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*) for 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 from 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 (total nitrogen, cellulose, hemi-cellulose, lignin, in vitro dry matter digestibility) and plant secondary chemistry (total phenolics, condensed tannins, cyanogenic glycosides, 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 undertake 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, while 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.

Key words: condensed tannin; Eucalyptus; *habitat patchiness; herbivory; intraspecific variation; phenolic;* Pseudocheirus peregrinus; *ringtail possum; sideroxylonal; terpene.*

INTRODUCTION

Patchiness in animal habitats is widely recognized as an important determinant of animal foraging. In studies of vertebrate foraging, patches are most often recognized in terms of the quantity of food available (Laca and Demment 1991). In contrast, the quality of the food resource is often harder to characterize, 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, Rei-

chardt 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. Failure to recognize, and measure, such variation in field studies may lead to an inability to address the questions of interest. If some individual plants of an important food species are well defended against herbivores while neighboring conspecifics in the same area are not, measurement of leaf chemistry of only one or few individuals of each plant species may severely over- or under-estimate food availability and thus affect the interpretation of the resulting data.

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 solely 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 her-

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bivores 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) which group all compounds in the sample that bear the assayed functional group. These measures are insufficient to capture the wide variation in deterrent activity due to subtle differences in molecular structure between compounds sharing the same functional group (Clausen et al. 1986, Waterman and Kool 1994).

2) Whatever our knowledge of relevant chemical constituents, field studies of foliage chemistry are limited to collections made at the scale of tree species 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, 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. 1998*a*). 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 that 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 were caught by hand in woodland dominated by *Eucalyptus* near Canberra, Australia and maintained on foliage from *E. polyanthemos* and *E. rossii* collected locally. The possums were housed individually in metabolism cages (0.6 m wide × 0.6 m high × 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 60-W incandescent bulbs during the day and by a single 40-W red incandescent bulb at night. Common ringtail possums are strictly nocturnal so food was offered at 1800 and removed between 0500 and 0800 the next day, depending on the particular experimental protocol.

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. 1998*a*). 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) was calculated using appropriate controls for evaporation. In this experiment food was offered at 1800 and removed at 0800. In order to reduce carryover effects and to ensure the welfare of those animals that fed little, 30 mL of 10% (mass/mass) 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 analyzed by analysis of variance (Ratkowsky et al. 1993). Note that in subsequent experiments the protocol differed, as detailed below and in Lawler et al. (1998*a*).

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 was measured after the experiments were conducted and their importance in determining feeding rates was 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. 1998*a*). Therefore, having shown in the preliminary experiment that there was an eightfold 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 nonexperimental 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 (until 0800 h) 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 analyzed 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 (R. Cunningham, *personal communication*). All intake data are expressed as grams of dry matter per (kilogram of body mass)$^{0.75}$ per day 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 of scaling of metabolic rate with body size as described by Demment and Van Soest (1985).

Analysis of foliage

On each day of the experiment, a control sample of foliage (to account for evaporative loss) 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. (1998*a*): nitrogen, cellulose, hemi-cellulose, lignin, cyanogenic glycosides, total polar phenolics, and con-densed 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 analyzed by gas–liquid chromatography for identification and quantification of individual terpenes (Edwards et al. 1993), including cineole, the predominant terpene in these species (Boland et al. 1991).

Sideroxylonals were extracted from 3 g of freeze-dried and ground leaf for 13 h using a Soxhlet apparatus with 125 mL of 20:80 (volume/volume) mixture of acetone and light petroleum spirit (40°–60°C boiling point). After extraction the solvent was evaporated off and ~10–15 mg of extract dissolved in methanol for high-pressure liquid chromatography (HPLC) analysis. Sideroxylonal concentrations were estimated by HPLC using a Waters Novapak C18 column (Waters, Milford, Massachusetts, USA) (3.9 × 1.5 mm) at a flow rate of 1.0 mL/minute using a solvent of 95% methanol, 4.9% water, and 0.1% triflouroacetic acid. Absorbance was measured at 275 nm. Although two stereoisomers, sideroxylon A and sideroxylon B, were resolved and quantified, sideroxylon B was present at concentrations <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 2-mm sieve and 1-kg batches were 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 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 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 (Pharmacia, Uppsala, Sweden) 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 (B. M. Eschler and W. J. Foley, *unpublished data*).

Correlative analysis of leaf experiments

Relationships between DMI for foliage from each tree and chemical characteristics of foliage 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 parameterization 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 ringtail possum food intake

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, as a percentage of wet matter, of 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 Weetabix (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 the 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 6×6 Latin square with treatment days alternating with nontreatment days on which only the basal diet was offered. On treatment days animals were offered the basal diet at 1800 to which was added one of six concentrations of sideroxylonal, and this was removed and replaced by untreated basal diet at 0500. To determine DMI of the animals the dry matter content of the diet offered was determined by subsampling the food offered, and the dry mass 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. This site was located near Queanbeyan in New South Wales,

adjacent to the Australian Capital Territory. The area surveyed was 100×50 m 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 individual trees 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 principles of near infrared reflectance spectroscopy (NIRS) are summarized 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. 2000).

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 minimize the interference of residual moisture with the near infrared (NIR) spectra of the samples. After cooling to room temperature ($\sim$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 (Foss NIR Systems, Silver Springs, Maryland, USA).

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. 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 et al. 1989). Optimal calibration was achieved by using the second derivative of the spectra with a gap size for its calculation of 10 nm. The range of wavelengths used
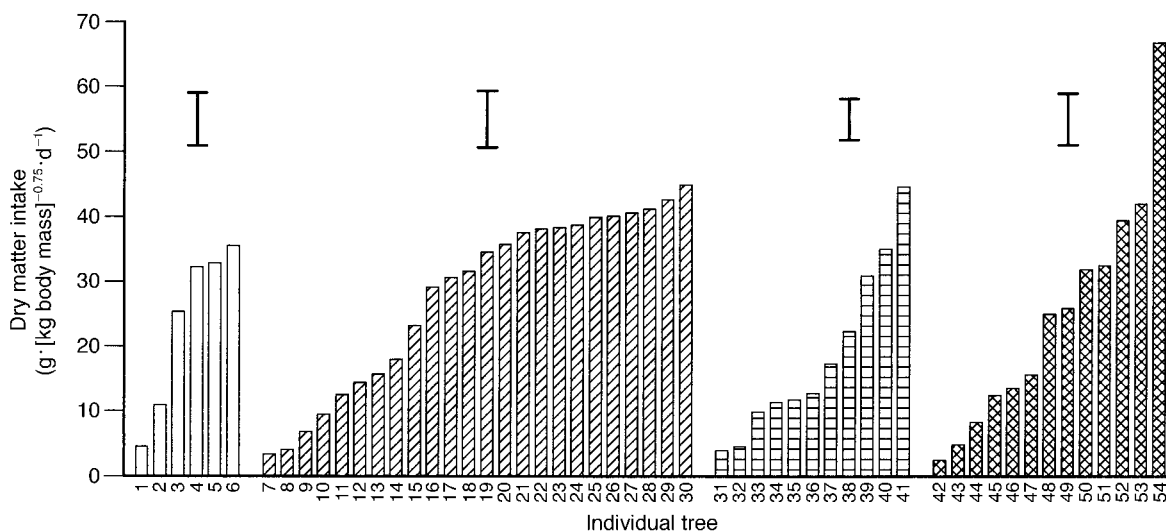
FIG. 1. Variation in dry matter intake by common ringtail possums fed *Eucalyptus polyanthemos* or *E. sideroxylon* foliage in no-choice experiments. Unshaded bars are *E. polyanthemos* from the preliminary experiment, diagonally shaded bars are *E. polyanthemos* from alpha design with 24 trees, horizontally shaded bars are *E. polyanthemos* from a Latin square design with 12 trees, and heavily shaded bars are *E. sideroxylon.* Vertical bars above each group of data show the magnitudes of least significant differences from ANOVA or REML analysis.

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. 1) 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 3.28–44 g·kg$^{-0.75}$·d^{-1} for *E. polyanthemos* and 2.4–67 g·kg$^{-0.75}$·d^{-1} for *E. sideroxylon* (Fig. 1). 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 char-

acteristics 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 (a terpene), significantly explained the decline in DMI seen across trees within species (Table 1, Figs. 2 and 3). 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),

TABLE 1. Summary of linear regressions of the relationships between dry matter intake (DMI) by common ringtail possums fed *E. polyanthemos* or *E. sideroxylon* foliage and chemical composition of the foliage and the relationship between chemical components. Parameters are presented with standard errors in parentheses.

Independent variables	Dependent variable	Regression equation	r^2
Sideroxylonal only	DMI	DMI = 37.60(1.64) − 6.55(2.47) × Es − 2.42(0.21) × [sideroxylonal]	0.75
Cineole only	DMI	DMI = 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

Notes: 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 a value of 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 that combine data for both species.
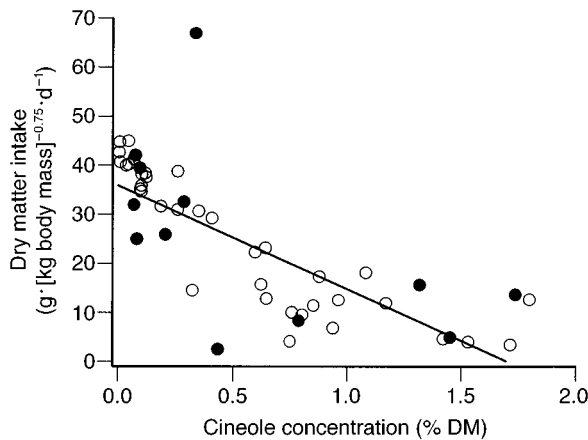
FIG. 2. Relationship between dry matter intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage (open circles) or *E. sideroxylon* foliage (closed circles) and cineole concentration of foliage. The regression equation (solid line) was not statistically different for the two species (equations summarized in Table 1). Note that tree no. 54 has been omitted from calculation of the regression equation (see *Results*).
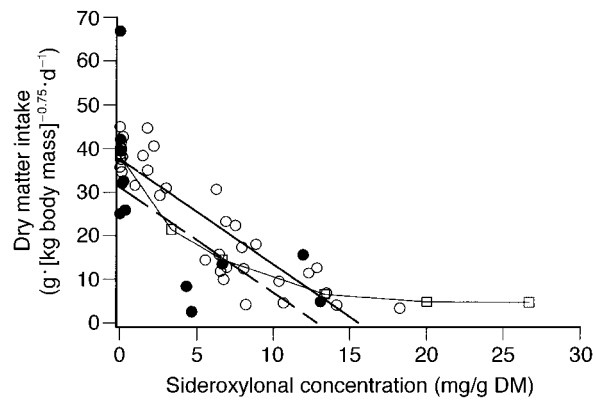

FIG. 3. Relationship between dry matter intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage (open circles), *E. sideroxylon* foliage (closed circles), or artificial diet with sideroxylonal added (open squares) and sideroxylonal concentration of foliage. The regression equations had the same slope but different intercepts for *E. polyanthemos* (solid line) and *E. sideroxylon* (dashed line) (equations summarized in Table 1). Again tree no. 54 was omitted from these calculations. Values for artificial diet are joined with lines to identify their positions more readily.

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. 4). 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. 3). 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. 3). 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:

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

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

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

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. 5) 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:
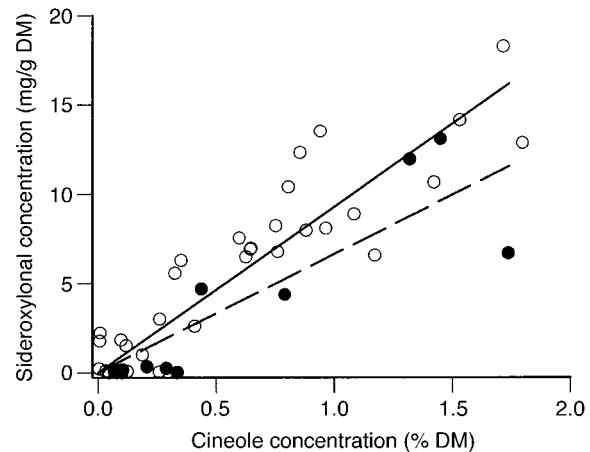

FIG. 4. Relationship between cineole and sideroxylonal concentration for *Eucalyptus polyanthemos* foliage (open circles; regression solid line) or *E. sideroxylon* foliage (closed circles; regression dashed line). Regression equations are summarized in Table 1.
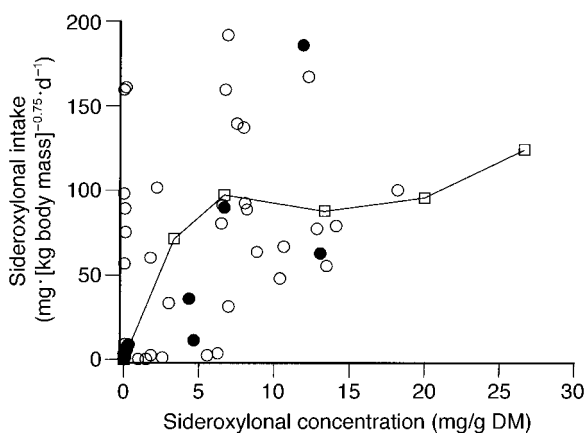
FIG. 5. Relationship between sideroxylonal intake of common ringtail possums fed *Eucalyptus polyanthemos* foliage (open circles), *E. sideroxylon* foliage (closed circles), or an artificial diet with sideroxylonal added (open squares) and sideroxylonal concentration of foliage.

$$DMI = 1 / (0.0094 (7.1 \times 10\text{--}4) \times [\text{sideroxylonal}])$$

or

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

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. 3). 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 >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).

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. 6a). Similarly, the potential DMI estimated by NIRS also varied markedly between individual trees (Fig. 6b). Foliar sideroxylonal concentrations ranged from zero to 12.6 mg/g, corresponding to a range of predicted dry matter intakes from 42.8 to zero g·(kg body mass)$^{-0.75}$·d^{-1}. The frequency distributions of both variables were approximately normal (sideroxylonal $-\chi^2 = 7.96$, 7 df, $P = 0.34$; DMI $-\chi^2 = 7.26$, 7 df, $P = 0.40$). In several cases, trees with high foliar sideroxylonal concentrations were the nearest neighbors of individuals with very low concentrations.

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 DFPCs show antifeedant action at foliar concentrations (Lawler et al. 1998*a, b,* 1999). 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. 1998*a,* 1999) which is the major terpene found in the species used here (Boland et al. 1991, I. R. Lawler, W. J. Foley, and Matsuki, *unpublished data*). In these *Eucalyptus* species, high foliar concentrations of sideroxylonal

TABLE 2. Summary of regression equations describing the relationship between dry matter intake (DMI) by common ringtail possums fed *E. polyanthemos* foliage and sideroxylonal content of the foliage.

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

Notes: 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. Model parameters are presented with standard errors in parentheses.
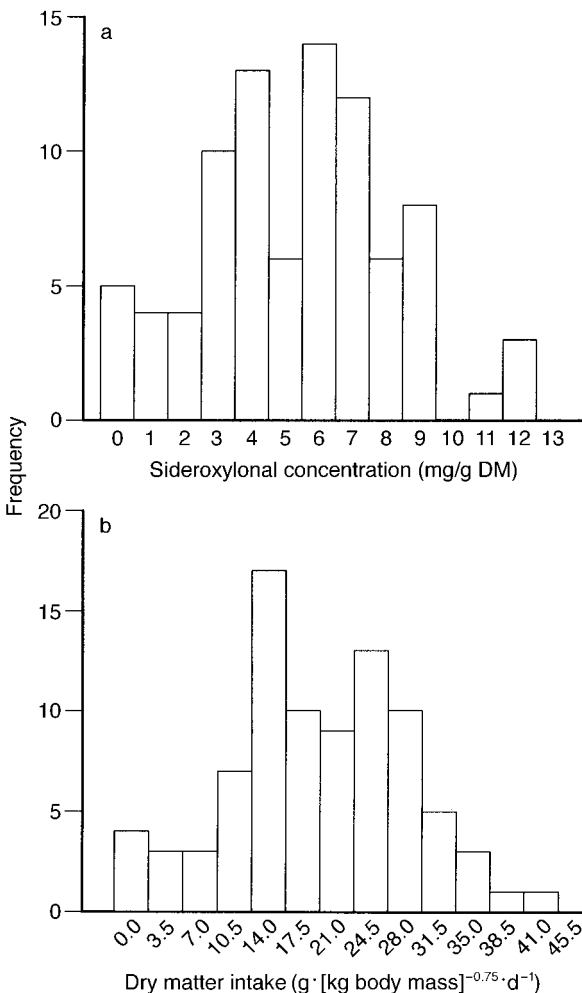
FIG. 6.   Frequency distributions of varying leaf chemistry between individual *E. polyanthemos* trees within one population: (a) foliage sideroxylonal concentrations; (b) near infrared spectroscopic prediction of dry matter intake of common ringtail possums.

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. 1999) 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 DFPCs 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 amounts of each group produced should correspond to each other. 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. 1999) that common ringtail possums and common brushtail possums (*Trichosurus vulpecula*) can develop a conditioned flavor aversion to terpenes via an association between terpenes and postingestive effects of a simple DFPC. We conclude that the 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

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 5HT3 receptors (Lawler et al. 1998b), and are enhanced by the conditioned aversion to terpenes, giving them preingestive feedback (Lawler et al. 1999). The data for the same animals feeding on the artificial diet with sideroxylonal added support to this contention and suggest a sideroxylonal threshold of ~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.

First, 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 5 g 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 postingestive 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 behavior 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 behaviors. Small errors in correcting for these behaviors 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 3 g on the highest sideroxylonal foliage would lead to an error in the sideroxylonal intake of >54 mg, or ~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 first requires the development 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; its utility is only now being recognized 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 analyzed 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 cali-
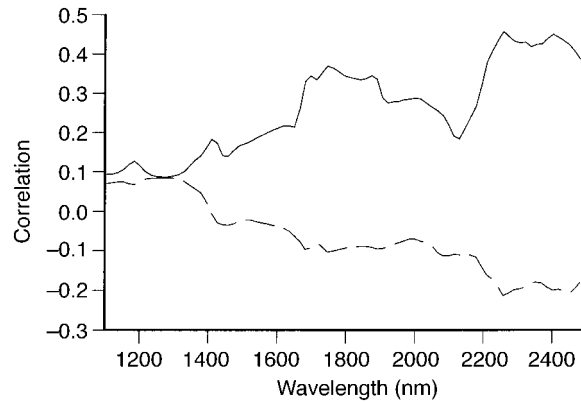
Fig. 7. Correlation between individual wavelengths of near infrared spectra of *E. polyanthemos* foliage and sideroxylonal concentration (solid line) and dry matter intake of common ringtail possums (dashed line).

bration equations are developed, over 150 samples can be scanned and analyzed 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. 2000). A number of studies have shown that other aspects of the plant–animal interaction that are difficult to define chemically (e.g., 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. 2000). 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. 7). Moreover, comparison of coefficients corresponding to each wavelength in the calibration equations for sideroxylonal content and possum intake showed that 6 of the 10 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

southeast 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 of > 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 et al. (1983) and Cork and Catling (1996) showed that for greater gliders 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 individual trees of favored 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 nonpolar phenols such as the DFPCs (I. R. 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 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 experiencing much greater herbivore damage than surrounding conspecifics where the herbivores are ringtail possums (Geritz 1987, Pahl 1987, I. R. 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, nutritionally adequate (and strongly herbivore-deterrent) conspecifics remained (W. J. Foley, *unpublished manuscript*). Measurement of foliar chemistry at the species level (i.e., sampling only a small number of individuals of each tree species) in these cases would have substantially overestimated the abundance of the food resource. 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. 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. First, the nutritional quality of a food for a herbivore is the product of how much food it can eat and its ability to digest or metabolize 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% to 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 among 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 inexpensive 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. 2000). This technique should now be applied to intensive field studies of animal foraging.

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