of microcapsules containing olive oil on food intake by common ringtail possums.

Day 0 represents the day before the experiment when animals were given the basal diet only. Circles represent animals given microcapsules on day 1 only and basal diet on subsequent days. Squares represent animals given microcapsules on days 1 and 2. Values are means ± standard error.

DISCUSSION

The use of microcapsules to present terpenes to folivorous marsupials in artificial diets in an ecologically realistic fashion is theoretically sound. However, the data presented here show that in practice the idea is much more complex. This study was conducted as part of a larger project studying the effect of deterrent compounds on the interaction between marsupial folivores and *Eucalyptus*. It is now becoming clear that a specific group of compounds other than terpenes is the major determinant of deterrency (Lawler *et al.* 1998a, b), and hence the work required to fully investigate the problems encountered here is not warranted. However, terpenes have been suggested to be important in a number other interactions, such as between deer and spruce (Duncan *et al.* 1994) and between leaf chewing insects and *Eucalyptus* (Edwards *et al.* 1993). It is likely that others will attempt to adopt microencapsulation in their studies for the reasons cited above, and hence this note is written as a caution to those wish to do so. Below we discuss some possible reasons for the problems encountered.

Perhaps the most significant factors in determining if this will be a viable technique are the ratio of oil (or terpene) to capsule wall (payload), and the concentration required in the diet to produce the desired effect. In their study of the effects of individual terpenes on performance of insect herbivores of Douglas Fir (*Pseudotsuga menziesii*), Clancy et al. (1992) had similar payloads of terpenes but required dry matter concentrations of not more than 0.8% and generally less than 0.4%. In contrast, we required a minimum of 7% dry matter, some ten times higher than that used by Clancy et al. (1992). Microencapsulation may be a more viable technique for dietary studies in those species with lower thresholds for the encapsulated compound. It is now known that ringtail possums can tolerate dietary terpenes at levels higher than found in most resistant *Eucalyptus* foliage (Lawler et al. 1998b) and hence the use of microencapsulated diets will not be viable, unless the terpene payload can be substantially increased. It should be noted however, that others have used microencapsulated oils (corn oil) in mammalian diets at high concentrations (approximately 3% of dry matter) without encountering such problems (M. D. Dearing personal communication).

Given that similar amounts of microcapsules of similar composition have been fed successfully to other mammals the effect of these capsules on intake of common ringtail possums is difficult to explain. The effect of the capsules on the nitrogen content of the diet is an unlikely explanation, because the diet with capsules was 1.7% N (dry matter) (cf basal diet 1.9% N), while common ringtail possums can easily maintain themselves on diets substantially lower in nitrogen. It is also unlikely that the olive oil itself caused the effects seen, as similar doses added freely to the diet had no such effect on intake.

We consider it likely that the particular digestive physiology of common ringtail possums is a factor in the problems encountered here. Ringtail possums cope with a fibrous diet by selective retention of fine particles in the caecum where fermentative digestion takes place (Cork and Foley 1991). The capsules are of appropriate size to be retained so one explanation may be that the use of capsules has allowed material to be carried through to the caecum, rather than absorbed earlier as is usually the case. This may in turn result in disruption of fermentation, perhaps through effects on the gut flora. If capsules are not ruptured or broken down previously than perhaps the olive oil is released into the caecum, or it may be a result of fermentation of the capsule wall material itself, if capsules are previously ruptured in the stomach. Animals often become sick if the site of digestion of diet components changes. McLean et al. (1995) found that when ringtail possums were dosed by gastric lavage with citronellal they showed no ill effect, but when the same compound was dosed in peanut oil, animals stopped feeding for 4-5 days and one died. In that case the peanut oil may have protected the citronellal from acid rearrangements in the stomach, causing unchanged citronellal to be absorbed from the small intestine. Cheeke and Patton (1980) also concluded that similar symptoms shown in rabbits eating grain-rich diets were due to the soluble carbohydrates being digested in the caecum and not the small intestine.

Unfortunately it is not possible to assess likely effects of the capsule material itself as proprietary arrangements with the company prevent our revealing the detailed nature of the capsules. Capsules produced by coacervation are frequently used in animal feeds for a wide variety of taxa (e.g. Clancy et al. 1992; Southgate and Lou 1995) and the capsules used here were approved by the United States Food and Drug Agency for human consumption. Hence we assumed they would be safe for this purpose, but cannot eliminate the possibility that the unforseen problems are a result of the very much higher concentrations given to these animals, relative to any human application.

In conclusion, for anyone who intends to use this technique for a similar purpose there are several problems to address. Firstly, it is important to determine both the active dose of the terpene (or other compound) required and the achievable payload so that some assessment can be made of whether the diet's appearance and palatability will be fundamentally changed in order to reach this concentration. Secondly, it is important to consider the likely effect of the capsules themselves, in order to prevent or allow for the problems encountered here. And finally, the question of cost is significant. In this instance, the capsules cost over US$3000 and inefficiencies in the encapsulation process required the use of over five times the amount of terpene required to be encapsulated. This will vary depending on the physical and chemical properties of the compound to be encapsulated, but may in many cases prove to be the most limiting factor.

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APPENDIX 3. COPING WITH CHEMICAL COMPLEXITY IN MAMMAL-PLANT INTERACTIONS: NEAR INFRARED SPECTROSCOPY AS A PREDICTOR OF FOLIAR NUTRIENTS AND OF THE FEEDING RATES OF FOLIVOROUS MARSUPIALS.

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ABSTRACT

We investigated the utility of near infrared reflectance spectroscopy (NIRS) as a means of rapidly assaying chemical constituents of *Eucalyptus* leaves and of directly predicting the intake of foliage from individual trees by greater gliders (*Petauroides volans*) and common ringtail possums (*Pseudocheirus peregrinus*). The concentrations of total nitrogen, neutral detergent fiber (NDF), condensed tannins and total phenolics could be predicted accurately by partial least squares regression models relating the near infrared reflectance spectra of foliage samples to analyses performed using standard laboratory procedures. Coefficients of determination (r^2) for all four constituents ranged between 0.88 and 0.98, and standard errors of prediction (SEP) between 0.80 mg g^{-1} dry matter (DM) for total nitrogen and 5.14 quebracho equivalents g^{-1} DM for condensed tannins. Near-infrared spectral-based models of food intake had r^2 values of 0.90 and 0.95 with SEP values of 3.4 and 8.3 g DM $kg^{-0.75}$ d^{-1} for greater gliders and common ringtail possums respectively. We used the predictive model of food intake for greater gliders to examine the relationship between leaf palatability and documented food preferences of animals in the wild. Ranked differences in leaf palatability across four *Eucalyptus* species were consistent with documented food preferences of greater gliders in the wild. We conclude that NIRS provides a powerful tool to predict foraging behaviour of herbivores where forage choices are determined by compositional attributes of food.

Key words: near infrared reflectance spectroscopy, leaf chemistry, folivory, plant secondary metabolite, *Eucalyptus*, *Petauroides volans*, *Pseudocheirus peregrinus*.

INTRODUCTION

The diets of mammalian browsers are chemically complex, containing a range of nutrients, indigestible tissues and plant secondary metabolites (PSMs) (Cork and Foley 1991; Hanley 1997). Understanding how these components interact to determine nutritional quality of the diet has been the subject of much research. Nutritional quality of browse is usually inferred indirectly from measures of nutrients such as total nitrogen and soluble carbohydrates, refractory components such as fiber and lignin and PSMs such as tannins (Oates et al. 1980; Waterman et al. 1980; Cork and Pahl 1984). Combinations of these measures have been used to develop indirect, predictive indices of feeding (e.g. Waterman et al. 1980; Cork 1992) but cannot address the two fundamental components of nutritional quality, food intake and the digestibility or metabolizability of that food (Robbins 1983).

Many studies using indirect techniques were performed in the 1980's but interest in this approach has declined for two reasons. First, it is apparent that variation in chemical composition both within and amongst individual trees is an important determinant of mammalian browsing (Ganzhorn 1988; Hjalten et al. 1996; Lawler et al. 1998; Pass et al. 1998). This means that sampling and subsequent nutritional analysis needs to be much more intensive than has been attempted in the past. However, to do so, often requires unacceptable time and laboratory resources and consequently, analytical constraints may limit the sampling intensity (Foley et al 1998).

A second problem is that the links between compositional analyses and nutritional quality are not well defined (Foley 1992; Hagerman et al. 1992; Waterman and Kool 1994; Hjalten et al. 1996). Consider for example, the interpretation of the concentration of total nitrogen (N) in plant tissue. If this is to be linked to animal foraging, we need to know the proportion of N that occurs as amino acids rather than nitrate or cyanide, whether any amino acids are limiting, the protein requirements of the animal, some knowledge of the animal's digestive physiology and the presence of other factors that might either enhance or diminish the availability of the N to the tissues. Synergisms with poorly defined components such as tannins make defining what is high and low quality food virtually impossible from compositional analyses alone.

Foley et al. (1998) have argued that near-infrared reflectance spectroscopy (NIRS) offers ecologists a powerful tool to address the problem of sampling intensity. Correctly calibrated, NIRS allows multiple components of plant samples to be measured quickly, non-destructively and with minimal sample preparation. Near-infrared spectroscopy has been used previously in agriculture to estimate the chemical components of pastures and other fodder species with a high degree of accuracy and precision (e.g. reviewed in Givens et al. 1997) but has been applied only rarely in nutritional ecology (e.g. Brooks et al. 1984). Spectral characteristics reflect the underlying chemical bonds that make up different nutrients and PSMs. Therefore, NIR spectra can "capture" chemical information about a plant sample accurately. However, correlations must be sought between spectra and samples of known composition in order to quantify this information, because peaks in an NIR spectrum cannot necessarily be assigned to single functional groups (Foley et al. 1998).

This paper presents evidence that NIRS is suitable for measuring a range of primary constituents and PSMs found in *Eucalyptus* foliage. We then argue that, if NIRS can predict foliar components known to influence foliage intake by marsupials, it may be possible to predict intake directly by modelling the relationship between spectral characters of foliage and a standard measure of the amount of food an animal is prepared to eat in the laboratory. We believe that this approach could enable ecologists to sidestep some of the difficulties of interpreting the effects of poorly defined foliar attributes on an animal's willingness to eat a particular foliage. We explored this approach by testing whether NIRS could predict accurately the intake of *Eucalyptus* foliage by two marsupial browsers, the common ringtail possum (*Pseudocheirus peregrinus*) and the greater glider (*Petauroides volans*). We then investigated whether NIRS could be used in field-based studies of feeding in folivorous marsupials by comparing our predictions of diet selection with those observed in free-living greater gliders.

MATERIALS AND METHODS

Chemical Composition of Eucalypt Foliage

Sampling of Foliage

Ninety samples of eucalypt foliage were collected from trees in both the north-eastern tropical zone (north-eastern Queensland) and south-eastern temperate zone (south-eastern and north-eastern New South Wales) of Australia, within the distribution ranges of both greater gliders and common ringtail possums. These collections spanned the young and mature foliage of 85 species of eucalypt, which were growing on a wide range of soil types and under of a range of different climates (Cork 1992; McIlwee, unpublished data). Each sample consisted of 30-50 g (wet mass) of foliage that was cut close to the central part of the crown of a single eucalypt tree. Each sample was frozen in the field either on a bed of solid CO_2 or else in a field-portable freezer and on return to the laboratory was stored at -20°C. All samples were freeze-dried and ground to pass a 1 mm screen in a Udy Cyclone grinder.

Analytical Procedures

We measured four chemical attributes of the foliage in duplicate and expressed all our results as a percentage of dry matter after correcting for residual moisture by drying a duplicate sample in a porcelain crucible at 80°C for 24 h. Not all samples were analysed for all constituents. Foliar nitrogen was determined by a semi-micro Kjeldahl procedure with selenium as a catalyst using a Gerhardt Vapodest nitrogen analyser. Recoveries of $(NH_4)_2SO_4$ standards were always between 99.5 and 100.7 %. Neutral detergent fiber (NDF), a measure of the cell wall constituents of the leaves, was determined as described by Van Soest et al. (1991) but using the filter bag apparatus described by Komarek (1994). Polar phenolic constituents were extracted from samples of ground foliage with 50% aqueous acetone, for 30 min, at 4°C, and in the dark (Cork and Krockenberger 1991). Total phenolics were assayed on portions of these extracts as described by Cork and Krockenberger (1991) and condensed tannins were assayed by the butanol-HCl method (Porter et al 1986). Quebracho, purified as recommended by Robbins et al (1991) was used as the standard for both assays and the results are expressed in quebracho equivalents. The standard error of the laboratory procedures (SEL) was calculated following the method of Smith and Flinn (1991) to give an estimate of the precision of laboratory procedures.

Measurements of the Dry Matter Intake of Greater Gliders and Common Ringtail Possums

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

Dry matter intake (DMI) of natural foliage diets was measured for greater gliders (*Petauroides volans*) and common ringtail possums (*Pseudocheirus peregrinus*) in a series of experiments with captive animals. Six greater gliders were caught by hand in eucalypt woodland near Taravale, in north-eastern Australia and eight common ringtail possums were caught at Kilcunda, in south-eastern Australia. Animals were housed in individual metabolism cages as described by Foley and Hume (1987a) and Foley (1992) and maintained inside a room maintained at 20-25°C under a 12h:12h light dark cycle. All animals were provided with fresh water *ad libitum* and maintained on foliage from several eucalypt species. Once branches of foliage were cut from trees, they were stored for up to seven days in large polythene bags in the dark at 4°C. Ends of the branches were left standing in water.

Greater gliders were fed foliage from six individual trees of five different eucalypt species (*Eucalyptus tereticornis*, *E. acmenoides*. *E. crebra*, *E. (= Corymbia) intermedia* and *E. grandis)* in a series of Latin square designs. Each experiment was restricted to the individuals of one species of eucalypt and so consisted of six individual trees fed over six days. Greater gliders are strictly nocturnal, have few energy reserves and must feed each night in order to survive (Foley 1987). Therefore, we fed each foliage overnight from 1730 h to 0500 h, after which it was replaced with high quality young tips of *E. tereticornis* foliage. This was necessary to ensure that each animal ate sufficient food each night during the experiment in order to minimise carry-over effects that might result from different degrees of hunger. All food was removed before mid day, to prevent animals from feeding at least six hours before the next set of experimental leaves were offered. Animals were weighed at 3 d intervals and their mass did not change by more than 5% throughout the course of the experiments. We measured the amount of dry matter consumed between 1730 and 0500 h as described by Foley (1992).

Common ringtail possums were fed six to eight individual trees of three eucalypt species. These were *E. ovata*, *E. viminalis* and *E. polyanthemos*. Four separate experiments were conducted in total with eight individual samples of tree foliage offered from *E. ovata*, six individuals of . *E. polyanthemos*, six individuals of *E. ovata* again and six individuals of *E. viminalis*. All experiments were designed as Latin squares (Lawler et al. 1998) and, as with greater gliders, test foliage was removed at 0500h and a palatable form of *E. ovata* foliage was offered. Under this regime, all animals maintained constant mass. Again, we measured the amount of dry matter consumed between 1730 and 0500 h as described by Foley (1992).

During experiments, additional bunches of foliage were placed in an empty cage and used to correct for any increase or decrease in the mass of the leaves due to changes in water content. These were insignificant. Bunches of foliage were sampled in a way consistent with the feeding of the animals. These samples were stored at -20°C and later freeze-dried and ground as described and used for chemical analyses and NIR spectroscopy.

Near infrared spectroscopy

Sub-samples of dried, ground foliage were allowed to equilibrate for one week in a room held at 15% RH and 22°C. This was done to ensure that the residual moisture content of each sample was similar and so reduce background signals or noise in the O-H band of the spectra. The spectrum of each sample was recorded by a NIR Systems 6500 Scanning Spectrophotometer with a spinning cup attachment. The instrument was housed in a room maintained at 22-24^{0}C and 55-60% RH and all samples were scanned in a custom-built, small-ring cup with an optical-grade quartz cover. At the beginning of each day, we checked the accuracy of the wavelengths, the repeatability of measurements and at regular intervals throughout the day, a standard sample of sugar cane leaf of known chemical composition was scanned to serve as a control. Near infrared spectra were measured as the log 1/R for each sample, where R= the reflectance of monochromatic light between 400 and 2500 nm at 2 nm intervals. We collected duplicate spectra of each sample and accepted duplicates only if the RMS (root mean square) of the difference between them was less than 50.

We modelled the relationship between spectral characters and our assays of the chemical composition and intake of the foliage using two different multivariate modelling procedures. Modified partial least squares (MPLS) regression (Shenk and Westerhaus 1991) was our preferred modelling method. It is recognized to be one of the more robust methods of modelling multidimensional data since it uses all available spectral information and far fewer samples are needed for calibration (Shenk and Westerhaus 1991). In contrast, multiple linear regression (MLR) models include only those wavelengths that have been selected and usually require more samples to derive useable models (Shenk and Westerhaus 1991). Raw spectral data were transformed to remove the possible influence of particle size differences between samples. Particle size can lead to spectral variation that is unrelated to the chemical composition of samples. To overcome this problem, we used standard normal variate (SNV) and detrend transformations (SNVD) as outlined by Barnes et al. (1989). These procedures separate physical and chemical variance between samples and leave transformed spectra free from effects of multi-collinearity and differences in particle size.

Two independent validation tests were used to assess the accuracy of all our NIRS-based models. These were: (1) Cross-validation of the whole sample set. In this procedure, the population is divided into arbitrary groups and a prediction is made of the values for one group based on calibrations developed from the remaining groups. It is a particularly useful method where sample sizes are limited, as all available measurements are used to derive the model without the need to maintain separate validation and calibration sets. It is also recommended for use with MPLS regression to prevent overfitting the model (Shenk and Westerhaus 1991). (2) Comparisons were made between the standard error of prediction for samples excluded from the calibration set and the standard error of prediction for each calibration set.

Predicted intake and food-tree use in free-living greater gliders.

Models relating foliar spectral characters to the intake of foliage from single eucalypt trees by greater gliders were used to assess the relationship between leaf palatability and the foraging preferences of animals in the wild. One hundred young and mature leaf samples were collected among four eucalypt species *Eucalyptus. acmenoides*, *E.(=Corymbia) intermedia*, *E. tereticornis* and *E. crebra* over 25 sites, all within the distribution range of greater gliders in north-eastern Australia (McIlwee, unpublished). A detailed study of foraging behaviour of greater gliders had been made at one of these sites by Comport et al. (1997) and these data were used to characterise the feeding preferences of greater gliders in the wild. A total of 440 independent foraging observations were available from Comport et al.'s (1997) study and we used these to determine food-tree use by greater gliders by comparing the total proportion of feeding observations for each eucalypt species (*Pf*) with its frequency of occurrence in the study area (*Pt*). Preferences are expressed as an index of feeding selectivity (SI) which are directly comparable between different eucalypt species, by means of the formula $SI= (Pf-Pt)/Pt$. This index produces a value of 0 when tree species are used in the same proportion as their occurrence, -1 when tree species are not used at all, and positive values indicate that animals forage on a species in a greater proportion than its occurrence.

The spectra of all samples was collected using a NIR Systems 6500 Scanning Spectrophotometer (as outlined above). The spectral variation of samples was assessed by calculating the Mahalanobis distance of each sample from the spectral mean of the greater glider food intake calibration set. All spectra were less than three 'H' distances from the calibration mean, which are within the standard limits over which predictions can be made (Shenk and Westerhaus 1993).

RESULTS

Chemical composition of Eucalyptus foliage

The precision of the analytical technique for each attribute is measured by the standard error of duplicate laboratory assays (SEL) for each sample analysed (Table 1). The semi-micro Kjeldahl technique proved the most accurate laboratory assay and the greatest source of error in the laboratory analysis was associated with the measurement of total phenolics.

There were highly significant ($p<0.0001$) relationships between the values derived by chemical analyses and the values predicted by NIRS for all four foliar constituents (Figure 1, Table 1). Slopes of the regression lines between values measured by chemical analysis and those predicted by NIRS did not differ significantly from 1, indicating that no significant bias existed in the prediction of any constituent by NIRS. Given that NIRS-derived predictions include both calibration and laboratory errors, a comparison of SEL and standard errors of prediction (SEP) for each constituent (0.50 - 4.47 % vs. 0.80 - 4.45 % respectively) suggests that calibration errors are similar to those of the laboratory. This demonstrates that NIRS is a reliable technique for quantifying various nutritional fractions of *Eucalyptus* foliage.

Dry matter of intake by greater gliders and common ringtail possums

Table 2 summarises the different predictive models that were tested in order to find the best-fit model that describes the relationship between food intake measured in standard feeding experiments and values predicted by NIRS. The relationship between food intake and NIRS predicted values were highly significant for all modelling approaches tested. The modified partial least squares (MPLS) model with SNV scatter correction provided the most accurate models of food intake for both greater gliders and common ringtail possums. Mathematical transformations further improved the performance of models, with the second derivative of log 1/R being most successful. Including data points from both visible and NIR parts of the spectrum also enhanced the accuracy of the models. However, we omitted visible wavelengths from the final models because our objective was to characterise food intake based strictly on chemical composition and not on aspects of colour.

The results for the highest ranked models for food intake for greater gliders and ringtail possums are illustrated in Figure 2 and summarised in Table 3. These data show that NIRS-based models of food intake are at least as good as the models derived for the chemical constituents of the leaves described in Table 1. Assessment of calibration performance using an independent validation set gave SEPs of 3.4 and 8.3 g DM kg$^{-0.75}$ for dry matter intake of greater gliders and ringtail possums respectively. This demonstrates that the models have a high predictive power outside the calibration set from which they are derived. However, for ringtail possums, despite a close match between actual and predicted intakes using the validation equation (as indicated by the high r^2 and SEC), the performance of the equation was low, as the SEP was approximately 2.5 fold greater than the SEC (8.3 vs 3.5 DM kg$^{-0.75}$). It is likely that the performance of the ringtail possum validation equation was limited by the low number of samples and the loss of important spectral variability from the calibration.

Comparison of food intake predicted by spectral modelling with dietary preferences of free-living greater gliders

There were significant differences in our NIRS-based predictions of food intake of greater gliders amongst tree species and leaf ages (Fig. 3a). Our models predicted that animals would consistently eat more young foliage than old for all four eucalypt species. *Eucalyptus acmenoides* had the highest potential intake for both young and mature foliage. *Eucalyptus (=Corymbia) intermedia* and *E. tereticornis* were predicted to be eaten in significantly lower amounts than *E. acmenoides*. However, the predicted intake of these species was significantly higher than that of *E. crebra.* This pattern reflected the feeding preferences of greater gliders reported by Comport et al. (1997), as shown in Fig. 3b.

Table 1. Performance of laboratory methods and NIRS predictive equations to measure attributes of leaf chemistry in eucalypts

Laboratory accuracy assessment: 'N^1' represents the total number of samples. Standard Error of the Laboratory (SEL) is: SEL = $\surd(\Sigma(y_1 - y_2)^2 / N)$ where y_1 and y_2 are duplicates of analyses. *NIRS equation performance*: Summary of Modified Partial Least squares linear regression statistics for NIRS predicted values for chemical attributes and actual measurements made by laboratory analysis. 'N^2' represents the number of samples in the calibration set (N^1 less laboratory outliers). The slope is the deviation of the regression line from the equation $y = x$ (where y is the NIRS predicted value and x is the laboratory measured value). The performance of NIRS equations is measured by (a) Coefficient of Determination (r^2) (b) Standard Error of the Cross-Validation (SECV) and (c) Standard Error of Prediction (SEP) for 30 samples independent of calibration set.

Laboratory accuracy				**NIRS equation preformance**							
				Regression Equation statistics							
	N^1	Mean	SEL	N^2	Mean	Range	Slope	r^2	SECV	SEP	Significance
N	85	12.37	0.52	84	12.79	7.17-20.1	0.964	0.961	0.759	0.79	***
NDF	85	39.78	1.46	81	39.35	14.6-59.8	0.980	0.978	3.166	3.62	***
CT	90	22.04	3.54	86	22.16	0.04-67.3	0.903	0.949	4.830	5.14	***
HP	90	40.90	4.47	85	40.45	14.2-60.2	0.871	0.877	4.511	4.45	***

Attributes include Nitrogen Concentration (N), Netural detergent Fibre (NDF), Condensed Tannins (CT) and Total Phenolics (TP). Concentrations expressed as mg g^{-1}drymatter (N), % dry matter (NDF), % quebracho equivalents g^{-1} drymatter (CT and HP). Coefficient of determination represented by r^2, with significance levels for regression at $p < 0.001$ (***).

Table 2: The effects of regression procedure, scatter correction, spectral transformations and inclusion of visible wavelengths on the performance of NIRS intake equations.

Effects of treatments on the performance of NIRS predictions are measured by the standard error of the calibration (SEC), standard error cross validation (SECV) and the coefficient of determination (r2)

	Greater Gliders			Ringtail Possums		
Treatment	SEC	SECV	r^2	SEC	SECV	r^2
Regression method						
PCR	6.786	6.822	0.791	8.460	9.420	0.726
PLS	3.198	5.343	0.820	7.025	9.005	0.811
MPLS	2.569	4.398	0.884	3.538	7.382	0.921
Scatter correction						
No correction	2.974	5.052	0.845	4.934	8.253	0.909
Detrend	2.899	5.341	0.852	4.538	7.382	0.921
SNV	2.519	4.398	0.884	3.785	6.732	0.945
Math treatments						
First derivative	2.569	4.398	0.884	3.785	6.732	0.945
Second derivative	2.118	4.296	0.921	3.469	7.299	0.954
Wavelengths						
408-2492nm	2.382	3.685	0.900	3.772	6.583	0.946
1108-2492nm	2.289	4.398	0.884	5.405	8.321	0.888

Regression procedures include Principal Components Regression (PCR), Partial Least Squares Regression (PLS), Modified Partial Least Squares Regression (MPLS). Scatter correction is used to remove the non-linearity in spectra due to effects such as differences in particle size and the scattering of light. Detrend is a baseline correlative method which removes the linear and quadratic curvature of each spectrum. Standard Normal Variate (SNV) correction scales each spectrum to a standard deviation of 1.0. First and second derivative algorithms perform optimal N-point smoothing of data before derivatives are calculated. Derivatives are taken on Log 1/R values (where R= reflectance of composite spectrum).

Table 3: Performance of NIRS equation predicting food intake (g DM kg $^{-0.75}$) for greater gliders (GG) and common ringtail possums (RTP).

a) Summary of observed voluntary intake statistics. **b)** Summary of Modified Partial Least Squares regression statistics for NIRS predicted voluntary intake. N^C and N^V represent the number of samples used in calibration and validation sets respectively. The slope is the deviation of the regression line from the equation y = x (where y is the NIRS predicted value and x is the observed value). Equation performance is measured by (a) the Coefficient of Determination (r^2), (b) the Standard Error of the Calibration (SEC), and (c) the Standard Error of Prediction (SEP) for 'N^V' number of samples.

			a) Observed intake (g DM kg $^{-0.75}$)			b) NIR equation performance			
Equation	N^C	N^V	Mean	Range	Std. Dev.	Slope	r^2	SEC	SEP
Calibration									
GG	30	-	26.9	12.8-46.9	7.8	0.96	0.943	1.80	n.a
RTP	25	-	22.6	1.3-50.12	16.2	0.95	0.945	3.79	n.a
Validation									
GG	20	10	26.9	12.8-46.9	8.0	0.99	0.901	2.51	3.42
RTP	16	9	24.1	2.5-50.1	16.0	0.85	0.953	3.47	8.29

Table 4: Summary of published NIRS empirical models predicting voluntary food intake (for mammalian herbivores.

Error between observed intake and NIRS predicted intake are measured by the Coefficient of Determination (r^2) and the Standard Error of Prediction (SEP) .

		Intake (g DM kg $^{-0.75}$)		**NIR Prediction**		
Forage	*N*	Species	Range	R^2	SEP	Reference:
Mixed samples	76	Sheep	40.-114	0.62	7.8	Norris *et al.* (1976)
Grazed pasture	21	Cattle	53-112.	0.72	9.6	Ward *et al.* (1982)
Faecal samples	36	Elk	40-113	0.80	8.9	Brooks (1984)
Grazed pasture	53	Cattle	75-129	0.66	7.8	Redshaw *et al.* (1986)
Grazed pasture	44	Sheep	66-116	0.55	8.4	Redshaw *et al.* (1986)
Grazed pasture	136	Cattle	n.a	0.86	5.5	Steen *et al.* (1995)
Foliage	30	GG	13-47	0.90	3.3	This study
Foliage	25	RTP	3-50	0.95	7.8	This study

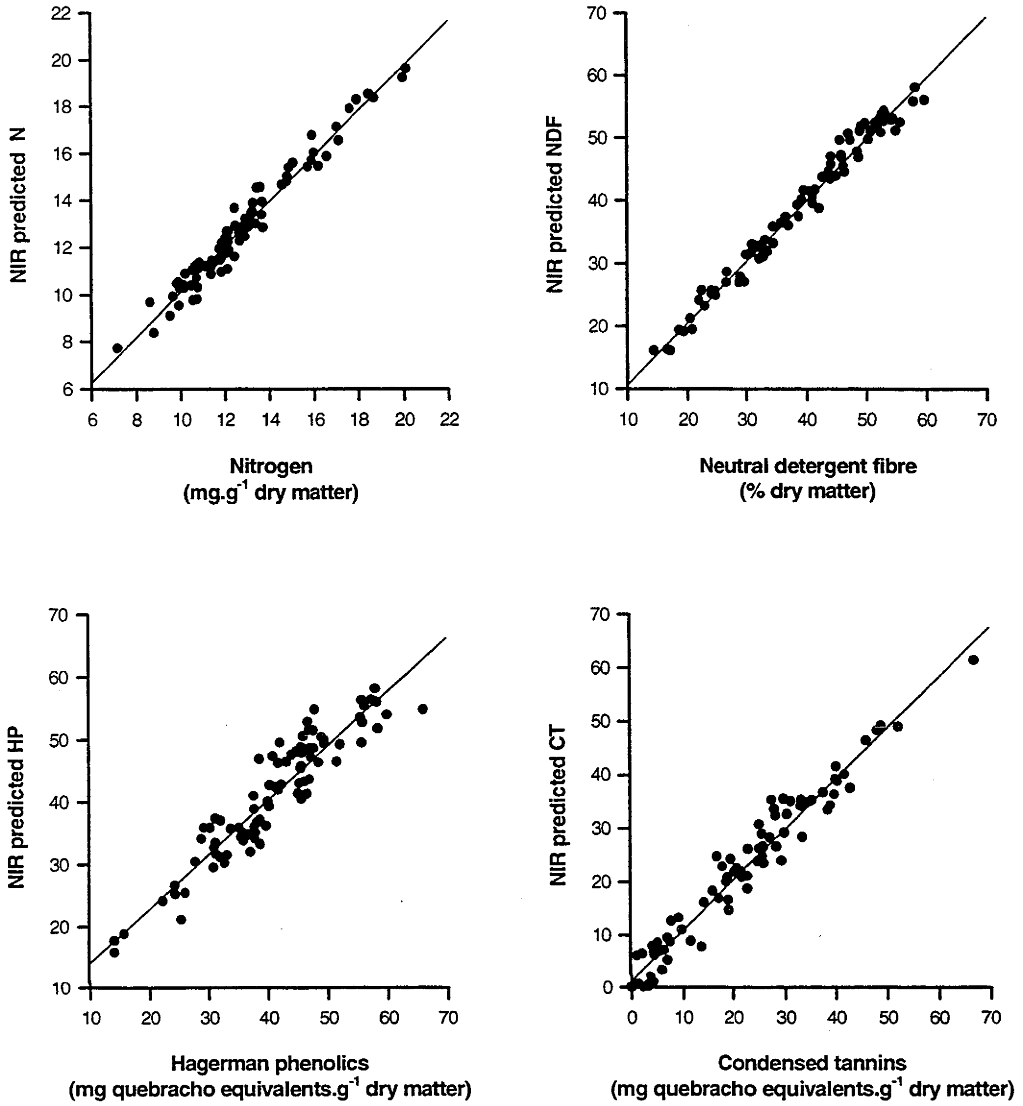

Figure 1: Relationship between values predicted by standard laboratory assays, and those predicted by a model based on partial least squares regression, relating near infrared reflectance spectra of *Eucalyptus* foliage to these standard assays for four components of *Eucalyptus* foliage.

Significant relationships were found between actual and predicted constituent values for nitrogen (r^2= 0.96, s.e.= 0.490, n= 84; $F_{1,83}$= 2161, P<0.0001), neutral detergent fiber (r^2= 0.98, s.e.= 1.64, n= 81; $F_{1,80}$= 3968, P<0.0001), condensed tannins (r^2= 0.95, s.e.= 3.08, n= 86; $F_{1,85}$ =1764, P<0.0001) and total phenolics (r^2= 0.87, s.e.= 3.46, n = 85; $F_{1,84}$= 618, P<0.0001). Concentrations are expressed as mg g^{-1} dry matter (N), % dry matter (NDF), mg quebracho equivalents g^{-1} dry matter (condensed tannins and total phenolics).

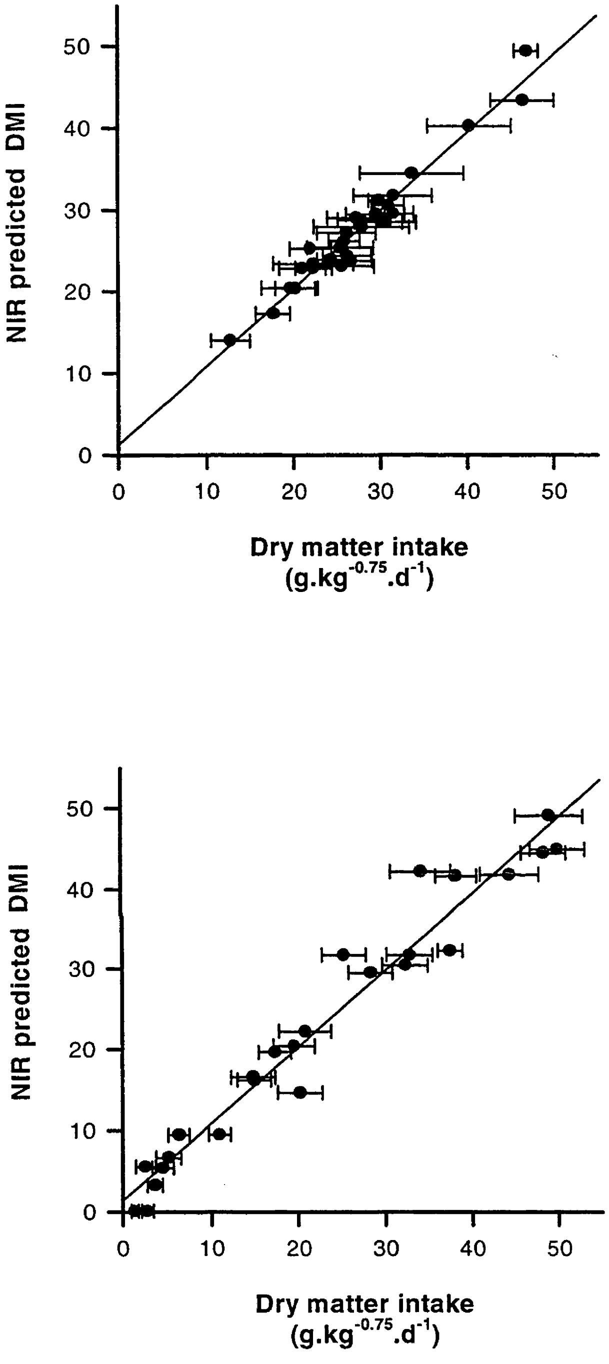

Figure 2: Relationship between mean voluntary dry matter intake (± standard errors) of *Eucalyptus* foliage for a) greater gliders and b) common ringtail possums and the intake predicted by a model based on partial least squares regression relating the near infrared spectra of *Eucalyptus* foliage to these measures of intake of *Eucalyptus* foliage.
Significant relationships between actual and predicted intake were found for both greater gliders (r^2= 0.94, s.e.= 1.80, n= 30; $F_{1,29}$= 272, P<0.0001) and common ringtail possums (r^2= 0.95, s.e.= 3.79, n= 25; $F_{1,24}$= 505, P<0.0001).

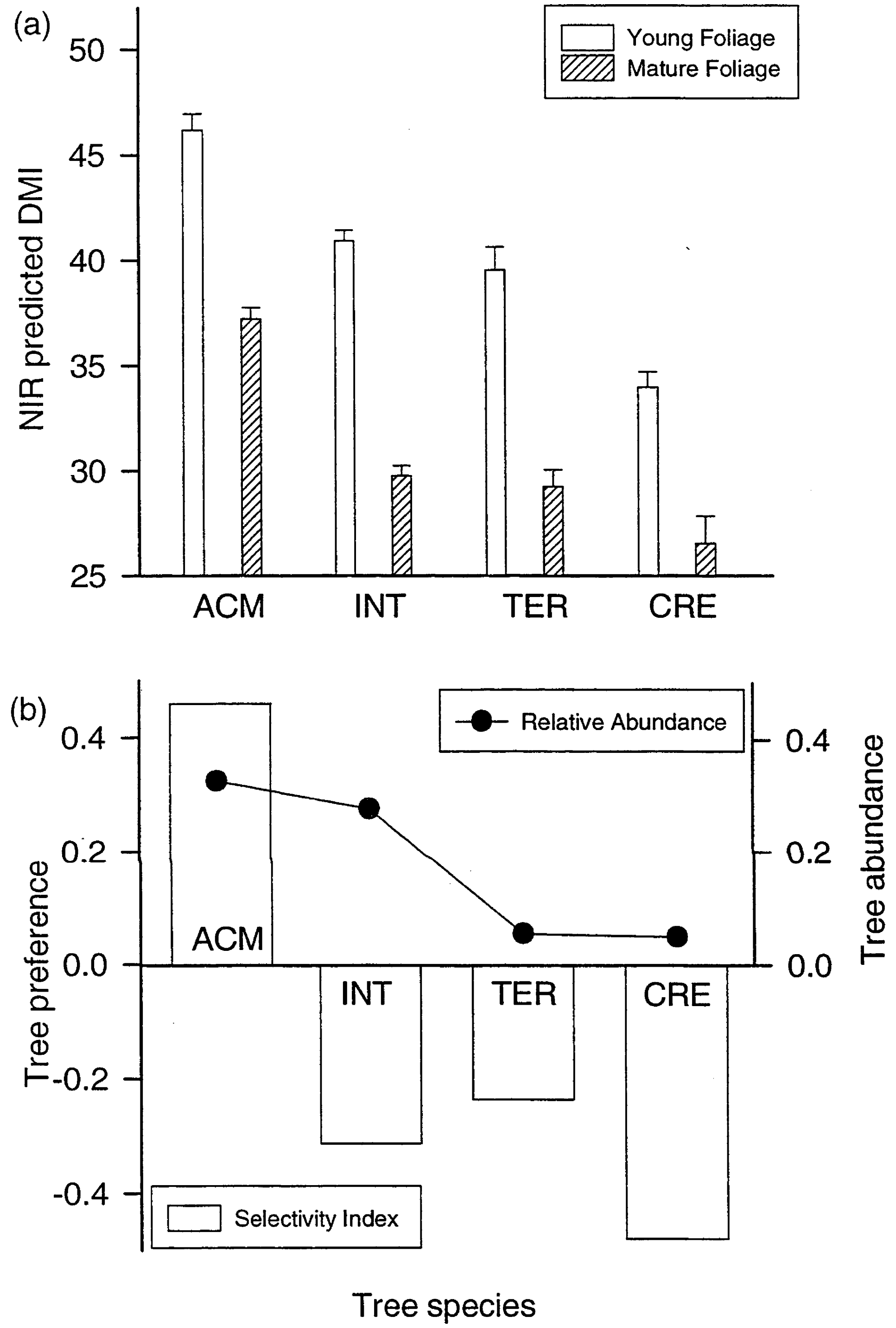

Figure 3: (a) Mean predicted dry matter intakes (g DM $kg^{-0.75}$ d^{-1}) of greater gliders with 95% confidence limits, for 100 young and mature foliage samples of each of four *Eucalyptus* species based on a model relating spectral characteristic of *Eucalyptus* foliage to its measured intake by captive greater gliders.

Predicted intakes differed significantly between tree species ($F_{3,792}$=277; P<0.0001) and leaf age ($F_{1,792}$= 1203; P<0.0001). **(b)** Observed feeding preferences greater gliders in the wild, expressed as a selectivity index (see text), and proportions of each tree species in the study area. Data of Comport et al. (1997). Abbreviations: ACM= *Eucalyptus acmenoides*, INT = E. (=*Corymbia) intermedia*, TER= *E. tereticornis* and CRE= *E. crebra*.

DISCUSSION

This study has shown that the chemical composition of eucalypt foliage can be predicted rapidly, and accurately using NIR spectroscopy. For example, up to 150 samples can be processed in a single day, and all four constituents measured here can be predicted simultaneously. This results in savings of at least 80% on normal laboratory costs and this effectively removes a major problem with intensive sampling of natural plant populations. Furthermore, NIRS provides a powerful method for dealing with chemical complexity of foliage diets as NIR spectra capture compositional data sufficiently well to allow robust models of food intake to be produced. Understanding the diet selection and foraging patterns of herbivores is complex due to interactions between physiological constraints and a wide range of dietary constituents (Hanley 1997).

Several studies have shown that NIRS is able to predict the composition of various plant materials with a high level of accuracy and precision (e.g. reviews in Givens et al. 1997; Foley et al. 1998). Consequently, we were not surprised that NIRS could predict these attributes in *Eucalyptus* foliage. Predictions of total polar phenolics were less accurate than other chemical components. However, given the enormous structural variation in compounds that are detected by the phenolic assay, as a group total phenolics are difficult to define chemically. Therefore, we were not surprised that the accuracy of NIRS was reduced for this constituent compared with analyses of total nitrogen. A comparison between the SEL of phenolics and the standard error of cross validation supports this conclusion.

Previous and on-going studies of the nutritional ecology of folivorous marsupials have argued strongly for the roles of leaf nitrogen, fiber, phenolic constituents and tannins in determining levels of food intake and diet selection (Cork and Pahl 1984; Foley and Hume 1987b; Cork and Sanson 1990; Kavanagh and Lambert 1990; Cork 1992; Pass et al. 1998; Lawler et al. 1998). Food intake is closely linked to chemical composition of the foliage in common ringtail possums irrespective of differences in digestibility of the foliage (Lawler et al. 1998; Lawler and Foley unpublished). This means that if NIR spectra can capture information about chemical makeup of a foliage, then it is likely that we can predict intake directly without necessarily measuring particular chemical components of the leaf. Our results show NIRS spectra alone are able to explain more than 90% of the variation in mean dry matter intake for both greater gliders and common ringtail possums.

The models that we derived to predict our standard measure of food intake for both greater gliders and ringtail possums show a similar or better accuracy than similar NIRS equations used to predict the intake of large grazing mammals, as illustrated in Table 4. This may be because compositional differences have a greater impact on the food intake of browsers than grazers. In grazers, animal-related factors such as mastication and overall diet digestibility can have a major effect on the level of digestibility of the diet and hence the subsequent intake (Cork and Foley 1991). Also, the small size and high mass-specific energy requirements of arboreal folivores (Cork and Foley 1991) should make them more sensitive to variations in diet quality than large herbivores. In this study, a substantially higher variation in food intake was observed in comparison to other feeding studies for these two marsupials (Foley 1987; 1992; McArthur and Sanson 1991) but this was because the earlier studies focussed more on differences in digestibility, and not differences in intake, as a cause of variation in nutritional quality of different foliages.

The ability of NIRS to capture the compositional features of foliage in a more holistic way than selected chemical analyses is, to our view, a major advantage of the method. This is because food intake for most herbivores is determined by a range of factors, both nutritional and anti-nutritional (many unknown), which are mediated by the specific digestive and toxicological constraints imposed by an individual animals physiology (Bozinovic and Del Rio 1996). Consequently, studies that seek correlations between selected compositional measures and animal feeding will rarely succeed. However, it is important to note that the intake values used to calibrate our models were derived with captive animals in a laboratory setting. Many non-forage factors influence food intake in the wild. These may include the effect of mixed-species diets (and hence the ability to choose between alternatives) and reproductive state. Based on this inherent limitation, our models might best be described as "potential intake" and an assessment of their utility in the field is required, as described above using the data of Comport et al. (1997).

Potential intake and greater glider feeding preferences.

It is difficult to transfer predictions of leaf palatability to animal foraging preferences in the wild, as animal preferences are no doubt influenced by a variety of circumstances. For example, most arboreal marsupials are known to track the seasonal availability of young foliage that is highly variable across space and time (Kavanagh and Lambert 1990). Nevertheless, studies have shown that folivorous marsupials also exhibit distinct feeding preferences for particular tree species (e.g. Hindell and Lee 1987) and individual trees within species and age classes of leaves. For example, Kavanagh and Lambert (1990) found that greater gliders show a strong preference for *E. viminalis* in New South Wales, which they associated with high concentrations of nitrogen and the low concentrations of fiber in the young foliage. Young growth generally has higher concentrations of available energy, moisture, N, P, K and a lower fiber content than mature foliage (Ullrey et al. 1981; Cork and Pahl 1984; Comport et al. 1997). However, these fractions are unlikely to determine potential intake alone (Cork and Foley 1991).

Given that there is a complex set of ecological circumstances that can influence the foraging choices of herbivores, one could probably not expect a precise match between foraging behaviour and estimates of nutritional quality. This may be particularly relevant to greater gliders if they are dependent on young leaf growth to meet their nutritional requirements, as variations in leaf production may have a large impact on foraging choices. Nonetheless, both the marked selection of greater gliders for *E. acmenoides* foliage over other tree species, and the strong avoidance of *E. crebra* were predicted by our model, suggesting that nutritional quality influences foraging patterns strongly in this species.

Our model of food intake in greater gliders predicted consistent differences between the intake of young and mature foliage, as would be expected from the field studies outlined above. The spectral variation between samples in this data set also suggests that intraspecific variation in leaf palatability is low compared to interspecific variation for these eucalypt species. However, the leaves used in the calibration come from sites in the same region, but not the same trees, as in the study of Comport et al. (1997). Their data also does not consider foraging preferences within eucalypt species. Field studies of koalas (e.g. Hindell and Lee 1987) and field and laboratory data of ringtail possum food preferences (Pahl 1987; Lawler et al. 1998; this study) suggest that with appropriate calibration and the increased sampling power made possible by NIR spectroscopy, a far greater understanding of the foraging decisions made by individual animals will be developed by examining spectral variation of all individual trees within a study site.

Empirical vs mechanistic models in nutritional ecology

This study has been a component of larger studies that have examined the chemical basis of food selection by folivorous marsupials (Foley 1992; Pass et al. 1998; Lawler et al. 1998). This knowledge has given us a sound appreciation of the role of different components in *Eucalyptus* foliage that constrain animal feeding. However, in cases where the underlying mechanisms behind feeding have not been clearly identified, we ask how valid is it to use simple empirical models to predict complex physiological and behavioural outcomes? It could be argued that food intake is the complex outcome of many factors that will always remain poorly understood but should a lack of complete understanding prevent us from attempting to quantify such attributes? We suggest that NIRS may confer valuable benefits in two areas.

Firstly, examination of NIRS calibrations may provide insights into the aspects of plant chemistry that determine the characteristic of interest, even though peaks in NIR spectra cannot be unequivocally be attributed to a single functional group. For example, Clarke and Lamb (1991) surveyed the use of different NIR wavelengths as predictors of dry matter digestibility in ruminants. They found that regardless of forage type, wavelengths associated with C-H groups typical of plant fiber were important. Similarly, in a study of resistance of sugar cane to stem borer attack, Rutherford and Van Staden (1996) used important wavelengths in calibration equations to implicate alcohols and carbonyl components in resistance. Therefore, an initial empirical approach became an analytical tool that substantially increased progress towards identifying resistance factors through many bioassays.

Applying this approach to our data, we used stepwise regression to identify the most important wavelengths used to predict both food intake and leaf constituents. Using just three wavelengths, stepwise regression was able to explain 76% of the variation in food intake in greater gliders and 82% in ringtail possums. However, most interesting was that wavelengths 1132 nm and 1572 nm identified in models of food intake by greater gliders were also highlighted by stepwise regression as being important for determining condensed tannin and total phenolic concentrations respectively (McIlwee and Foley, unpublished). Although these compounds may be important determinants of foliage quality it is worth noting that these connections do not demonstrate any causative relationships. However, they do provide some indication of where further research efforts could best be directed. No correlation was found between important wavelengths used in models describing food intake in common ringtail possums and models of any of the four foliar constituents measured. However this is not surprising since food intake in common ringtail possums is largely controlled by a single group of non-polar phenolics that were not measured in this study (Pass et al. 1998; Lawler et al. 1998).

A second reason for adopting an empirical approach to predicting animal performance is the great rapidity with which a useable predictive model can be developed relative to a mechanistic understanding. In an era when many habitats are being lost or altered by anthropogenic activities in unmeasurable ways, such as the perceived effects of elevated CO_2 (e.g. Lawler et al. 1997), it is essential to be able to understand, or to predict, which effects are likely to be important for animals. If a full understanding of the underlying chemistry is demanded before decisions can be made, then irreparable damage may occur before the information is forthcoming (if it can ever be known). The stark difference in the rate of development of predictive equations using NIRS, as opposed to attempts using mechanistic approaches is clearly evident. For example, the effect of secondary chemistry of *Eucalyptus* on the foraging behaviour of ringtail possums has been the subject of much study over the past two decades (e.g. Southwell 1978; Foley and Hume 1987; Hume and Esson 1993). However, the complexity of the secondary chemistry and the unknown effects of this on different marsupial folivores meant that little progress was made. Recently, however, detailed chemical studies finally identified compounds that explained 80% of the variation in feeding between individual trees by common ringtail possums, and koalas (Pass et al. 1998; Lawler et al. 1998; Lawler, Foley, Eschler unpublished; Moore and Foley unpublished). This required the combined efforts of five chemists and zoologists and four years of full-time work using bioassay-guided fractionation. In contrast, we were able to develop the initial predictive NIR models in a single afternoon, which estimate the feeding rates of folivorous marsupials with greater accuracy than our detailed chemical investigations have so far produced. Furthermore these models incorporate all leaf attributes thought to affect rates of food intake.

If caution is used, models can potentially be developed for management purposes without any knowledge of the underlying chemistry (although we emphasise that it will also begin to identify aspects of the chemistry as outlined above). If samples of all food species within a defined habitat are represented in the spectral variation of a calibration set then application of that calibration over the entire area will be valid. Extension of that calibration to other areas or species will require expansion of the calibration set, depending on the purpose for which the calibration is to be used.

CONCLUSION

In conclusion, NIR spectroscopy can be applied to the measurements of defined leaf chemical characteristics and functional attributes for herbivores. The great savings of time and cost conferred through reduced sample preparation and analysis offer the chance to release researchers and managers from the constraints of conventional laboratory analyses and poor mechanistic understanding of herbivores and their foods. Many studies postulate differences in the nutritional quality of the diets of animals as a basis for differences in population densities, social organisation or evolutionary success. However, testing these hypotheses is generally limited by the difficulties of measuring "nutritional quality" for free-living animals. We believe that the adoption of NIRS in field studies will provide a better understanding of the foraging decisions of herbivores, and the chemical attributes on which they are based. However, in closing we caution that in adopting this approach for large scale assessments of habitat quality, ecologists and land management advisers must be aware of the limitations of their models.

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APPENDIX 4. CHEMICAL ECOLOGY OF HERBIVORY IN *EUCALYPTUS*: INTERACTIONS BETWEEN INSECT AND MAMMALIAN HERBIVORES AND PLANT ESSENTIAL OILS

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INTRODUCTION

Eucalyptus dominates more than 90% of Australian forests and woodlands and supports a wide range of endemic vertebrates and invertebrates. Nonetheless, few insects, and even fewer mammals, eat the foliage to any appreciable extent (Landsberg and Cork 1997). *Eucalyptus* foliage contains low amounts of dietary nitrogen, which is essential for the maintenance and reproduction of all animals (Cork and Foley 1991), but also appreciable amounts of essential oils and phenolic compounds. Both these groups of compounds are believed to have a significant influence on the acceptability of foliage as food and the nutritional quality of that foliage (Hume 1982).

There has long been speculation about the degree and nature of the influence of *Eucalyptus* oils on the food choice of herbivores. This interest has been driven by a number of considerations. Firstly, the restricted diet of koalas suggests that a knowledge of its nutrition and food choices will contribute substantially to its conservation and the conservation of other sympatric species (Cork and Sanson 1990).

Secondly, efforts to establish plantations of *Eucalyptus* for wood fibre and sawn timber are plagued by the damage inflicted on plants by pest insects and mammals (Montague 1994, Patterson *et al.* 1996). Although this is countered by extensive poisoning campaigns, current research is seeking to incorporate naturally resistant genotypes into plantation management systems (Farrow 1993). Determining the role of essential oils and related compounds in conferring resistance to herbivores has substantial economic consequences.

Finally, the dominance of *Eucalyptus* in the Australian environment, a variable level of herbivory and the substantial quantities of plant secondary metabolites contained in the foliage, has spurred a number of studies seeking a more fundamental understanding of the evolutionary interactions between plants and herbivores (Morrow and Fox 1980, Morrow *et al.* 1976, Cork and Foley 1991). For example, Morrow and Fox (1980) used *Eucalyptus* and its volatile oils to test whether herbivory was controlled by the metabolic costs of detoxification. These types of studies have made important contributions to broader ecological theory.

In this chapter we review studies which have examined the role of *Eucalyptus* oils in determining the feeding of Australian insects and mammals or which have measured the consequences of ingested oils. We conclude by evaluating the role of essential oils in conferring natural herbivore resistance on *Eucalyptus* and the prospects of selecting high yielding genotypes for use in breeding programmes.

COMPONENTS OF *EUCALYPTUS* OILS RELEVANT TO HERBIVORES

Previous chapters have reviewed the nature of *Eucalyptus* oils and discussed the diversity of mono- and sesquiterpenes present in different species. Several different techniques have been used to extract *Eucalyptus* oils from plants eaten by herbivores, but most studies have used steam distillation. However, steam distillation can volatilise compounds other than essential oils and may also lead to some rearrangements or hydrolysis of naturally occurring compounds. Since some of these non-terpene compounds are involved in significant ecological interactions we review their nature and effects below.

Benzaldehyde

Benzaldehyde (Figure 1a) almost certainly arises from the hydrolysis of cyanogenic glycosides in leaf tissue and in some species (e.g. *E. yarraensis*, Boland *et al.* 1991), can dominate the steam-volatile extractives. The only cyanogenic glycoside that has been isolated from *Eucalyptus* is prunasin (Figure 1b) (Finnemore *et al.* 1935, Pass *et al.* 1998, E.E. Conn pers. comm.). Concentrations in the young expanding tips of *E. cladocalyx* can exceed 10 mg/g dry mass (Gleadow *et al.* 1998). Sheep have been reported to be poisoned after eating *E. cladocalyx* foliage (Everist 1981) and koalas after eating *E. viminalis* foliage, but in neither case has the link with cyanogens been proven (Southwell 1978).

Of potentially more importance ecologically is the observation that many eucalypts are polymorphic for prunasin. These include *E. viminalis*, *E. orgadophila*, *E. ovata* and *E. polyanthemos*. This provides a powerful tool for investigating fundamental questions of the cost of maintaining plant secondary metabolites and may provide explanations of variable herbivore behaviour (R. Gleadow and I. Woodrow pers. comm.).

Acylphloroglucinols

Eucalyptus and several other genera of the Myrtaceae contain fully substituted acylphloroglucinols, some of which are steam volatile and frequently isolated during phytochemical investigations aimed principally at the volatile essential oils (Boland *et al.* 1991, Ghisalberti 1996). The most widespread compound is torquatone (Figure 1c) (Bowyer and Jeffries 1959) which can comprise up to 40% of the steam-volatile constituents of *E. torquata* (Bignell *et al.* 1994). A range of other compounds varying in the level of oxidation of the nuclear carbons have been described including jensenone (Figure 1d), which dominates the steam-volatile extract of *Eucalyptus jensenii* leaf (Boland *et al.* 1991, 1992). These compounds, together with the acylphloroglucinol-terpene adducts described below, can have substantial biological activity in a number of different systems.

Acylphloroglucinol-Terpene Adducts

In the past 10-15 years a number of compounds have been identified which result from terpenes bonded to fully substituted acylphloroglucinol derivatives (Ghisalberti 1996, Pass *et al.* 1998). These are known as euglobals (e.g. euglobal III, Figure 1e) (Kozuka *et al.* 1982a, b) and macrocarpals (e.g. macrocarpal G, Figure 1f) (Yamakoshi *et al.* 1992). None are steam volatile but, given that common essential oils form part of the structure, and given their apparent importance in some ecological interactions (Pass *et al.* 1998, Lawler *et al.* 1998a), some weight will be given to them in this chapter.

Although there are undoubtedly more compounds to be discovered, those already known are formed with only a restricted number of terpenes. The euglobals contain a monoterpene moiety derived most commonly from β-pinene, although compounds with sabinene and β-phellandrene have also been identified. A second group of euglobals are formed with sesquiterpenes, most commonly bicyclogermacrene. Macrocarpals differ from euglobals in that they lack the ether linkage between the aromatic and terpenoid parts of the structure. All terpene adducts of known macrocarpals are derived from sesquiterpenes.

Dimeric Acylphloroglucinols

A limited number of compounds have been described which can generically be termed dimers of the simple acylphloroglucinols. These include sideroxylonal A (Figure 1g) and B, robustanol A and grandinal. Of these, only the effect of sideroxylonals on insect and mammal feeding has been investigated, with strong indications of high deterrency (see below).

Biosynthesis of Acylphloroglucinol Compounds

Little is known of the biosynthesis of the simple acylphloroglucinol structure itself. The euglobals and dimeric compounds are presumed to be formed by Diels-Alder condensations but there have been no formal synthetic studies to date (Ghisalberti 1996). The generation of a macrocarpal is thought to involve a carbocationic species which acts as a cationic initiator in the cyclisation of the sesquiterpene precursors (Ghisalberti 1996). Acylphloroglucinols occur in all subgenera of *Eucalyptus* (Eschler, Pass, Willis and Foley unpubl.).

ECOLOGICAL FACTORS AFFECTING THE CONCENTRATION OF OILS IN *EUCALYPTUS* FOLIAGE

The concentration of volatile essential oils in foliage may be influenced by a number of extrinsic factors including soil type, light and atmospheric CO_2. Knowing how essential oils and other plant secondary metabolites respond to these factors is important because (a) we need to be able to predict how climate change will affect animal-plant interactions, and (b) several key theories of animal-plant interactions are couched in terms of the relationship between the level of herbivory and the resources available to the plant.

Most influential of these theories are those of resource availability or carbon-nutrient balance (Bryant *et al.* 1983, Coley *et al.* 1985). These propose that the concentration of carbon-based plant secondary metabolites is governed by the availability of carbon to other nutrients. That is, under conditions where carbon is plentiful relative to other nutrients (e.g. high light, low soil nutrients) growth will be limited by these other nutrients, leaving the excess carbon to be diverted to activities other than growth, such as carbon-based defences (Coley *et al.* 1985).

The few available data suggest that *Eucalyptus* phenolics behave in a manner consistent with these theories but that the same is not true for essential oils. Lawler *et al.* (1997) grew seedlings of *E. tereticornis* under conditions of varying carbon and nutrient supply and measured the concentration of steam-volatile essential oils in the foliage (Figure 2). The concentration of leaf essential oils in *E. tereticornis*, and also in *E. citriodora* (Lawler, Foley and Woodrow unpubl.), actually increased with high soil nutrients. Increased light also increased foliar essential oil concentrations but only under high soil nutrient conditions (Lawler *et al.* 1997). Elevated atmospheric CO_2 had no effects on foliar essential oil concentration in *E. tereticornis* or *E. citriodora* but Adamson and Woodrow (pers. comm.) found elevated foliar essential oils in *E. nitens* seedlings grown under elevated CO_2.

The oil content of seedlings differs both quantitatively and qualitatively from that of the foliage of the mature plant (Doran this volume, Boland *et al.* 1982). For example, seedling leaves of *E. delegatensis* contained only about 0.1% (dry weight basis) of steam-volatile essential oils, whereas mature foliage contains 10-20 fold more oil (Boland *et al.* 1982). These differences are significant because seedlings may often be more susceptible to herbivory than adult plants. Attempts to select for, or breed, plants resistant to herbivores on the basis of their foliar terpene concentrations may founder unless the relationship between the concentration of essential oils and herbivory in seedling plants is considered. The differences between seedling and adult plants are most probably a consequence of the low energy reserves contained in seedlings, together with the relatively high metabolic cost of synthesis and maintenance of essential oils (Gershenzon 1994), but this is an area that needs further research.

DIET CHOICE IN VERTEBRATE HERBIVORES

The vertebrate herbivores that eat *Eucalyptus* foliage extensively are all marsupials. These include arboreal folivores such as koalas (*Phascolarctos cinereus*), greater gliders (*Petauroides volans*), common ringtail possums (*Pseudocheirus peregrinus*) and common brushtail possums (*Trichosurus vulpecula*), and some macropodines (kangaroo family) such as the swamp wallaby (*Wallabia bicolor*), the Tasmanian pademelon (*Thylogale billardieri*) and the red-necked wallaby (*Macropus rufogriseus*). Although kangaroos can inflict severe damage on *Eucalyptus* plantations (Montague 1994), there are no data examining their interactions with *Eucalyptus*. In contrast, the reliance of koalas and the other arboreal folivores on *Eucalyptus* foliage, and in particular that of a restricted subset of species, has led to many studies of the relationship between the volatile oil content of the foliage and food choice. The rationale for these studies has ranged from the distinctive odours of the oils, to their alleged thermogenic properties and their potential germicidal effects on the microflora of the gut (Pratt 1937, Fleay 1937).

However, to date there has been no convincing or conclusive evidence that simple essential oils play any role in diet selection by koalas or any other marsupial herbivore. For example, Southwell (1978) could find no association between the level of defoliation of individual *Eucalyptus* trees and their concentration of the total steam-volatile oils or the proportion of cineole in these oils.

Betts (1978) claimed that the ratio of cineole to sesquiterpenes in *E. rudis* was an important determinant of food choice in koalas eating that species but his data explained only 18% of the observed variation. Furthermore, there was no explanation as to why this fraction or ratio should be more or less important than any other component. Pratt (1937) argued that cineole had a thermogenic effect, whereas α-phellandrene exerted a cooling effect on the animals which ate the compound, and that these effects correlated with food choice in koalas in different locations. Neither the supposed thermogenic effects nor the alleged pattern of food choice has stood up to closer examination (Southwell 1978).

Hume and Esson (1993) argued that koalas required a minimum threshold of oil in the diet but there was no clear correlation between the total steam-volatile concentrations and the relative feeding effort by koalas. In this case, feeding effort was allocated to one of four categories but animals always had some favoured foliage available. Zoidis and Markowitz (1992) found no correlation between the concentration of cineole in foliage and food intake by koalas in the San

Diego Zoo. In contrast to the study of Hume and Esson (1993), Lawler *et al.* (1998a) compared koala feeding on different individual trees of *E. ovata* and *E. viminalis* and found that intakes of foliage were higher when the essential oil content was significantly lower than Hume and Esson's suggested threshold. For individual trees within each *Eucalyptus* species, intakes decreased in a manner strongly consistent with the total essential oil concentration (Figure 3). A similar pattern was also observed for common ringtail possums feeding on the same trees (Figure 4a).

Although these correlative approaches appear to be a reasonable way to test the basis of food choice in mammals, they are unlikely to succeed where the essential oil profile of the foliage is complex, as in *Eucalyptus*. This is because we have to decide *a priori* what dietary components are important, and simply because a foliage smells strongly to man does not mean that it smells the same way to an animal. In addition, what is toxic to man may not be toxic to a particular herbivore. For example, black colobus monkeys (*Colobus satanus*) can eat large quantities of toxic alkaloid-rich leaves which would be fatal to non-adapted species (McKey *et al.* 1981). Similarly, while we have shown above that ingestion of *Eucalyptus* leaves correlates strongly with the essential oil content of those leaves, for their body sizes, the marsupial folivores of *Eucalyptus* can eat over 10 times the amount of essential oils known to cause fatality in humans (McLean and Foley 1997), and can do this for sustained periods (Lawler unpubl.). Correlative evidence does not imply any causal link; a correlation may arise simply because the concentration of oils is correlated with the true limiting factor.

Most studies of the role of essential oils in regulating feeding in other vertebrate herbivores have been similarly inconclusive (e.g. Duncan *et al.* 1994) or do little to demonstrate causal relationships. Clearly a better option is a bioassay involving the use of pure compounds added to an artificial diet in isolation. In this way, other unknown differences between diets can be excluded and cause and effect can be identified conclusively.

Bioassays with Marsupials Fed Isolated Essential Oils

The few studies that have fed isolated *Eucalyptus* oils to marsupial herbivores do not support the notion that essential oils, in themselves, are important influences on diet selection. Pass *et al.* (1998) isolated oils from both palatable and unpalatable forms of *E. ovata*. The main difference was quantitative: unpalatable trees had higher total oil contents though the compositions of the oils were similar, with cineole comprising about 80% of the total extract. Although animals preferred not to feed on either the whole extract or cineole-treated food when given a choice, there was no reduction in food intake when only cineole-treated food was offered.These results are consistent with studies of Krockenberger (1988) in which he fed cineole to both common ringtail and common brushtail possums. No effect on food intake, total diet digestibility or nitrogen metabolism was detected. M. Harvey and I.D. Hume (unpubl.) fed common brushtail possums volatile monoterpenes and sesquiterpenes extracted from *E. haemostoma* leaves at 3.5-4.0% (dry matter) levels in the diet but they, too, could detect no effect of these fractions on food intake except when the animals were given a choice between oil-rich diets and the basal diet alone.

The study by Lawler *et al.* (1998a) mentioned earlier has presented perhaps the strongest evidence of a negative correlation between food intake by marsupial folivores and the essential oil content of the leaves. This was achieved by looking at the differences in intake of individual trees within each *Eucalyptus* species. This reduced the interference of qualitative differences (i.e. differences in the constituent compounds of the oil) with the quantitative differences (i.e. actual amounts) in feeding between subject trees which may occur when several species are used. However, the same study also refuted the role of essential oils as true toxins. Using no-choice experiments they showed that the amounts of essential oil required to be added to an artificial diet to reproduce the decrease in food intakes seen in leaves were much higher than those found in leaves (Figure 4). Hence the bioassay showed that the essential oils were not the cause of the effect, as may have been implied by the correlation. This is further emphasised by data which show that both common ringtail possums and common brushtail possums can rapidly be conditioned to eat diets containing greater than 12% cineole (dry weight basis) (Lawler *et al.* 1998b - see below, Boyle unpubl.).

Although the difference between two-choice and no-choice experiments suggests that there is some metabolic cost involved in ingesting essential oils, this cost is either so small, or dissociated sufficiently from the ingestion process, that it has no effect on food intake. Integrating the above observations, a possible role for essential oils in diet choice of herbivores was suggested by Lawler *et al.* (1998a). There is a strong smell and taste associated with essential oils and food intakes are strongly correlated with leaf essential oil levels. However, since they apparently do not present an insurmountable challenge to the animals after ingestion, perhaps they are used by the animals as a cue to the toxin levels of the leaves.

Lawler *et al.* (1998b) tested whether the avoidance by animals of high essential oil diets could be the result of a conditioned food aversion, i.e. does the aversion result from the animals learning to associate the sensory effects of the essential oils with the negative effects of another compound also found in *Eucalyptus* leaves? An initial test was made on 12 ringtail possums of reduction in food intake when cineole was added to the diet (again in a no-choice situation): all possums were found to reduce food intake significantly (Figure 5a). Half the animals (treatments) were then 'taught' to eat cineole by increasing the amount of cineole in the diet daily until it reached over 10% of the dry weight of the diet after 12 days. The remaining animals (controls) were fed only the basal diet over the same period. At the end of this time they were again tested: those exposed to increasing cineole showed little reduction in intakes, while the control group matched their previous levels (Figure 5b). The treatment animals were then taught the aversion to cineole again by adding both cineole and jensenone (an acylphloroglucinol derivative) in corresponding amounts to the diet so that the effects of the jensenone would be associated with the smell and taste of the cineole. Finally, both groups were tested again with only cineole added to the diet: the treatment group again behaved in a manner not significantly different from the controls (Figure 5c). Lawler *et al.* (1998b) also present matching data for common brushtail possums fed cineole in artificial diets. It is clear from these experiments that the avoidance of *Eucalyptus* essential oils by marsupial folivores can be the result of a learned association with negative effects not caused by the essential oils themselves. The value of essential oils as deterrents of these animals appears to lie in their strong, overriding smell and taste, which is easily associated with negative effects.

Effects of Ingested Essential Oils on Vertebrate Herbivores

Even if essential oils do not actually deter mammals from eating *Eucalyptus* foliage, they may still impose a metabolic cost on the animal and lead to some disruption of metabolic and digestive processes. The germicidal effects of many constituents of *Eucalyptus* foliage (Boland *et al.* 1991) have suggested to some workers the potential for deleterious interactions with intestinal bacteria (Fleay 1937).

The marsupial herbivores that eat *Eucalyptus* foliage rely to a small extent on the digestion of foliar cell walls in the hindgut for part of their energy intake, but the great majority of digestible energy is derived from the constituents of the cells. For example, fermentation measured *in vitro* in the caecum and/or proximal colon of koalas (Cork and Hume 1983) and greater gliders (Foley *et al.* 1987) contributed only about 10% of both species' digestible energy intake. Nonetheless, if ingested essential oils disrupt this process by killing the caecal bacteria, then the animal might find itself unable to digest an essential part of its diet. Early *in vitro* studies in deer by Nagy *et al.* (1964), Oh *et al.* (1968) and Connolly *et al.* (1980) showed that many components of the oils of Douglas Fir significantly inhibited the growth and activity of rumen microbes derived from sheep and deer *in vitro.*

It is difficult to sustain an analogous argument for marsupials, namely, that the microbial populations of koalas, greater gliders, and ringtail and brushtail possums are all found in the caecum and colon. Indeed, Foley *et al.* (1987) showed that most oils were absorbed in the simple stomach and small intestine, thus avoiding for the most part interactions with microbes.

Eucalyptus essential oils could still have a deleterious effect in those species which house their microbial population in the stomach (e.g. foregut fermenters such as goats and wallabies) but the evidence from studies of similar essential oils in sheep and deer (Cluff *et al.* 1982, White *et al.* 1982) suggests that the oils are again rapidly absorbed, or else volatilised and eructated from the rumen very soon after they are ingested. There is little reason to suspect that the situation would be different with wallabies and *Eucalyptus*. Overall, the evidence suggests that *Eucalyptus* essential oils have little direct effect on the digestive processes of animals that normally eat the foliage.

Metabolic Transformations of *Eucalyptus* Essential Oils by Mammals

Absorbing ingested oils from the gut avoids interactions with microbes but these materials must still be detoxified and excreted, and doing so can be energetically expensive. Several authors (Cork *et al.* 1983, Foley 1987) have argued that the poor conversion of digested energy from a diet of *Eucalyptus* foliage into metabolisable energy is due, in part, to the excretion of oils and their metabolites in the urine.

There have been a number of studies of the metabolic fate of ingested *Eucalyptus* oils in folivorous marsupials. Part of the rationale for these studies has been a curiosity about the pathways of degradation (e.g. Bull *et al.* 1993) but amongst ecologists the interest has been driven by the notion that understanding the metabolites might help in measuring the cost of ingesting oil components.

In koalas, brushtail possums and greater gliders 95-98% of volatile oils are absorbed from the digestive tract. These oils are believed to be modified in the liver by oxidation or hydrolysis, possibly conjugated with a small molecule, and then excreted via the urine or bile (Hume 1982). Several recent studies have been reviewed by McLean and Foley (1997). The outstanding trend in studies by McLean *et al.* (1993, 1995, 1997) is that mammals which are capable of including *Eucalyptus* foliage in their diets appear to have developed a significantly greater capacity to produce highly oxidised metabolites of ingested monoterpenes than have other animals. These highly polar products can then be excreted in the urine without the need for conjugation with glucuronic acid or glycine.

For example, McLean *et al.* (1995) studied the metabolic fate of dietary citronellal in greater gliders and common ringtail possums. They found that this monoterpene was cyclised and oxidised to *trans*-3,8-dihydroxy-*p*-menthane-7-carboxylic acid, in contrast to rabbits which do not oxidise the cyclised product. McLean *et al.* (1997) examined the metabolism of *p*-cymene in rats, common brushtail and ringtail possums, and greater gliders. They observed eleven urinary metabolites and grouped these according to the number of oxygens added per molecule of *p*-cymene. Rats excreted metabolites containing from one to four oxygens, whereas brushtails excreted metabolites with two to four oxygens, and common ringtails and greater gliders only excreted metabolites with three or four oxygens. This trend is also apparent when considering metabolites of α- and β-pinene formed by common brushtails and koalas (Southwell *et al.* 1980). One advantage of excreting unconjugated products is that the loss of carbohydrates and amino acids which are used as conjugates is avoided. This may be a significant advantage in the case of nutrient-poor diets such as *Eucalyptus* foliage.

Recently, Carman and co-workers (Carman and Klika 1992, Bull *et al.* 1993, Carman and Rayner 1996) made a detailed study of the fate of cineole in common brushtail possums. Common brushtails produce a range of metabolites, principally 9-hydroxycineoles and cineole-9-oic acids, as well as other alcohols and diols. Of particular interest is the chirality of the major metabolites because females produce a higher ratio of S/R enantiomers than males. Carman and Klika (1992) argue that these could be involved in pheromonal signalling. Clearly there remain a number of exciting avenues to explore in our understanding of the metabolism of *Eucalyptus* essential oils by folivorous marsupials.

DIET CHOICE IN INSECT GRAZERS OF *EUCALYPTUS*

Feeding by insect herbivores on *Eucalyptus* foliage is just as variable as feeding by vertebrate herbivores. Several studies have identified marked interspecific (Morrow and Fox 1980), intraspecific (e.g. Journet 1980, Edwards *et al.* 1993, Stone and Bacon 1994, Patterson *et al.* 1996) and even intra-individual (Edwards *et al.* 1990) differences in the amount of defoliation. A review by Ohmart and Edwards (1991) details the nature of the plant-animal interaction. They concluded that plant secondary metabolites of *Eucalyptus* have very little effect on patterns and amounts of insect herbivory and on the survival and performance of insect herbivores. However, since the time of Ohmart and Edwards' review, continuing studies have gathered strong correlative evidence to suggest that insect herbivory is affected by foliar essential oils, in particular cineole (Edwards *et al.* 1993, Stone and Bacon 1994). We briefly review the accumulation of this evidence below.

Morrow and Fox (1980) were the first to suggest that there was an association between the foliar essential oil content and the level of damage in two alpine eucalypts, *E. stellulata* and *E. pauciflora*. However, their study of five *Eucalyptus* species showed that the distribution of damage, eggs and insects, as well as the feeding rates, growth and survival of insects, demonstrated few consistent effects of total essential oil content (Table 1). The average oil yield of the species used in the experiments ranged from 1.2% to 11.6% (dry matter basis) with yields in some individual plants as high as 20%. In *E. dives* and *E. viminalis* there was an association between yield and damage. The plants that suffered low levels of damage had oil yields significantly greater than those suffering low levels of damage. However, there was no significant difference in total oil yields between the two levels of damage for the other three species, *E. pauciflora*, *E. bridgesiana* and *E. melliodora*. In fact, there was a tendency toward lower oil yields for lightly damaged trees in the latter two species. Morrow and Fox (1980) argued that antiherbivore effects may only become apparent if a threshold in total oil concentration is exceeded. However, it can be seen from Table 1 that the heavily grazed *E. dives* had more than twice the total oil yield of the lightly grazed trees of *E. pauciflora*, *E. bridgesiana* and *E. melliodora*.

Recent studies by Edwards *et al.* (1993) and Stone and Bacon (1994) have shown a strong negative association between insect herbivory and leaf essential oils, while another (Patterson *et al.* 1996) has shown no relationship. Recent work has taken into account the composition of the oils, whereas Morrow and Fox only considered the total oil yield. For example, Edwards *et al.* (1993) found that the proportion of cineole in the leaf essential oil best explained differences in levels of herbivory by Christmas beetles (Figure 6). Although Patterson *et al.* (1996) found no effect of essential oils in *E. regnans* on herbivory by *Chrysophtharta bimaculata*, the concentration of cineole in that species is very low. The authors also suggested that perhaps paropsine beetles are less affected by *Eucalyptus* essential oils than are other insect herbivores. It should be noted also that a high proportion of the *E. camaldulensis* trees examined by Edwards *et al.* (1993) had both a high proportion of cineole in the leaves and a high level of defoliation by *Anoplognathus*, a non-paropsine beetle (Figure 6c).

The above studies suffer from the same shortcomings as do those relating mammal feeding preferences to leaf essential oils, namely, the studies are all correlative and do not show cause and effect. If essential oils, including cineole, are to be conclusively shown to be deterrents to insect feeding, then bioassays with a range of insect species need to be performed. To date we are not aware of any studies in which either whole oil extract or isolated cineole have been fed to any foliage-feeding insect. Previously it might have been argued that direct addition of cineole would lead to an unnatural situation because the headspace vapour pressure would be excessive. It has been shown that high vapour pressures of *Eucalyptus* essential oils are highly toxic to insects (Sarac and Tunc 1995, J. Seymour pers. comm.). However, there are now a number of methods available, such as microencapsulation (Clancy *et al.* 1992), that may overcome these difficulties and make these sorts of experiments more feasible.

It may be that cineole is only effective in synergy with other oil components or it may serve as a cue to the presence of some other toxic molecules. Cineole is clearly metabolised by some of the major insect herbivores of *Eucalyptus* (see below). However, it remains to be seen whether this need to metabolise cineole is limiting the ability of insects to ingest cineole-rich diets, as appears now not to be the case for marsupial folivores of *Eucalyptus* (see above).

Metabolic Transformations of *Eucalyptus* Essential Oils by Insects

Insects use a diverse range of strategies for coping with the essential oils they encounter in their food. Some insects cope with the high essential oil content of *Eucalyptus* leaves by feeding around oil glands (Ohmart and Edwards 1991) or sequestering them within the body (Morrow *et al.* 1976). Others may simply tolerate the oils, while still others possess mechanisms for metabolising and detoxifying them.

Morrow and Fox (1980) showed that the gas chromatographic profile of faeces from both *Paropsis atomaria* and *Anoplognathus montanus* fed on a range of *Eucalyptus* species was not significantly different from that of the ingested oils. They concluded that the oils are tolerated but not detoxified, even though many of the oil components induce polysubstrate membrane oxidases in other species. Similarly, Southwell *et al.* (1995) found that *Paropsisterna tigrina* produced frass with an essential oil profile almost identical to that of the ingested leaf when fed *Melaleuca* leaf, unless cineole was a significant component of the diet. For high-cineole diets, the beetles (adults and larvae) oxidised the bulk of the cineole to (+)-2β-hydroxycineole while other oil components were unchanged. Ohmart and Larsson (1989) found that larvae of *P. atomaria* absorbed or converted the majority of essential oils of *E. blakelyi* leaves, of which most (> 75%) was again cineole. They found no evidence of sequestration of the oils and concluded that the oils are metabolised by the larvae. However, proportions of the two other main components of the oil (limonene and α-pinene) were similar in the frass and the leaves, which appears to be consistent with the findings of Southwell *et al.* (1995) that cineole is metabolised while the other components pass through the gut unchanged. Oil budgets were provided for total oils and for cineole alone, and it was not clear whether the absorption of cineole entirely explained the effect seen in the total oils. Ohmart and Larsson (1989) also drew attention to the fact that when Morrow and Fox (1980) fed leaf of *E. elata* to *P. atomaria* the major component of the oil, piperitone, was removed and presumably metabolised.

Just how the essential oils are metabolised by insects is still unknown. Monoterpene compounds are known to induce mixed function oxidase (MFO) activity (Moldenke *et al.* 1983) which accords with studies by Rose (1985) who found that insects with high MFO activity invariably fed on hosts (including *Eucalyptus*) containing monoterpene compounds. The study of Southwell *et al.* (1995) is the most recent study in this area and concludes with the statement that the oxidase source (microbial or gut enzymes) is unknown. Clearly then, there is much to be learnt about insect metabolism of *Eucalyptus* essential oils.

THE ROLE OF TERPENE-ACYLPHLOROGLUCINOL ADDUCTS IN FOOD CHOICE OF MAMMALS AND INSECTS

Data on the role of essential oils in mediating feeding by folivorous mammals and insects on *Eucalyptus* have not been clear and we are now beginning to realise that perhaps their role has been overstated. Nevertheless, as described above, they may play a very important role due to their very close relationships with another group of compounds, the acylphloroglucinols, which have only relatively recently been discovered, and for which evidence of an antiherbivore role is accumulating rapidly, for both mammalian and insect herbivores.

Earlier, we described the structures of these compounds and their molecular relationship with the essential oils. It is unfortunate that our current state of knowledge is such that we cannot discuss in greater detail the biosynthesis of these compounds or provide a mechanistic explanation of the relationship. Nevertheless, we do know that all of the currently identified active deterrent acylphloroglucinol compounds contain either a terpene side chain or, at the very least, an isoprene unit (the basic building block of terpenes). There is a strong correlation between the concentrations of terpenes and of acylphloroglucinol compounds in four *Eucalyptus* species that have been examined closely (Foley, Matsuki and Floyd unpubl.). In mammalian herbivores, at least, the secondary correlation between food intake and essential oil concentration is due to the primary relationship between the oils and the acylphloroglucinols. The development of the evidence for this is discussed in this section.

Pass *et al.* (1998) investigated the difference between palatable and unpalatable forms of *E. ovata* by a process of bioassay-guided fractionation. They found that the differences between the two forms in their ability to deter feeding by common ringtail possums were principally due to the higher concentration of a compound called macrocarpal G (Figure 1f), an acylphloroglucinol with an adduct of bicyclogermacrene - a relatively uncommon sesquiterpene found in *Eucalyptus*. This led Lawler *et al.* (1998a) to conduct more comprehensive experiments with ringtail possums, examining a range of palatabilities of individual *E. ovata* trees to the animals. As discussed above, the resultant range of intakes correlated strongly with the essential oil content of the leaves (Figure 4a) but bioassay experiments showed that essential oils could not cause the effects seen (Figure 4b). In contrast, bioassays with purified macrocarpal G reproduced the range of reduced intakes in concentrations that corresponded closely with those found in the leaves (Figure 7) and it was concluded that macrocarpal G was the primary cause of feeding deterrence in *E. ovata*.

Difficulties in the extraction and purification of macrocarpals led us to investigate the deterrent properties of related compounds. The initial focus was on jensenone (Figure 1d) as it was listed by Boland *et al.* (1991) as a major component of the steam distillate of *E. jensenii*, and was thus likely to be relatively easy to extract and purify in suitable quantities. This was achieved and a number of experiments have been carried out with jensenone added to the diets of common ringtail and common brushtail possums. These have shown jensenone to be a potent antifeedant in molar quantities very similar to those found for macrocarpal G. Further work has indicated that their mechanism of action is due to stimulation of the animals' emetic systems (Lawler, Pass, Foley unpubl.).

More recent work has focused on sideroxylonals (dimers of jensenone) as they are found in variable concentrations in a number of *Eucalyptus* species, are often the predominant acylphloroglucinol in those species (Eschler, Pass, Foley unpubl.), and methods have been developed to quantify precisely the amounts found in the leaves. These compounds, too, show strong correlations with the range of food intakes by common ringtail possums (Figure 8) and preliminary evidence suggests that this applies also to koalas (Moore and Foley unpubl.). Bioassay experiments have again supported the conclusion that sideroxylonals are responsible for feeding deterrency in those plants where concentrations are sufficiently high (Lawler, Eschler, Foley unpubl.). Once again we see the correlation between essential oils and both food intakes and sideroxylonal concentrations, suggesting that the oils do not have a causal antifeedant effect.

There are, so far, few data on the effects of acylphloroglucinols on insect feeding. Correlations between defoliation and sideroxylonal concentrations of leaves have been shown (Foley and Floyd unpubl.*)*, but bioassays have not yet been conducted. Hence the same problems as described previously with correlative studies are encountered, and we cannot yet say that acylphloroglucinols are the cause of deterrency in insect-resistant trees. Work in this area should advance rapidly since the problems of headspace concentrations associated with essential oils do not occur with acylphloroglucinols.

EFFICACY OF SELECTING HIGH ESSENTIAL OIL GENOTYPES IN CONFERRING NATURAL HERBIVORE RESISTANCE ON *EUCALYPTUS* PLANTATIONS

The selection of herbivore-resistant genotypes for *Eucalyptus* plantations may be a practical approach to protecting them from damage by both vertebrate and invertebrate herbivores (Floyd and Farrow 1994). However, we conclude with a word of caution on selecting for genotypes containing high concentrations of essential oils to confer this resistance as suggested by Farrow (1993) and Stone and Bacon (1994).

There is now some evidence that for mammalian folivores essential oils are not the actual deterrents, but appear so because their concentrations are correlated with acylphloroglucinol compounds. Whether this is also the case for insects cannot currently be assessed, due to the difficulties associated with mixing free essential oils into basal diets. There is no doubt that essential oils are absorbed and metabolised, in some cases, by both insect and mammalian folivores, though there is little compelling evidence that the rate of metabolism is limiting to food intakes.

In any case, the selection of resistant genotypes is a sound approach. Difficulties may arise, however, if this resistance is then attributed to the wrong compound and selection is based on that criterion rather than resistance *per se*. It appears that the concentrations of essential oils and of acylphloroglucinol compounds are closely related, so selection of high essential oil genotypes may be successful, as long as that selection also selects for correspondingly high acylphloroglucinol contents. However, our knowledge of the biosynthesis of acylphloroglucinols, and its relationship with the biosynthesis of essential oils, is sorely lacking at present. There is a distinct danger that at some point in the artificial selection process the relationship between concentrations of the two groups of compounds may break down, and that high essential oil genotypes may no longer contain high acylphloroglucinol concentrations. This danger is especially significant where genetic engineering may be used to manipulate a single factor, such as cineole production, unless flow-on effects on related compounds are also assessed.

We believe that in this instance mammalian folivores at least, and perhaps insects, would rapidly learn, within a local area, that high essential oil contents are not associated with negative effects and will commence to feed on those plants at a high rate. It now appears that mammalian folivores of *Eucalyptus* avoid high essential oil diets as the result of a conditioned food aversion, and there is strong evidence to suggest that these aversions are lost rapidly where there is repeated exposure to the taste stimuli in the absence of the negative feedback. This would be the case for a high essential oil/low acylphloroglucinol *Eucalyptus* plantation.

Whether this effect would be seen for insect herbivores of *Eucalyptus* is less certain. For the reasons discussed above, the actual role of essential oils in determining resistance of foliage against any insect herbivore remains uncertain. Furthermore, if, indeed, essential oils prove not be the causal agents then there is less information on the insect's ability to learn to eat these leaves over either a single generation or evolutionary time. That is, there may be an immediate behavioural response to encountering edible high essential oil plants, or there may be a reduction in selection for avoidance due to reduced mortality or increased performance of those insects feeding on high essential oil plants. It was shown by de Little and Madden (1975) that *Chrysoptharta bimaculata,* a significant pest of Tasmanian *Eucalyptus* plantations, may choose to oviposit on species on which larval growth and survival rates were subsequently shown to be lower (Baker 1995) than those on other species present. Thus there may be the opportunity for at least the larval stages to encounter plants rich in essential oils and to learn that these plants are a suitable food source, again undermining the resistance conferred by the essential oils.

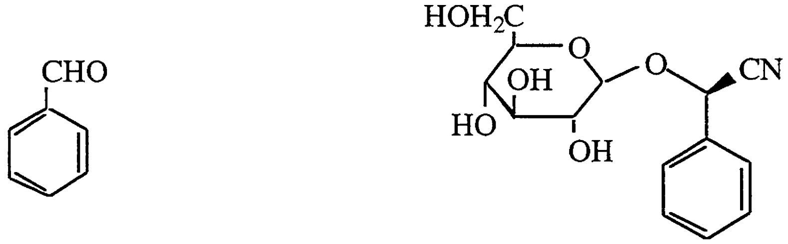
It is important then, that in the attempts to develop these herbivore-resistant plantations the questions and gaps in our knowledge outlined in this chapter are addressed. At the very least, caution is advised in this endeavour, and the resistance of genotypes intended for use should be checked regularly and directly on the animals concerned, until we have confidence in the attributes to which we attribute herbivore resistance.

Table 1. Relationship between essential oil content of leaves and grazing damage by insect herbivores of *Eucalyptus* (from Morrow and Fox 1980).

Species	Essential oil content[a]		p value of difference
	Heavy grazing	Light grazing	
E. pauciflora	1.149 ± 0.150	1.253 ± 0.253	ns[b]
E. bridgesiana	3.866 ± 0.423	3.214 ± 0.363	ns
E. melliodora	4.223 ± 0.700	2.973 ± 0.546	ns
E. viminalis	3.740 ± 0.658	6.864 ± 1.466	$0.025 < p < 0.05$
E. dives	9.909 ± 0.870	13.389 ± 1.512	$0.013 < p < 0.025$

a % w/w, dry matter basis.

b Not significant.

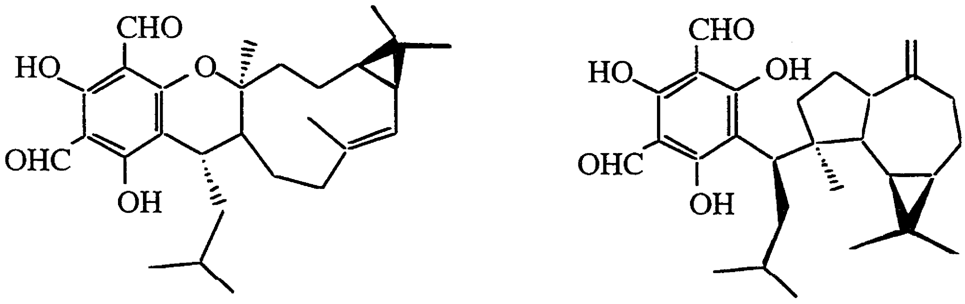


(a) benzaldehyde

(b) Prunasin

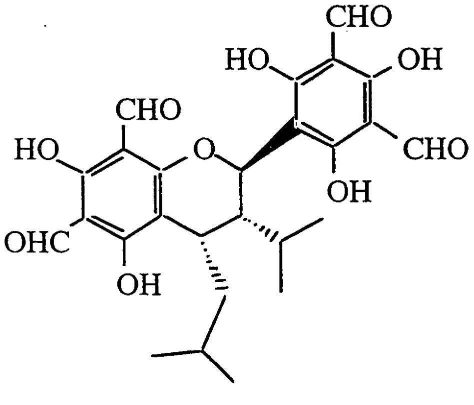
(c) Torquatone

(d) Jensenone



(e) Euglobal III

(f) Macrocarpal G



(g) Sideroxylonal A

Figure 1. Structures of some non-terpenoid compounds extracted from *Eucalyptus* leaves by conventional steam distillation for essential oils and of possible antifeedant compounds with similar structures.

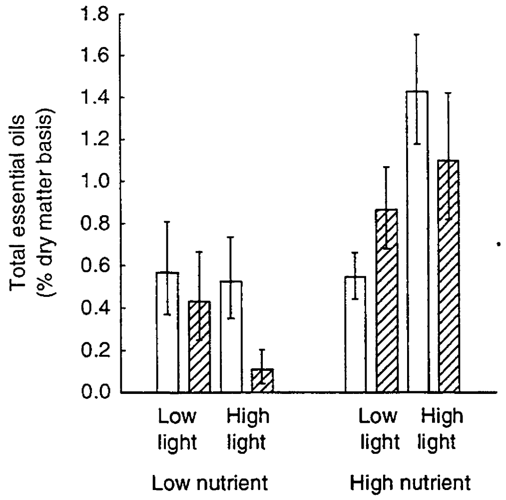


Figure 2. Effect of variation in soil nutrients, light and atmospheric CO_2 on essential oil content of *Eucalyptus tereticornis* leaves.
Unshaded bars represent plants grown at ambient (350 ppm) CO_2 levels while shaded bars represent plants grown at elevated (800 ppm) CO_2 levels (after Lawler *et al.* 1997).

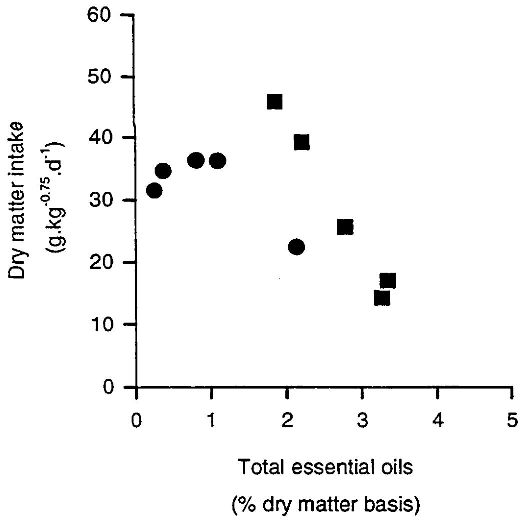
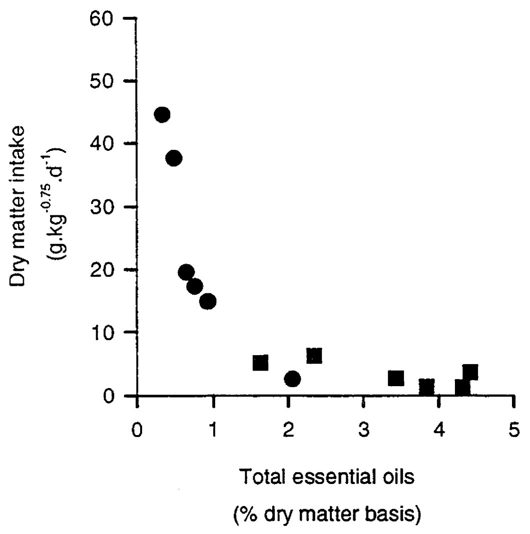


Figure 3. Relationship between food intake of koalas and essential oil content of leaves. Circles represent *Eucalyptus ovata* and squares represent *E. viminalis* (after Lawler *et al.* 1998a).

a.



b.

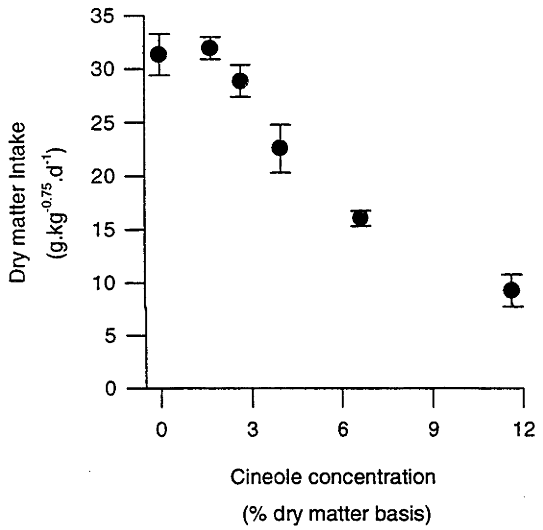


Figure 4. Relationship between food intake of common ringtail possums and essential oil content of (a) leaves and (b) an artificial diet.
In (a) circles represent *E. ovata* and squares represent *E. viminalis*. Note that in leaf diets the total essential oils are given, while in the artificial diet cineole only was used as a representative essential oil, as in a range of species, including *E. viminalis*, it is the predominant component of the oils. (Derived from Lawler *et al.* 1998a)

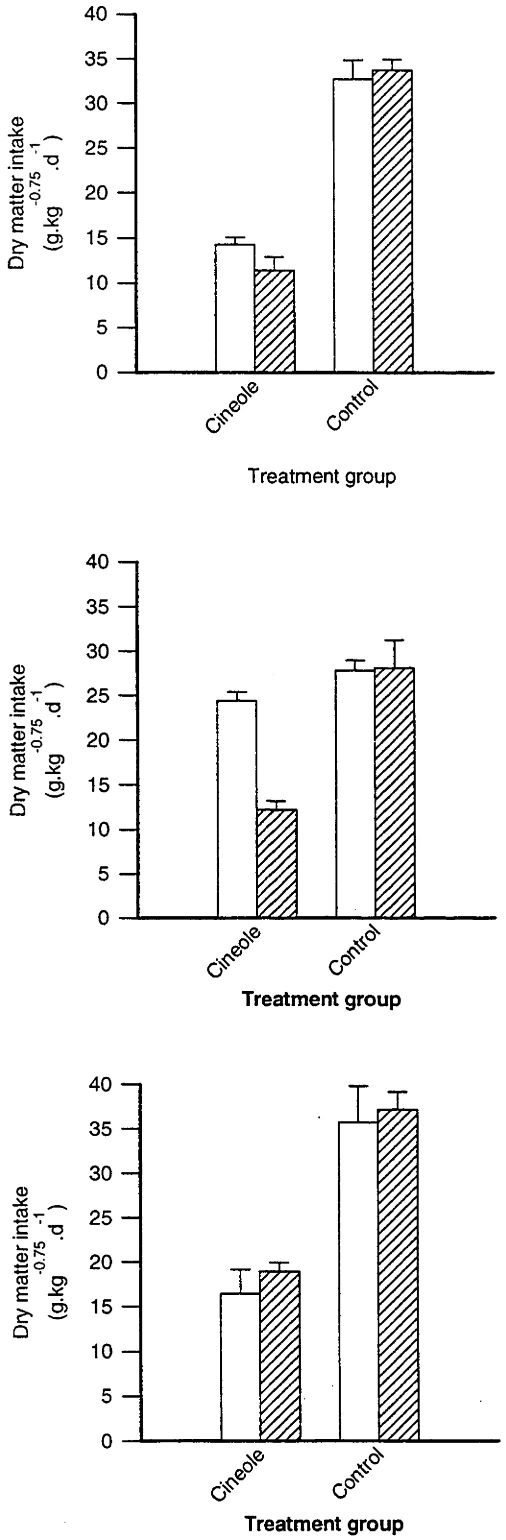


Figure 5. Evidence for a conditioned food aversion to cineole in common ringtail possums. Data shown are food intakes by the same animals of an untreated diet and one to which cineole was added. Data are for two groups of animals, one of which (treatment) was acclimated to a high-cineole diet while the other (control) was fed only the untreated diet throughout. Three stages of the experiment are represented: (a) initial test, (b) post acclimation, (c) after reconditioning (see text for explanation). Unshaded bars represent treatment animals and shaded bars represent control animals (after Lawler *et al.* 1998b).

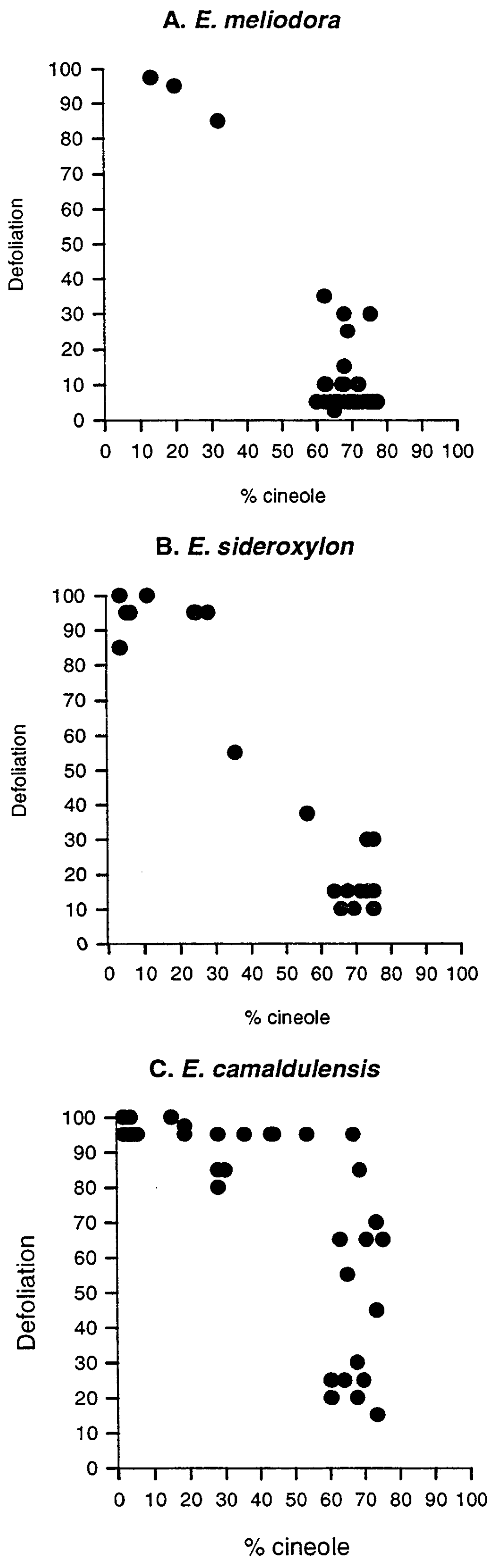
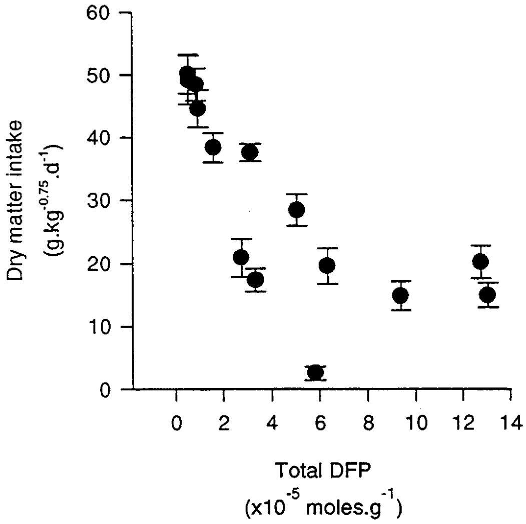


Figure 6. Relationship between tree defoliation by insects and proportion of cineole in the essential oil of the leaves of three *Eucalyptus* species: (a) *E. melliodora*, (b) *E. sideroxylon*, (c) *E. camaldulensis* (after Edwards *et al.* 1993).

a.



b.

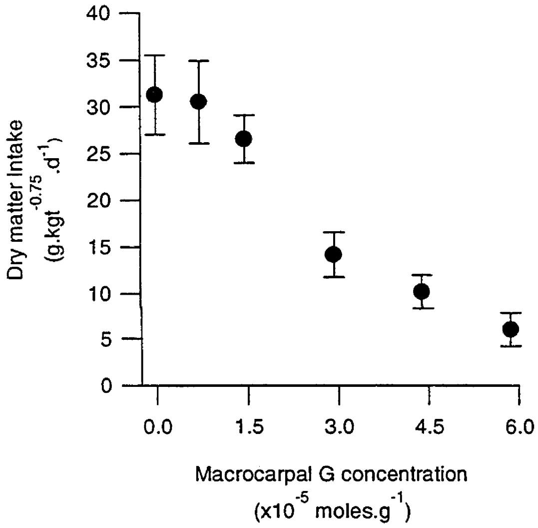


Figure 7. Relationship between food intake of common ringtail possums and total DFP/macrocarpal G content of: (a) *Eucalyptus ovata* leaves, (b) an artificial diet (after Lawler *et al.* 1998a).

Note that data for leaves are total acylphloroglucinols as there is no existing assay specifically for macrocarpals (although mass spectrometry shows that acylphloroglucinols in *E. ovata* are predominantly macrocarpals).

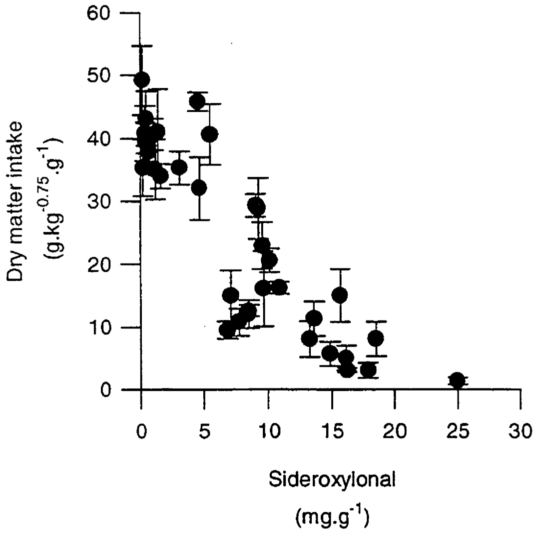


Figure 8. Relationship between food intake of common ringtail possums and sideroxylonal content of *Eucalyptus polyanthemos* leaves (Lawler unpubl.).

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APPENDIX 5. ECOLOGICAL APPLICATIONS OF NEAR INFRARED REFLECTANCE SPECTROSCOPY - A TOOL FOR RAPID, COST-EFFECTIVE PREDICTION OF THE COMPOSITION OF PLANT AND ANIMAL TISSUES AND ASPECTS OF ANIMAL PERFORMANCE.

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ABSTRACT

Many ecological studies rely heavily on chemical analysis of plant and animal tissues. Often, there is limited time and money to perform all the required analyses and this can result in less than ideal sampling schemes and poor levels of replication. Near infrared reflectance spectroscopy (NIRS) can relieve these constraints because it can provide quick, non-destructive and quantitative analyses of an enormous range of organic constituents of plant and animal tissues. Near infrared spectra depend on the number and type of C-H, N-H and O-H bonds in the material being analyzed. The spectral features are then combined with reliable compositional or functional analyses of the material in a predictive statistical model. This model is then used to predict the composition of new or unknown samples. NIRS can be used to analyze some specific elements (indirectly - e.g. N as protein) or well defined compounds (e.g. starch) or more complex, poorly defined attributes of substances (e.g. fiber, animal food intake) have also been successfully modeled with NIRS technology. The accuracy and precision of the reference values for the calibration data set in part determines the quality of the predictions made by NIRS. However, NIRS analyses are often more precise than standard laboratory assays. The use of NIRS is not restricted to the simple determination of quantities of known compounds, but can also be used to discriminate between complex mixtures and to identify important compounds affecting attributes of interest. Near infrared reflectance spectroscopy is widely accepted for compositional and functional analyses in agriculture and manufacturing but its utility has not yet been recognized by the majority of ecologists conducting similar analyses. This paper aims to stimulate interest in NIRS and to illustrate some of the enormous variety of uses to which it can be put. We emphasize that care must be taken in the calibration stage to prevent propagation of poor analytical work through NIRS but used properly, NIRS offers ecologists enormous analytical power.

Key Words: near infrared reflectance spectroscopy, leaf chemistry, plant-herbivore interactions, plant defense, food intake, decomposition

INTRODUCTION

The analysis of nutrient concentrations in samples of plant and animal tissues is an integral part of many ecological studies. These analyses (e.g. total nitrogen, carbohydrates and lipids) are frequently so time-consuming and expensive that sampling strategies adopted are less than ideal. For example, variations of nutrients within individuals of a plant species often may be an important component of animal foraging that is ignored in many studies simply because the extra analytical work cannot be accommodated.

Another typical problem for the analyst involves the large amount of sample required for some analyses; these may be difficult to obtain (e.g. from seedling plants). Alternatively, the analyst may have to bulk replicate samples to get sufficient material for all necessary analyses and again compromise an ideal experimental design.

Often ecologists are forced to use a range of indirect measures in place of a more suitable direct measure of the attribute of interest. This situation has often arisen in studies of the foraging behaviour of folivorous primates and marsupials (Waterman et al. 1980). Typically, investigators measure the concentrations of a diverse range of elements and attributes such as "fiber" when what is actually needed is an estimate of relative food intake by the animal (Poppi 1996; McIlwee, Lawler and Foley unpublished).

Near infrared reflectance spectroscopy (NIRS), offers ecologists enormous flexibility in meeting these sorts of challenges. The analysis of multiple constituents of plant tissues can be made rapidly in a single operation using NIRS technology (up to 150 samples can be processed in a day by a single operator) and with minimal sample preparation (Marten et al. 1989). The use of NIRS is low-cost (savings of at least 80% of normal laboratory costs are easily achievable; Aragones et al. 1997) and amenable to small sample sizes (multiple analytes can be obtained on as little as 0.2 g of material; Aragones et al. 1997). Finally, NIRS analysis is non-destructive and produces no chemical wastes which should be an important consideration for all ecologists.

Use of NIRS has been adopted enthusiastically by many agricultural and manufacturing industries. Those concerned with evaluating and improving the nutritive value of feedstuffs for livestock are some of the biggest users (see Shenk and Westerhaus 1994). Some NIRS methods have been established for more than 20 years and have achieved "official" status (e.g., AOAC 1990). Much of the early use of NIRS centered on exploiting the technique as a time and cost-effective method of nutrient analysis. However, in recent years, there have been many innovative uses of NIRS and it is becoming an increasingly accepted tool for routine analysis in many research areas.

Many of the analytical techniques used in ecology are based on those used in agricultural industries but NIRS has been little used in ecological studies (but see Brooks et al. 1984; Joffre et al. 1992; Malley et al. 1996; Nilsson et al. 1996 for notable exceptions). NIRS offers an excellent opportunity for ecologists to escape the constraints of laboratory analyses on the design of their investigations and to apply many laboratory-based measurements to free-ranging animals.

In this article we summarize some recent studies using NIRS in ways that we believe will be useful to ecologists. Our aim is to stimulate interest amongst ecologists in NIRS, to bring together a scattered literature and to widen the applications of the methodology. We are less concerned with describing detailed methodologies for applying the technique to different materials that might be of interest to experienced users of the technique. In our own work (nutritional ecology of herbivores), we have found that NIRS has allowed us to focus our research on landscape-scale questions and to apply laboratory findings to the field. Perforce, many of the examples we cite are related to our own interests, but we stress that our belief is that the potential applications of NIRS are enormous.

Basis of the technique

The composition of plant or animal tissues is ultimately reflected in the types of bonds between the atoms or groups of atoms (functional groups) that make up those tissues and information about these functional groups can be sought through many different forms of spectroscopy. When a sample of organic material is irradiated, the bonds continually vibrate which causes stretching and bending. This in turn causes a type of wave motion within the bond at a frequency that is characteristic of the functional group. The frequencies of the incident light that match the frequencies of the vibrational waves are absorbed whereas other frequencies are reflected or transmitted. The process is the same as that which allows us to see colored objects that intercept white light (Van Kempen and Jackson 1996).

Near-infrared radiation (750- 2500nm) (Fig. 1) is absorbed mainly by C-H, N-H and O-H bonds (Osborne et al. 1993) which are the primary constituents of the organic compounds of plant and animal tissues. The chemical constituents of the tissue determine the nature and number of bonds present and therefore the wavelengths and amount of light that is absorbed. Therefore, the spectrum of light that is reflected from the sample contains detail on the chemical composition of that material (Shenk et al. 1992; Shenk and Westerhaus 1994).

However, the peaks in an NIR spectrum are not distinct or sharp because they consist of overtones and combinations from primary absorptions in the mid-infrared region and also because some of the light is scattered (Shenk and Westerhaus 1993). Consequently, there are few if any regions of the NIR spectrum where absorbance can be due to only one type of functional group and direct interpretation of the spectral absorbances is very difficult for complex mixtures (Figure 2). Given the limited number of functional groups present in plant and animal tissues (due to limited numbers of metabolic pathways) an analyst could assume that absorbances in the spectral region characteristic of the amide bond of a protein is, in fact, largely due to the protein in the tissues. However, one could never be certain about this (see Fig 2) and so NIRS relies on applying statistical tests to that assumption. In other words, the analyst derives a statistical model that tests the intensity of the relationship between a particular absorbance and an independent laboratory assay of protein content in a range of different plant or animal tissues. Since a different group of plant or animal tissues might contain different functional groups that also absorb in the spectral region of interest, the soundness of the relationship between spectral absorption and laboratory assays should be assessed for each type of tissues of interest. Near infrared analysis is thus an indirect or secondary method that estimates chemical composition by comparing spectra with samples of known composition (Shenk et al. 1992; Shenk and Westerhaus 1994). This procedure is known as calibration.

In practice, the analyst develops a multivariate statistical model to describe the relationship between the NIR spectral absorbances and the chemical components or characteristic of interest (Shenk and Westerhaus 1993). The statistical model is then used to predict the composition of unknown samples that are part of the same population. Samples which fall outside the population can be analyzed by traditional means and iteratively included in a new model (Shenk et al. 1992; Shenk and Westerhaus 1994). These procedures are discussed in more detail below.

Mechanics of NIR spectroscopy and sample presentation - importance of particle size.

A NIR spectrophotometer consists of a light source, a means of selecting particular wavelengths (NIR spectroscopists traditionally use wavelengths (nm) rather than wave-numbers as favoured by those working in the mid IR) within the spectrum, a detector for collecting the reflected radiation and a signal and data processing computer (Fig.3). The sample (which in most current applications is dried and ground - but NIRS can be used with any other sample form - see section below on analysis of fresh forages), is usually packed manually into a sample cup which has an optical grade quartz glass cover on one side. Care is required to ensure that the sample is spread evenly and packed to a consistent degree of compression. The sample cups can be small ring cups of between 20 and 50 mm diameter or alternatively larger cells that hold up to 50 g of coarser material. These are then placed in the spectrometer and the reflectance spectra collected.

In some applications, fiber optics are used to bring the light to either a remotely presented sample and the information is conducted back along the optic cable to the detector and microprocessors or captured by remotely placed detectors and conveyed electronically to the microprocessor.

Particle size of the sample has a major effect on the NIR reflectance spectrum (Casler and Shenk 1985, Windham 1987). Increasing particle size results in an increased apparent path length for the incident light and so the measure of reflectance also increases (Fig. 4). Consistent particle size between samples is therefore important and analysts using NIRS techniques with dried, ground samples need to be aware of the performance of their laboratory grinders (Shenk and Westerhaus 1993). The fineness of the particles is less important than the distribution of sizes and many grinders simply cannot produce a uniform particle size. Depending on the nature of the samples being ground, analysts may have to regularly replace impellers and other grinding parts more frequently than expected to ensure that this optimum is maintained.

Calibration procedures.

The spectroscopic and chemometric principles used in developing calibration models are beyond the scope of this short review. Nonetheless it is appropriate to provide a broad outline of approaches which are most typically taken by analysts so that readers can appreciate the strengths and limitations of the whole technique. Those interested in specific procedures should consult some of the many excellent references on this subject (e.g. Shenk and Westerhaus 1991a,b; Shenk and Westerhaus 1993; Smith and Flinn 1991; Osborne et al. 1993; Baker et al. 1994). Figure 5 summarizes the most common steps taken to develop calibrations for plant and animal tissues (Shenk and Westerhaus 1993).

The essence of any calibration procedure is to ensure that the range of spectral variation found in the whole population is represented in the samples selected for analysis for calibration development. Generally, the entire population is ranked in terms of distance from the average spectrum. Prior to ranking, a variety of mathematical treatments are applied to the spectra to emphasize only those data that are relevant. Particle size and structure of a sample can cause spectral differences that are unrelated to chemical composition. These transformations usually include scatter correction that may use standard normal variate procedures and mathematical derivative transformations.

Once the population has been structured in this way, samples with either extreme spectra or those that have very similar spectra are eliminated so that those remaining represent a defined degree of spectral variation. Various algorithms have been developed for doing this. The

CENTER and SELECT algorithms of Shenk and Westerhaus (1991a) have proved among the most popular for forage scientists but a variety of other methods have been developed (Shenk and Westerhaus 1991b).

In theory selection of samples that cover the range of spectral variation in the data set should be sufficient but we believe that most ecologists would be more comfortable if the calibration set also covered the range of taxonomic variation or included all types of treatments applied in field experiments. For example, in our work on herbivore foraging in tropical savannas, a number of common grass species were not included in the original calibration set chosen by the SELECT algorithm. Consequently, we expanded the set by selecting samples from our collection of those particular plant species (Woolnough and Foley unpublished).

Once sufficient samples have been selected, the traditional compositional analyses are performed on this subset. These may include measures such as concentration of nitrogen or protein, carbohydrates, lipids or even individual fatty acids and amino acids. However, as we describe in more detail below, other more integrative attributes may be measured such as the digestibility and intake of food by herbivores, grain yield during milling (Welsh et al. 1996), susceptibility to insect attack or pulp yield in the paper industry. Given the importance of these assays or measures, it is vital that the analyst be confident of the degree of accuracy and precision of these analyses (see Conventional laboratory assays).

The actual model is then constructed by developing a regression equation between the spectral absorbances and the traditional laboratory analyses (Shenk and Westerhaus 1991c; Shenk and Westerhaus 1993). This involves any of a number of multivariate regression procedures including multiple linear regression (MLR), principal components regression (PCR) and partial least squares (PLS) regression. Although many early studies were performed with MLR, this approach uses only a few wavelengths whereas the other methods use the full spectrum. Greater computing power is one reason why a modification of the PLS procedures (MPLS) is one of the most popular regression procedures (Shenk and Westerhaus 1993) but there are other even more sophisticated approaches available involving neural networks and wavelet theory. All these options are provided in various statistical packages but the analyst needs a clear view of the purpose of the calibration before choosing one of these methods and should operate within the bounds of standard practices (Anon 1995).

Validation of the calibration can be done in several ways. Previously the most common method used involved an independent set of samples for which compositional data was also available. In such cases, the calibrations derived from a "calibration data set" could be tested by predicting values in a “validation data set”, the relationship between these being assessed by simple regression analysis. More recently, this approach has been superseded by a procedure called cross validation, a form of Monte-Carlo simulation, in which the population is arbitrarily divided into a small number of groups and a prediction is made of the values for one group based on calibrations developed from the remaining groups. In turn, predictions are made for all groups with the average of predictions for all groups (Shenk and Westerhaus 1993). Depending on the amount of computing power available, these "groups" may contain as few as one sample. This procedure is useful because all available chemical analyses for all individuals can be used to determine the calibration model without the need to maintain separate validation and calibration sets (Shenk and Westerhaus 1993). Nonetheless, some analysts still maintain separate validation sets for verifying these models.

Conventional laboratory assays - what NIRS has revealed.

Most scientists who have embarked on studies using NIRS have found that conventional analyses involve as much work as the NIRS technique in providing an assessment of the calibration model. Because NIRS is so critically dependent on the quality of the data used to build predictive models, analysts often need to check and evaluate their assays in more detail than previously, as unknown sources of error become identified (Windham et al. 1988). NIRS can be used, to some degree, to provide an independent check of laboratory values. For example, in our laboratory we were confident from earlier work that we could predict total nitrogen in plant material with a high degree of accuracy so when we obtained a set of samples and data from another laboratory that resulted in a poor calibration, we first questioned the accuracy of the original analyses. Re-analysis for total nitrogen in our laboratory with blind duplicates resulted in a much better calibration that doubled the precision of the NIRS analysis.

Some debate exists as to whether NIRS calibrations can be more accurate than the underlying reference method on which they are based. NIRS predictions contain both the underlying laboratory errors as well as NIR instrument errors so it is unlikely that this could be the case. However, what may be most important is the kind of laboratory errors that are made. During the development of calibration models, most software packages will highlight erroneous mixes between spectra and laboratory values which can be of use in helping to identify potential sources of error. It is also possible in theory, that unbiased but randomly inaccurate sources of error may be averaged-out in large calibration sets. Whether a calibration is used for analysis depends very much on the discretion of the analyst. However, in practice, a good NIRS method should have a prediction error which is close to the standard error of the laboratory reference data.

Several studies have also highlighted the fact that inter-assay variation can also be a potential problem when trying to construct calibration equations (e.g. Shenk et al. 1992; Shenk and Westerhaus 1994). This variability may need to be assessed by re-analyzing series of blind duplicate samples on successive occasions. Exchange of samples between laboratories conducting the same analyses, benefits all parties.

Linking wavelengths to chemical constituents

Although the statistical models built on NIRS data are essentially empirical, users must realize that the wavelengths used in the equations relate to the underlying chemical composition. Ecologists will have greater confidence in NIRS if they can see that the wavelengths used in calibration models are those characteristic of the components of interest. For example, several studies have shown that the prediction of crude protein in temperate grasses depend on absorbances at 2120-2160 nm. The spectral region between 2100-2200 nm corresponds to N-H stretching in amide bonds (Shenk and Westerhaus 1993) and so wavelengths of 2120-2160 nm should be suitable for estimating crude protein. Not surprisingly, many calibration equations for crude protein emphasize wavelengths in this region. Plant secondary metabolites (PSMs) such as phenolics are expected to have a major absorbance around 1650nm (Flinn et al. 1996) and this wavelength has been used to separate forage samples containing high and low concentrations of phenolic constituents (Fig 7; Flinn et al. 1996) but again we caution that lignin could also absorb in this region and so the 1650 nm spectral region is not necessarily diagnostic for phenolics. Calibration is required to make quantitative estimates.

Assignment of wavelengths for attributes such as dietary fiber, total digestibility or food intake to particular chemical compounds is not so easy because these entities are either poorly defined chemically, depend on a great number of individual compounds or vary from forage to forage. One would expect however, that wavelengths for plant fiber estimation should correspond to regions where C-H bonds absorb and this is what has been found (Clark and Lamb 1991). Assigning wavelengths to components of interest remains an important research goal for many using NIR spectroscopy, particularly in agricultural industries because of the extensive databases that have already been built to describe aspects of plant quality on which producers are paid.

Ecological applications

The main purpose of this section is to highlight some of the recent innovative studies using NIRS that have potential applications to ecologists. From these studies, we synthesize implications for its possible use in the future. We conclude the review with a list of new challenges which NIRS might help to overcome. However, we stress that in many of these "new-areas" much work needs to be done in testing the limitations and potential of NIRS before the technique can be universally adopted in these areas of research. Despite this, we are hopeful that given time and the growing enthusiasm and support for NIRS, that this will happen in most, if not all, of the fields mentioned below.

In assessing the potential use of NIRS in each of these areas, it is important to bear in mind that the main benefit of NIRS lies not as a replacement tool for traditional analyses, but rather as a complementary tool which can be used alongside conventional analyses to improve the efficiency and cost of studies, or alternatively to explore areas which are otherwise impossible using alternative means. As with the application of any technology into new areas, there is a potential danger to over-use or misapply NIRS and any predictions that are made should be carefully evaluated before they are applied. The following examples below illustrate some of the current and emerging applications of NIRS in the field of ecology.

Resolution of complex mixtures

Measuring the botanical composition of different plant communities is integral to many ecological studies, especially those concerned with foraging in grasslands. In the past this information was obtainable either by hand-sorting clipped quadrats or by a variety of visual estimation. Hand sorting is slow and laborious and although visual estimation is rapid, there may be many unquantifiable errors (Coleman et al. 1985).

Shenk et al. (1979) originally showed that NIRS could be used to determine the proportion of legumes in legume-grass mixtures to an accuracy of about ±10% and several studies have attempted to expand this concept to more complex pastures (i.e. Coleman et al. 1990; Pitman et al. 1991; Atkinson et al. 1996). Artificial mixtures of three to four different components were accurately predicted by Coleman et al. (1990), but subsequent work showed that the predictive equations were too sensitive to the source of material used to make up the mixtures. Single sources of each component led to acceptable calibrations whereas calibrations involving many sources of each component, as would occur with natural pastures, were not sufficiently robust for routine use. Nonetheless, acceptable calibrations have been generated from pasture samples that were meticulously separated by hand (Petersen et al. 1987; García-Criado et al. 1991).

NIRS has been used to assess the botanical diversity of grassland habitats by comparing the spectral similarity or "composition" between samples (Hill et al. 1988). For example, Atkinson et al. (1996) used NIRS to monitor changes in the composition of vegetation samples across time and space, which could be traced back to hand-sorted component species. In this study, NIRS was used as a discriminant tool which could be used to group samples at varying levels of detail, in order to identify the overall similarity in composition between samples (see "Emerging Applications" section for more detail).

A second example of how NIRS can be used to resolve differences in complex mixtures is a study by Downey and Boussion (1996) who tested the ability of NIRS to discriminate between different blends of coffee. They obtained a 95% successful resolution based on differences in spectra alone. These differences were presumed to be predominantly due to variation in the concentration of caffeine or another related alkaloid.

Overall, it seems that NIRS has the potential to save researchers substantial time and effort in monitoring the make-up of mixtures. From past studies, it is evident that calibrations for narrow or closed populations can be expected to yield more precise information than predictions across multiple landscapes and a range of environmental conditions. However, before this prospect is adopted, a greater effort needs to be devoted to understanding the factors that affect the robustness of the predictive equations in individual situations.

Determination of plant nutrients

By far the widest use of NIRS has been in the measurement of nutritional components in animal feeds (e.g. Norris et al. 1976; Brown et al. 1990; Coleman and Murray 1993). Components measured include total nitrogen, moisture, fiber (neutral and acid detergent fiber and acid lignin), starch, individual sugars, amino acids and plant tannins. To give but one example of the potential time and cost benefits of using NIRS to analyze such components, Jin et al. (1994) used NIRS to simultaneously measure concentrations of sucrose, glucose, fructose, citric acid, malic acid and vitamin C in intact strawberries. NIRS gave a similar accuracy to conventional HPLC techniques, but with the advantage of near-instantaneous, non-destructive and chemical-free analysis. The prediction of crude protein in grain remains the most common application of NIRS in agricultural industries but plant nutrients and carbohydrate fractions have been successfully predicted in a range of different shrub and tree leaves as well (Meuret et al. 1993; Martin and Aber 1994).

Plant secondary metabolites and anti-nutritional components

Components of several PSMs such as the hydroxyl groups of plant phenolics, have significant absorbances in the NIR region and successful calibrations have been developed for these constituents. For example, Windham et al. (1988) predicted the content of "total tannin" (Folin-Denis assay of 50% methanol extractives) with a co-efficient of determination of 0.91 in

Lespedeza cuneata. Given the errors of conventional laboratory assays for these poorly defined compounds these levels of accuracy may well be the best achievable. However, there is no reason to suspect that other types of tannin analyses would not yield calibrations that are satisfactory for most ecological studies (Roberts et al. 1993).

Less work has been directed at other PSMs. Clark et al. (1987a) developed a robust calibration to predict total alkaloids in larkspur (*Delphinium occidentale*) and lupine (*Lupinus leucophyllus*). Both morphine and nicotine have been predicted with a high degree of precision in poppies and tobacco (McClure and Williamson 1988), and glucosinolate concentrations have been reliably measured in rapeseed (Mika et al. 1997). In many of these studies, examination of spectral characteristics has revealed strong relationships between functional groups and specific wavelengths, potentially a valuable means of screening samples for the presence of these groups.

The wide range of plant types and chemistries involved in these examples gives confidence that NIRS can be used successfully to analyze large numbers of samples for PSMs. However, all these examples are of organic compounds and the ability of NIRS to predict functional aspects of PSMs such as astringency or protein-precipitating capacity is unknown.

Given the clear potential of NIRS to investigate the chemical composition of plants, we believe the technique has a wide application for investigating many ecological questions. Examples where NIRS might be particularly useful is in understanding the effects of resource availability on plant growth and chemical defense, the role of nutrition in influencing the population dynamics of herbivores and in understanding the specific foraging patterns of animals in relation to the nutritional heterogeneity and complexity of their surroundings.

Mineral analysis

The estimation of mineral elements by NIRS is usually dependent on the occurrence of those elements in either organic or hydrated molecules (Clark et al. 1987b; Vasquez de Aldana et al. 1995). For example, key wavelengths used in detecting Mg were similar to the peaks of the chlorophyll spectrum and Ca peaks were similar to those of pure calcium pectate which may be a component of cell walls. Mineral analysis studies have suggested that K, P, Mg, Ca, S, Al and possibly Si exist in forms detectable by NIRS at least in some grasses and legumes (Clark et al. 1987b; 1989; Saiga et al. 1989).

Despite the reports of many satisfactory calibrations for mineral elements, it is difficult to apply their results to other plants because the form of organic molecule-mineral compounds can vary seasonally or among species and genera. This may lead to unstable calibrations, inconsistent results and difficulty in expanding calibrations beyond a well characterized population. For example, phosphorus in plants exists mainly in organic forms frequently as phytate, phospholipids and nucleic acids. The proportion of total P in this form varies between closely related cereals and forages and also seasonally (De Boever et al. 1994). Calibrations obtained for total P in forages were considered acceptable by Clarke et al. (1987b) but were not successful in similar studies by Vasquez de Aldana et al. (1995). Caution needs to be exercised when using NIRS to analyze mineral elements. However, the technique can be useful for broad first approximations and for selecting samples for more accurate analyses.

Soil analysis

Due to the large inorganic fraction of material in soil, NIRS has not been as widely used for soil analysis (Mayer 1989). However from the few studies for which there are data, it is evident that at least for carbon and nitrogen, calibration equations in size specific soil fractions can yield an accuracy similar to that recorded for plant tissues (Morra et al. 1991).

A potential application of NIRS to soil biologists is its ability select soil samples from populations to maximize the variation in particular soil properties in a minimum subset of samples from the population (Stenberg et al. 1995). This is a powerful application of NIRS, as it allows one to select samples over the full range of variation in any one or combination of parameters. In this way, extreme or peripheral samples may be chosen which might otherwise fail to be represented in a sample set for analysis. In addition, a specific distribution of samples can be chosen in reference to a particular parameter. Soil parameters used by Stenberg et al. (1995) included clay content, cation exchange capacity, base saturation and pH . Cost savings of 70% were achieved when the technique was compared to the most appropriate sampling strategy involving only wet chemistry analysis.

Prediction of functional attributes

NIRS has enormous potential as a holistic tool for investigating natural systems. Although reductionist-type approaches are useful in understanding how individual components of a system function, what is often more desirable is an understanding of how these connected parts function as a whole. For example, at times the prediction of chemical composition may be the ultimate aim of the analyst. However, in many situations ecologists are interested in more complex attributes and are constrained to use compositional measures as proxies for these more complex phenomena. Examples of complex attributes which have been examined using NIRS include features such as the susceptibility of plants to insect attack (Rutherford and Van Staden

1996), the yield of pulp and biological degradability of sawlogs (Wright et al. 1990, Hoffmeyer and Pedersen 1995) and the prediction of the nutritional quality of wild foods for free-ranging herbivores (McIlwee, Lawler and Foley unpublished).

Understanding the organization of complex systems is an important objective of ecology. However, because there is an almost unlimited variety of ways that biotic and abiotic components can be assembled into complex ecological systems, our understanding of the structure and dynamics of these systems has remained inherently limited due to traditional approaches that have thus far dominated ecology (Brown 1997). Many systems are inherently non-linear which limits our ability to make ecological predictions. However, much time and effort can be saved from looking at these systems from a holistic approach and deciding whether they are in fact capable of being modeled. NIRS offers the potential for a shift towards whole system empirical modeling in several areas of ecology. This methodology can still be used alongside more formal experimental approaches to generate new insights into how complex systems function.

We see the prediction of functional attributes as being one of the most important applications of NIRS in ecology. Any attribute that is suspected of being influenced by the composition of plant or animal material could potentially be modeled in this way. As well as aspects of animal performance, NIRS could also be used to measure attributes of plant performance, such as innate mean relative growth rate (see Cornelissen et al. 1996). Although this is partly an empirical approach, it is similar to the many successful bioclimatic models that have been used to predict the limits of animal distribution. Below, we provide a range of examples which illustrate the power of NIRS.

Insect resistance

A good example of this approach is in studies of the resistance of different sugarcane cultivars to attack by stem borers. Rutherford and Van Staden (1996) developed a MLR model of NIR data to predict resistance of sugarcane cultivars to *Eldana saccharina*. They found that they could account for 54% of the variation for resistance but, more significantly, wavelengths in their model indicated that alcohols and carbonyl components of the stalk surface wax correlated with an important part of this resistance. In this case, NIRS became not just a tool to build an empirical model but indicated an underlying mechanism that could be further investigated. This approach can save substantial time in targeting a research program to enhance resistance.

A second example where NIR has been used to predict a poorly defined attribute is the variable susceptibility of *Eucalyptus* trees to insect attack. Generally this is caused by variations in both primary and secondary chemistry of the foliage (Edwards et al. 1993) but the most important features have not yet been identified. However, it was still possible to develop a model to account for 88 % of the variation in resistance to defoliation of individual trees based solely on the spectral characters of the foliage (W. Foley, P. Edwards, W. Wanjura and M.Matsuki unpublished).

Prediction of animal performance

Many studies postulate differences in the nutritional quality of the diets consumed by animals as a basis of different population densities, social organization and evolutionary success. However, testing these hypotheses is limited by an inability to measure "nutritional quality" in free-living animals (Hanley 1997). Nutritional quality has most often been estimated indirectly by measuring the concentration of nutrients including nitrogen and fiber and PSMs such as condensed tannins. In most cases, whether tannins affect the intake or digestibility of foods in these species, or whether the crude protein is in a usable form or not is not known. In fact, crude measures of plant composition tells us little about how well a particular food will sustain an animal.

Nutritional quality is reflected in two parameters that can be measured in feeding experiments with captive animals; the level of food intake and the digestibility/metabolizability of that food. These estimates have been made by NIRS using both samples of the food being eaten (Redshaw et al. 1986; Givens et al. 1991; 1992) or alternatively from samples of the feces excreted (e.g. Lyons and Stuth 1992). Prediction of diet quality from feces samples means that it is the actual diet chosen by the animal that is being evaluated, not that which the ecologist decides is being eaten. Brooks et al. (1984) developed a number of calibrations for use with both food and feces samples of white-tailed deer but most innovations in this area have been from Stuth and co-workers (Lyons and Stuth 1992; Leite and Stuth 1994).

Use of feces samples to predict the quality of diets ingested by free-ranging herbivores means that the researcher need not know what the actual diet ingested by the animal comprised. Examination of feces has also been used to examine the digestive efficiency of herbivores on range of natural diets through a simple comparison of difference spectra between diet and feces (e.g. Coleman and Murray 1993). Although there are several assumptions that need to be addressed using this method, namely the effects of microbial residues and sloughed animal tissue, it does provide a simple and potentially valuable means of evaluating the nutritional value of foods and looking at the associative effects of food ingestion and nutrient assimilation.

When combined with geographic information systems, estimates of the nutritional quality of herbivore diets can become powerful tools for monitoring pastoral degradation at many scales. For example, Stuth (personal communication) has used NIRS to predict diet quality from feces samples of cattle and deer and by plotting the position of each feces sample has identified differences in the quality of different habitats to support these herbivores. Knowledge of the spectral stability of feces exposed to field conditions adds to its power as a management tool (Leite and Stuth 1994).

Isotope discrimination - water use efficiency and herbivore diets.

Stable isotopes are used in many areas of ecology, including estimation of water use efficiency, separation of C4 and C3 plants and in studies of foraging and food webs. Modern isotope ratio mass spectrometry (IRMS) is highly accurate and precise, it is relatively expensive, laborious and time consuming. NIRS may be able to predict relative abundances of ^{13}C. For example, Okano et al. (1993) found that NIRS could predict abundance of ^{13}C in a range of C3 and C4 plants to within 97% of measured values. This degree of accuracy could be sufficient for many studies of herbivore foraging where the aim is to measure the proportion of tropical grass in the diet.

Clark et al. (1995) used NIRS to predict ^{13}C discrimination in a range of grasses and legumes so that they could select genotypes with high water use efficiency (WUE), based on the well established relationship between water use efficiency and ^{13}C carbon ratios in plants (Salisbury and Ross 1992). They found that NIRS could identify up to 82% of samples with low WUE, and concluded that accuracy may be sufficient to identify promising genotypes in the early selection stages. Clearly NIRS is not expected to replace IRMS as a means of determining isotope abundance but we believe that NIRS may have a role in rapidly screening a large number of samples of plant tissues so that the more expensive analysis can be targeted on those samples that are of interest.

Litter decomposition studies

Mineral cycling in forest and aquatic ecosystems has been investigated using NIRS. The chemical composition of leaf litter is a key factor regulating its rate of breakdown and the speed and non-destructive nature of NIRS has been used by several groups. McClellan et al. (1991) showed that total N, C and ash could be accurately measured by NIRS as well as several components of plant cell walls. Following on from this, Gillian et al. (1993) showed that NIRS was able to directly predict the stage of decomposition (expressed as the percentage of ash-free litter mass remaining) without relying on indirect estimates based on chemical composition.

Cornelissen and Thompson (1997) provide a range of potential functional attributes of living leaves (for both monocots and dicots) which can be used as a basis for predicting the decomposition rate of leaf litter. Such attributes could be used by people using NIRS to examine the impact of anthropogenic activities and environmental changes on decomposition processes.

Limnology and freshwater ecology

NIRS has been used to measure C, N and P in suspended sediments and in seston by collecting the material on glass-fiber filters and then obtaining NIR spectra (Malley et al. 1993; 1996). Agreement between the NIRS measures and those obtained by traditional methods was excellent in both cases. However, the use of NIRS is not restricted to these types of compositional assays. Korsman et al. (1992) and Nilsson et al. (1996) have shown that NIRS can be used to predict the past chemical composition of lake water from models of the surface sediments and current water chemistry. A record of the current water chemistry is contained in the surface sediments and once a relationship is established between these parameters, past histories can be evaluated by taking further sediment samples. Nilsson et al. (1996) found that they could predict 83 to 85% of the variance in total phosphorus and pH, respectively, and 68% of the variance in total organic carbon. This approach could become a powerful tool for monitoring past events in water chemistry.

Biochemical markers and rates of biological activity.

NIRS can be used to detect biochemical markers, such as chitin (Roberts et al. 1994) which is used to estimate levels of fungal infection in crops and pastures. Direct estimates of fungal infection have also been made by NIRS (Roberts et al. 1988). Dysprosium (Dy), a pulse-dosed marker used to measure rates of digesta flow in herbivores has also been successfully measured using NIRS in studies on cattle (Reeves and Glenn 1995). Although NIRS was capable of determining Dy in labeled forages, interference by a second co-marker limited the accuracy of predictions.

In addition to detecting markers, NIRS also has the potential to measure rates of biological activity. For examples Hall et al. (1991) used NIRS to measure bloodmeal size and its disappearance rate in live disease-carrying mosquitoes, NIRS has been used as an non-intrusive means of monitoring changes in blood composition (Hinckley et al. 1995, Delpy and Cope 1997) and Palmborg and Nordgren (1993) applied NIRS to the measurement of microbial biomass and activity of forest soils.

Analyses of high-moisture materials- prospects for analysis of fresh samples.

Although some of the earliest applications of NIRS involved analysis of fresh meat samples, most applications currently involve dry and ground materials. Even this limited sample preparation increases analysis time and cost substantially. The imbalance between the number of applications for dried and fresh samples discredits the real advantages offered by the direct analysis of fresh materials.

Samples which contain substantial quantities of moisture present particular difficulties for NIRS. The hydrogen bonds in water absorb significant amounts of NIR radiation and results in broad peaks that obscure spectral information derived from other compounds (Abrams et al. 1988). Wet samples tend to be more heterogeneous and moisture can be expressed from the sample when it pressed against the glass window of the sample cell. A major difficulty is to generate samples for instrument standardization that will not degrade over time. Currently this is a serious weakness in applying NIRS to high-moisture materials (Berding and Brotherton 1996).

There is no single solution to these issues and each case has to be treated individually. The difficulties are not insurmountable since many robust calibrations have been developed for moist materials including dairy products, fish, fresh, intact fruits and vegetables, meats and sugarcane (Blosser and Reeves 1986; Mathias et al. 1987). In calibration development for example, use of high absorbance spectral regions characteristic of high moisture materials are best avoided because of the non-linearity associated with these regions.

Emerging applications: Challenges and prospects for the future use of NIRS.

Use of NIRS as a discriminant tool - chemotaxonomy and beyond

Ecologists often wish to classify elements of populations into groups for a range of different reasons, even if it is as simple as determining whether an animal is one sex or another. NIRS has been successfully used in this fashion to predict whether pupae are male or female (Jin et al. 1995) and to predict whether individual seeds or grains have been infected by larval insects (Ridgway and Chambers 1996). In such applications, the spectral data is often acquired by fiber optic probes that can be focused on very small targets such as single insect larvae.

NIRS is also finding use as a tool for discriminating among products from different provenances or of different species. The wealth of data available in NIR spectra means that the digitized spectra are particularly suited to the variety of multivariate statistical procedures that are typically used in discriminant analyses. For example, Schimleck et al. (1996) acquired the NIR spectra of a range of timber samples. They were able to build models based on principal components analysis of NIR spectral data that successfully discriminated between woods from *Eucalyptus* and *Pinus*, between woods of different species of *Eucalyptus* and between woods of the same species of *Eucalyptus* grown at different sites. Other similar applications have involved identifying adulterated food products, such as contamination by fungi (Davies et al. 1987) or determining the infection levels of diseases such as anther smut disease in crops (Nilsson et al. 1994). These approaches can easily be transferred to ecological applications.

Discriminant analyses are very common in ecology and typically require substantial amounts of chemical or physical measurements to be successful. Collecting these can be time-consuming and expensive and we believe that many ecological applications could profit from using NIRS data as an input to discriminant procedures.

On-line processing and portable and hand-held NIRS

Most NIR spectrophotometers that are currently used for research are dedicated laboratory instruments which are housed in carefully controlled conditions. However, the greatest use of NIRS is as an on-line analytical tool in industrial plants ranging from sugar mills and wheat silos to pharmaceutical manufacturing plants (for examples see Bellon and Boisde 1989, Osborne et al. 1993; Schulz and Losing 1995). In these environments the instrumentation is exposed to much more rugged conditions and usually involved in the analysis of fresh, intact samples. This provides the potential for more exposed environmental monitoring.

In the future we can expect NIR instruments to be become smaller and more portable. There are already a number of hand-held instruments that have been used to measure the nitrogen concentration of the leaves of crop plants as an aid to fertilizer management. For example, Blakeney et al. (1996) successfully used a hand-held battery operated instrument for nitrogen measurements in rice leaves.

In the past year, portable instruments that can scan the whole NIR spectrum have become available. These instruments use a variety of fiber optic probes and coupled with the speed of advances in optics and micro-processor technology should lead to even more robust portable instruments in the future that offer ecologists the possibility of real-time analyses of plant and animal composition in the field. In particular we could expect closer alliances to be formed between those interested in traditional airborne remote sensing and those interested in finer resolutions as well as advances in statistical processing which will allow a better description of spectral features.

CONCLUSION

Although we are very optimistic about the future of NIRS, we conclude with the warning that NIRS cannot turn poor analysts into good ones. Used uncritically, it can allow poor results to be propagated. Ultimately, the quality of NIRS based predictions depends entirely on the quality of the measurements used to generate the statistical model and this must guide any analytical endeavour.

NIRS offers several special advantages such as speed of determination, minimal or no preparation of sample, non-destructive analysis, no consumption of reagents, and low costs of analysis. However, the capital cost of NIR spectrophotometers is high (ca $US40-80,000) so ecologists might first consider developing collaborations with existing users of NIRS in agricultural industries or research stations where the technique is widely used. In this way, they can evaluate the usefulness of the method for particular samples and analyses before committing to purchase. As NIRS moves forward into the future, we look forward to witnessing the integration of the technique into applied ecology.

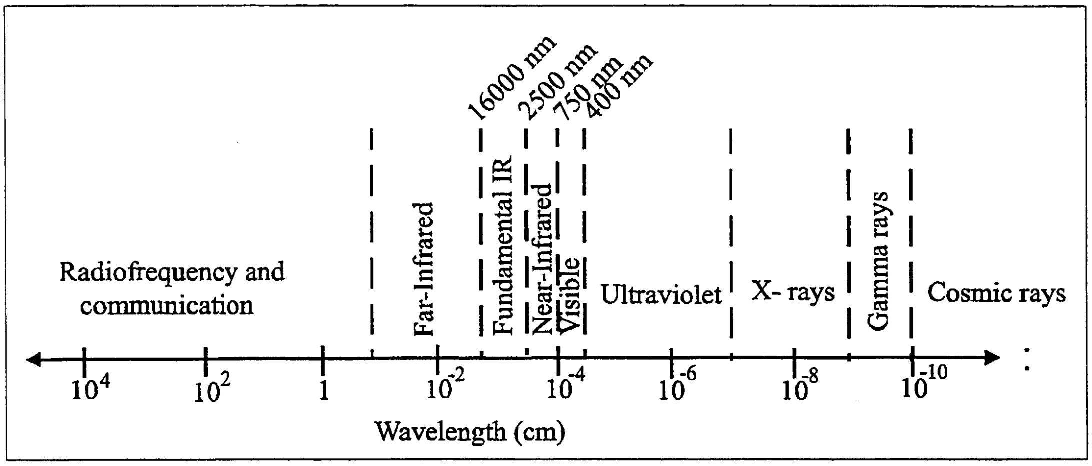

Figure 1. The electromagnetic spectrum showing the position of near-IR.

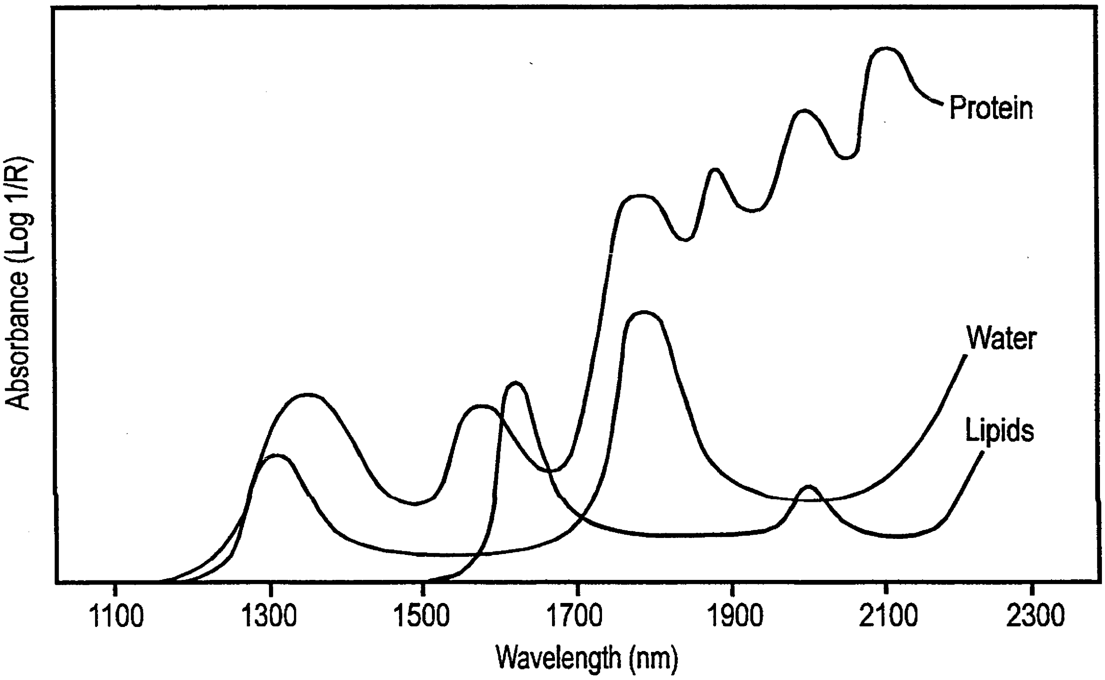

Figure 2. Broad and overlapping peaks attributable to different constituents are characteristic of a near infrared reflectance spectrum (adapted from Osborne et al. 1993; Givens et al. 1997).

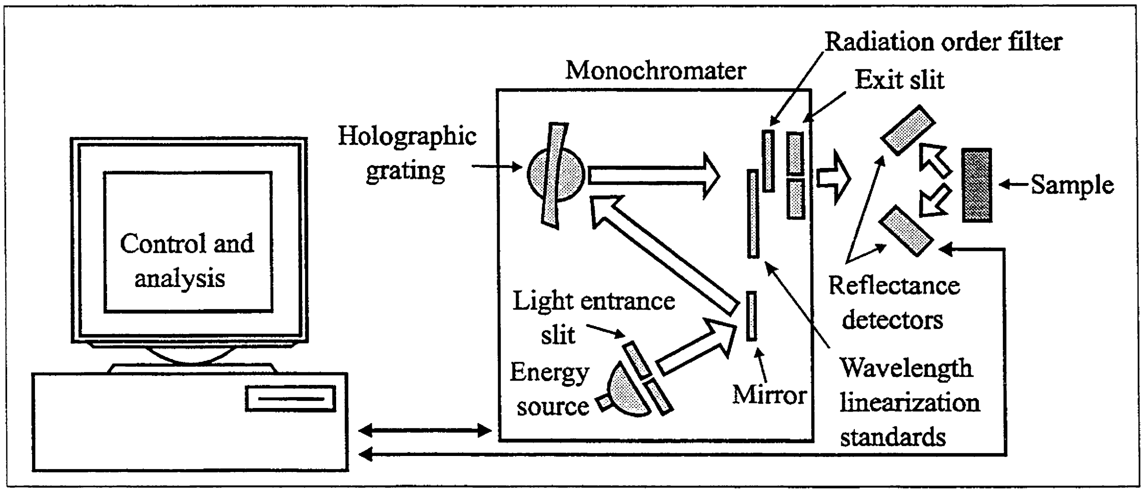

Figure 3. Components of monochromator-based near-IR spectrometer

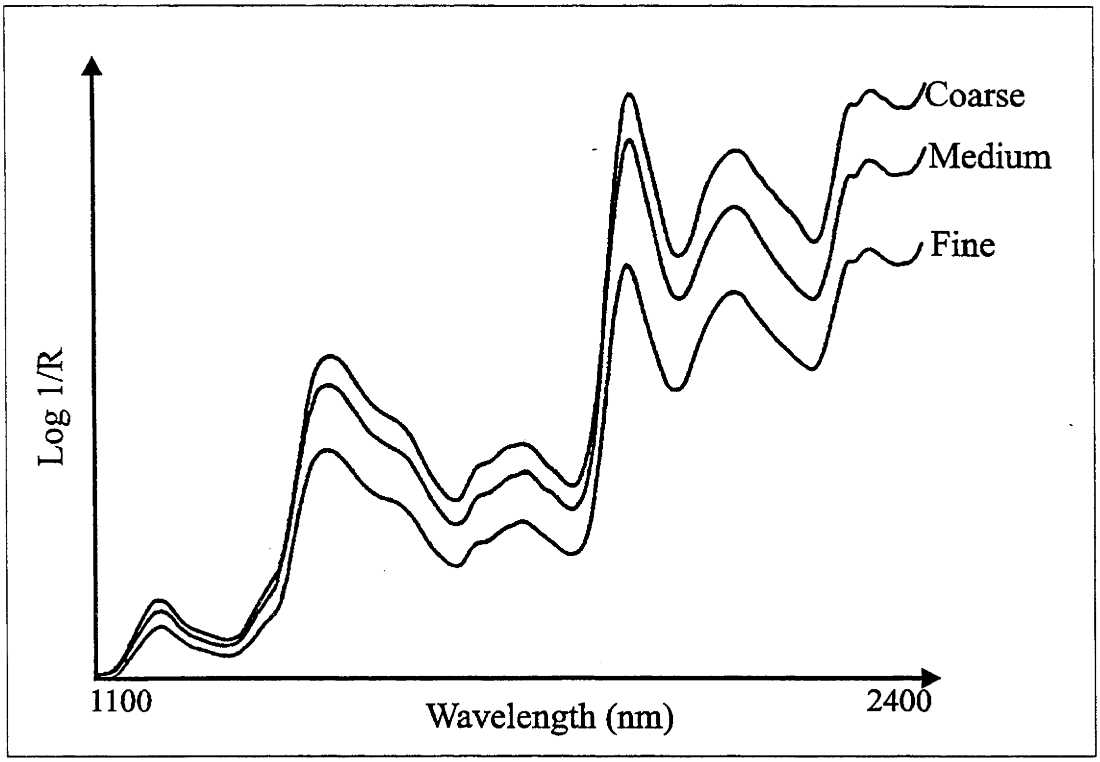

Figure 4: The effect of particle size on spectral absorbance in the near-IR region. (adapted from Shenk et al. 1992)

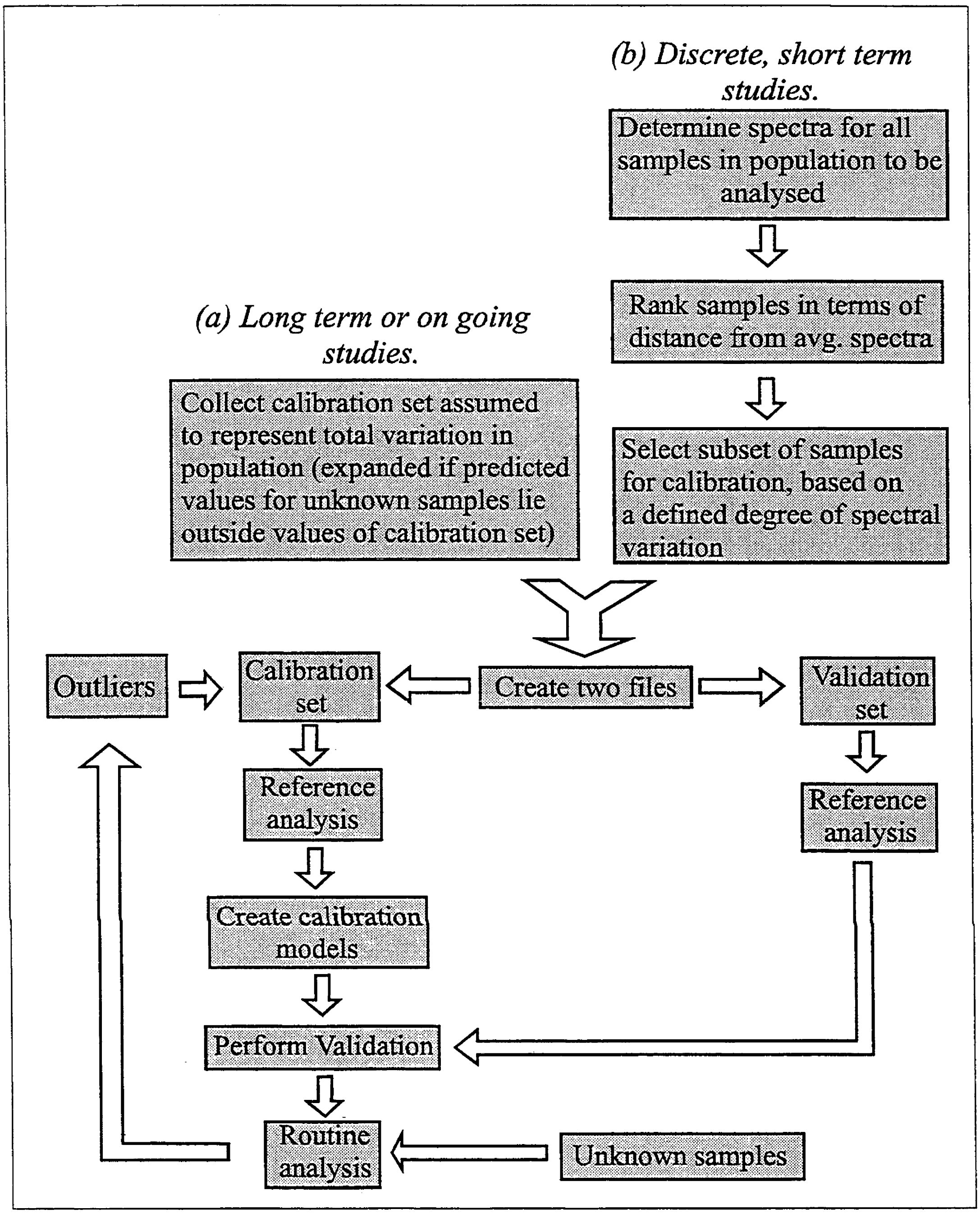

Figure 5: Alternative strategies for the development of calibration equations needed for the routine analysis of unknown plant or animal samples

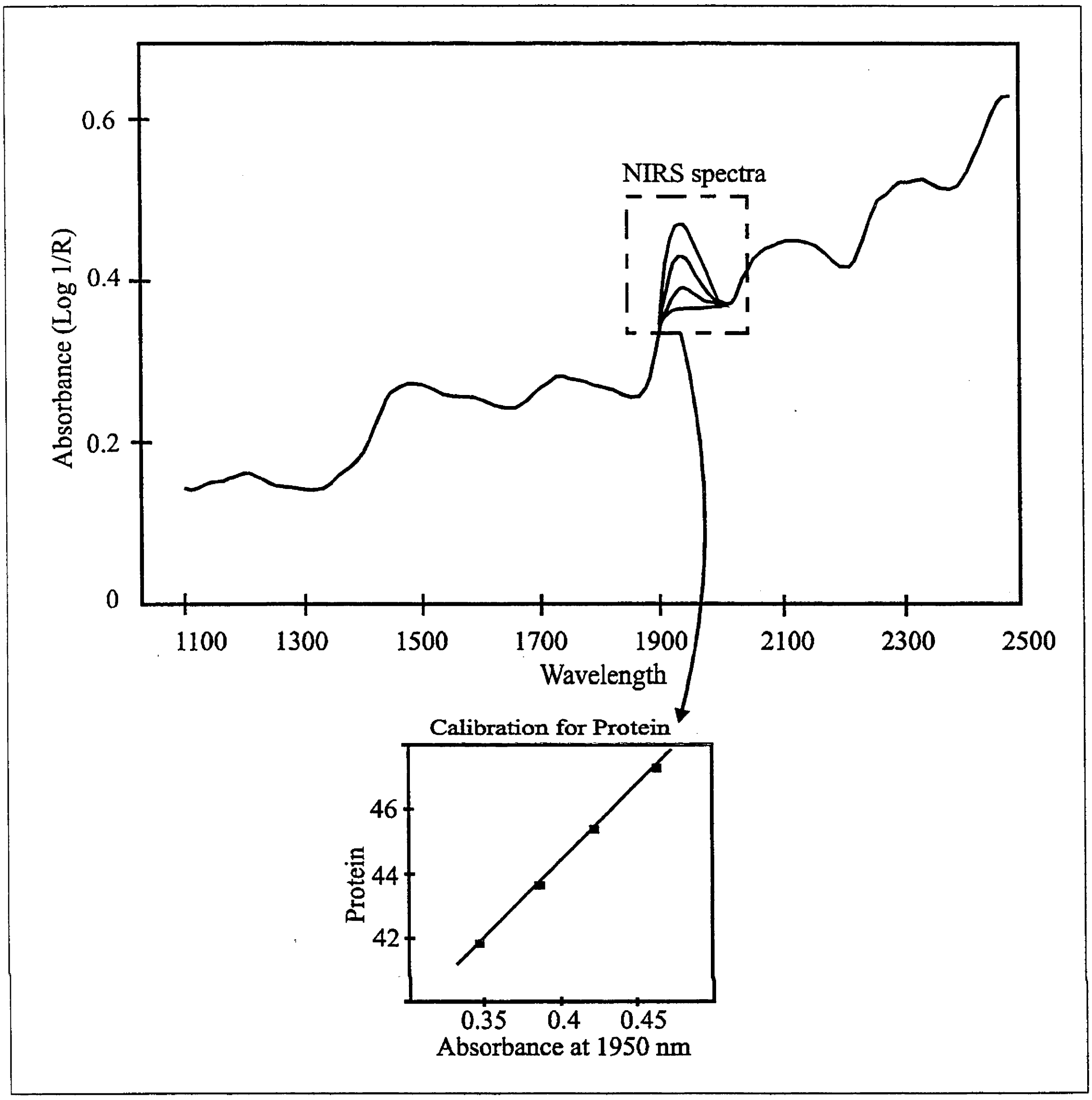

Figure 6. Fictitious example illustrating how spectral information is correlated with analytical information in order to develop a calibration equation (adapted from Van Kempen and Jackson 1996).

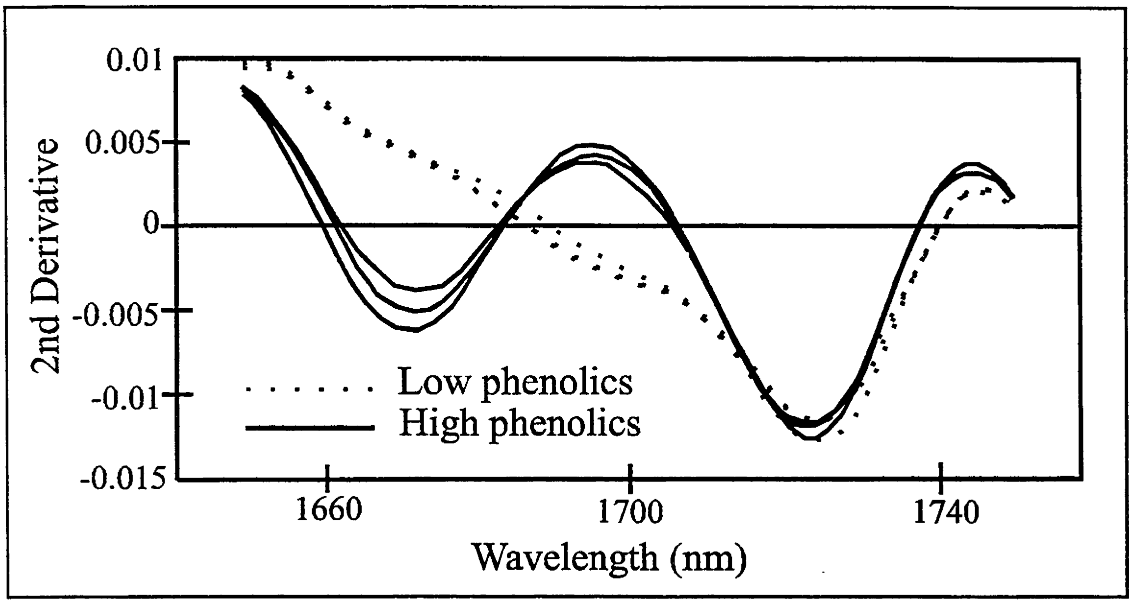

Figure 7: Example showing the allocation of wavelengths to specific chemical bonds (taken from Flinn et al. 1996).

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