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CONFLICTING DEMANDS ON DETOXIFICATION PATHWAYS INFLUENCE HOW COMMON BRUSHTAIL POSSUMS CHOOSE THEIR DIETS

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Abstract. Most herbivores eat more and survive better when they have access to a variety of foods. One explanation involves the detoxification of plant secondary metabolites (PSMs). By feeding from a variety of plants that contain different classes of PSMs, animals can use multiple detoxification pathways and presumably consume more food. Although popular, this theory is difficult to test because it requires knowledge of the detoxification pathways of each PSM in the diet. We established that common brushtail possums (*Trichosurus vulpecula*) use various combinations of oxidation, hydrolysis, and conjugation with glucuronic acid (GA) or glycine to detoxify six PSMs. Compared to their ingestion of a single PSM, possums ate more when offered a choice between two diets containing PSMs that require apparently independent detoxification pathways (benzoate and 1,8-cineole, benzoate and *p*-cymene, benzoate and orcinol, benzoate and salicin, or orcinol and 1,8-cineole). However, possums still did not eat as much of these diets as they did of a basal diet free of PSMs. This suggests that detoxification pathways are never independent, but are separated instead by degrees. In contrast, possums offered a choice of two PSMs that require competing detoxification pathways (1,8-cineole and *p*-cymene, 1,8-cineole and salicin, or orcinol and salicin) ate no more than when offered diets containing one of the compounds. There was an exception: even though both rutin and orcinol are detoxified via conjugation with GA, the feeding behavior of possums did not suggest competition for detoxification pathways. This implies that the supply of GA is not limiting. This study provides the first convincing evidence that herbivorous mammals can eat more by selecting mixed diets with a diversity of PSMs that make full use of their detoxification potential. It also emphasizes that other behavioral and physiological factors, such as transient food aversions, influence feeding behavior.

Key words: Australia; common brushtail possum; detoxification limitation hypothesis; diet choice; diet mixing; generalist herbivore; glucuronic acid (GA); glycine; plant secondary metabolites; Trichosurus vulpecula.

INTRODUCTION

Mammalian herbivores are most often generalists, eating a variety of foods (Freeland 1991). Moreover, when mammalian generalists are restricted to a single food, they tend to eat less than when fed a mixed diet (Dearing and Cork 1999, Burritt and Provenza 2000, Wiggins et al. 2003) and, if studies were longer, would probably grow more slowly and have lower survival rates, as generalist herbivorous insects do in this situation (Bernays et al. 1994, Hagele and Rowell-Rahier 1999, Miura and Ohsaki 2004). However, there is no universal explanation for why mixed diets are more suitable than a single plant species for most mammalian herbivores. Instead, varied diets seem important for different reasons, depending on the particular herbivore and plant.

Two prominent explanations for a generalist diet involve the nutritional requirements and detoxification limitations of herbivores. Often, an animal cannot meet its nutrient requirements from a single plant or plant part and must select foods with nutrients that complement each other (Westoby 1978). In contrast, the detoxification limitation hypothesis predicts that the amount of food that a herbivore can safely ingest depends on the rate at which it can detoxify any plant secondary metabolites (PSMs) that the food contains (Freeland and Janzen 1974). It follows that a herbivore should be able to eat more if it selects multiple foods with PSMs whose detoxification requires different rate-limited pathways (Freeland and Janzen 1974).

Although both nutrients and PSMs are likely to influence the foraging strategies of mammalian herbivores, the detoxification limitation hypothesis has gained increasing popularity with ecologists (Dearing and Cork 1999, Foley et al. 1999, Sorensen and Dearing 2003, Wiggins et al. 2003), with only little experimental

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support. We know that many herbivores regulate the quantities of PSMs that they ingest (Lawler et al. 2000, Mangione et al. 2000, Stapley et al. 2000) and that the concentrations of PSMs in plants can restrict feeding (Jakubas and Gullion 1990, Lawler et al. 2000, Wallis et al. 2002). However, animals may eat more of a mixed diet than a single diet due to the variety of flavors and sensations available, rather than the constraints of detoxification pathways. For example, humans eat more when given several different foods rather than the same food multiple times (Rolls et al. 1981), as do rats (Treit et al. 1983), hamsters (DiBattista and Sitzer 1994), and cattle (Ginane et al. 2002). Therefore, in order to separate the effects of food variety from those of detoxification, we must know how an animal detoxifies PSMs.

A detoxification pathway is a series of reactions that transforms a PSM into a product suitable for excretion. Herbivores may use either or both of two phases to detoxify PSMs. In phase I reactions, compounds are hydrolyzed, oxidized, or reduced; in phase II, they are conjugated with compounds such as glucuronic acid (GA), glycine, or sulfate (Katzung 2001). Detoxification of a single compound can involve multiple pathways and can produce many metabolites. In addition, each pathway can process many different compounds (Katzung 2001). Pathways for different PSMs overlap when they use the same enzymes or cosubstrates for at least part of the detoxification process. For example, two PSMs might be metabolized by separate phase I processes, but might both be conjugated with GA in phase II. Saturation occurs when the supply of toxin exceeds the capacity of enzymes or cosubstrates at any point in the detoxification pathway. The detoxification limitation hypothesis assumes that intoxication will then occur, unless the herbivore stops ingesting the toxin.

The common brushtail possum (*Trichosurus vulpecula*) is an ideal generalist mammalian herbivore with which to test the detoxification limitation hypothesis. Brushtail possums eat foliage, fruits, and flowers from a range of species (Freeland and Winter 1975, Nugent et al. 2000). In addition, the detoxification pathways utilized and the urinary metabolites produced by brushtail possums have been identified for several naturally consumed PSMs. We chose six PSMs (1,8-cineole, *p*-cymene, benzoic acid, salicin, orcinol, and rutin) that are found in natural forage. Both cineole and cymene are detoxified by brushtail possums via serial oxidation, and are sometimes conjugated with GA (Boyle et al. 1999, 2000). In contrast, most benzoic acid is conjugated with glycine (Awaluddin and McLean 1985, Marsh et al. 2005). The phenolic glycoside, salicin, is first hydrolyzed to its aglycone and glucose, after which oxidation and conjugation with GA or glycine may all occur (McLean et al. 2001). The other PSMs that we tested, orcinol and rutin, are phenolic compounds for which the detoxification products are unknown in possums. In humans, hydrolysis of rutin, followed by oxidation, produces hydroxyphenylacetic acids (Olthof et al. 2003). These compounds are further metabolized to 4-methyl catechol glucuronide in sheep (Martin 1982). We expected that brushtail possums would detoxify rutin via hydrolysis, oxidation, and glucuronidation because eucalypt foliage contains rutin (Conde et al. 1997) and possums eating *Eucalyptus* produce 4-methyl catechol glucuronide (S. McLean, *unpublished data*). Orcinol is mostly found in lower plants, such as lichens, but can occur as a glycoside in higher plants (Martin 1982). Sheep metabolize it by conjugation with GA (Martin et al. 1983), and we anticipated that possums would do the same.

Although many different forms of detoxification enzymes catalyze the same reaction, a few forms process most compounds. For example, at least 17 families of cytochrome P450 enzymes (CYPs) exist, but only three are responsible for the oxidation of most drugs and PSMs (Lin and Lu 2001). Thus, we assumed that those PSMs whose metabolites require the same process (e.g., oxidation or GA conjugation), compete for detoxification.

We were interested in the feeding behavior of possums confronted with foods containing PSMs that either require the same detoxification pathway, or use independent pathways. Specifically, we asked the following. (1) How much can possums eat of a basal diet containing individual PSMs? (2) Can possums eat more when given a choice of PSMs? (3) Do the detoxification pathways of the PSMs influence how much possums eat? We predicted that if PSMs saturate detoxification pathways, then giving possums a choice between two diets containing PSMs detoxified by independent pathways would allow them to eat more than they could of a diet with a single PSM or two PSMs requiring competing pathways. Conversely, if detoxification constraints do not influence feeding decisions, possums might eat more when offered a choice of foods, regardless of whether detoxification pathways of PSMs in those foods compete.

Methods

Nine male common brushtail possums (body mass 2.73 ± 0.10 kg, mean $\pm$ SE) were captured in cage traps on the Australian National University campus, Canberra, Australia. They were held in pens measuring $1.8 \times 2.2 \times 3.2$ m for all experiments other than the urine collections. For three weeks following capture, they were offered foliage collected from the capture area and a basal diet (55.5% apple, 28% banana, 5.5% ground rice hulls, 4.7% ground lucerne, 4.7% ground Weet-Bix [a whole wheat breakfast cereal from Sanitarium, Berkeley Vale, NSW Australia], and 1.6% acid casein on a wet matter basis) as a wet mash. The dry matter (DM) content of the basal diet was 31% and the nitrogen content was 1.9% of DM. We gradually reduced the foliage so that possums were eating only the basal diet by the end of the third week. The PSMs were added to

the basal diet to make each experimental diet. Possums were offered food between 1700 and 0900 hours daily. Dry matter intake (DMI) was calculated by drying a subsample of the food offered and all uneaten food at 60°C to constant mass. Water was available ad libitum.

Materials

Compounds added to the food or used as analytical standards came from Sigma-Aldrich (Sydney, Australia; sodium benzoate, hippuric acid, salicin, 4-methyl catechol, salicyl alcohol, 3-hydroxyphenylacetic acid, cumic acid, 1,8-cineole, orcinol, rutin, salicyluric acid, and benzoic acid), BDH analaR (Merck Ltd., Lutterworth, UK; salicylic acid) and Fluka (Ronkonkoma, New York, USA; *p*-cymene, *N*,O-bis(trimethylsilyl) trifluoroactetamide [BSTFA]). Cineole metabolite standards (9-hydroxycineole, 9-cineolic acid, and 7-cineolic acid) were isolated from possum urine (Boyle et al. 2000). The extract of *Helix pomatia* (a mixture of β-glucuronidase at 141 000 units/mL and aryl sulfatase at 3950 units/mL) that was used to hydrolyze glucuronide metabolites in urine came from Boehringer (Mannheim, Germany).

Overview of experiments

The experiments in this study can be divided into three parts: (A) limiting-dose experiments, to determine the concentrations of PSMs used in later experiments; (B) experiments to establish detoxification pathways of PSMs; and (C) comparisons of food intake of possums offered diets containing PSMs requiring independent or competing detoxification pathways. Part C was a series of experiments examining (1) the food intake of possums offered paired and single diets; (2) the extent to which possums maintained their preferences through time; (3) the food intake of possums offered diets containing two PSMs or a choice between diets containing each of these separately; and (4) whether the supply of glucuronic acid (GA) limits feeding.

Part A: Establishing limiting doses of PSMs

Testing the detoxification limitation hypothesis relied on knowing how much PSM a possum could ingest before food intake declined (the "nonlimiting dose") and the concentrations that limited intake (the "limiting dose"). The limiting dose was defined as the dietary concentration of a PSM that reduced food intake by 50% from that on the basal diet (80–100 g DM/d). In separate 6×6 Latin square experiments, we measured food intake for six brushtail possums offered six evenly spaced concentrations of cineole (0–1.1 mmol/g DM), cymene (0–0.6 mmol/g DM), benzoate (0–0.8 mmol/g DM), and orcinol (0–1.2 mmol/g DM). Possums were fed PSMs in the week prior to each experiment to allow the induction of detoxification enzymes. Then, possums received every treatment for one night. Similarly, five evenly spaced concentrations of rutin (0–0.4 mmol/g DM) were offered to five possums for one night each.

TABLE 1. The concentrations of PSMs (plant secondary metabolites) added to brushtail possum diets in several experiments.

PSM	PSM concentration (mmol/g DM)	
	Nonlimiting	Limiting
1,8-cineole	0.21	0.64
p-cymene	0.19	0.61
Sodium benzoate	0.21	0.60
Salicin	0.06	0.23
Orcinol	0.23	0.89
Rutin	0.15 (low)	0.30 (high)

Notes: The limiting concentrations resulted in a 50% reduction in feeding compared to the basal diet, while nonlimiting concentrations did not reduce feeding and were below the concentration at which a plateau in PSM intake was reached (Appendix A). Rutin did not reduce intake at any concentration studied, so we chose low and high concentrations instead.

The limiting and nonlimiting concentrations of salicin were determined from a previous study (Pass and Foley 2000) because we did not have enough of the compound available for this experiment. Possums were provided with the basal diet, free of PSMs, for at least one week following each experiment.

Part B: Detoxification pathways of PSMs in possums

It was necessary to identify, or confirm, the detoxification pathway(s) for each PSM in order to design feeding experiments with PSMs requiring competing and independent pathways. To do this, nine possums were placed into metabolism cages measuring $50 \times 37 \times 45$ cm with an external nest box ($21 \times 37 \times 22$ cm), in a room kept at 18°C on a 12 h:12 h light : dark cycle.

We randomly divided the possums into three groups of three and fed each group sodium benzoate, salicin, or *p*-cymene over five nights, during which we measured dry matter intake (DMI) to calculate PSM intake. On the first night, possums were given the basal diet; on the second and third nights, the basal diet supplemented with a nonlimiting concentration of their PSM; and on the final two nights, they got the limiting concentration (Table 1). On the first, third, and fifth nights of each period, we collected urine for 24 h into plastic bottles placed in a thermos with solid pellets of CO_2. A subsample (~20 mL) was stored in a glass vial at −20°C for later analysis. A two-day rest period, when possums received only the basal diet, followed the experiment. This was usually enough time for possums to excrete all of the metabolites. Following this, the experiment was repeated using the remaining PSMs: 1,8-cineole, rutin, and orcinol. We tried, where possible, to ensure that PSMs fed consecutively were metabolized by differing pathways. Thus, possums got benzoate and then cineole, or cymene and then orcinol, or salicin followed by rutin.

To confirm that possums ingesting two PSMs simultaneously produce the same metabolites as those ingesting them separately, we collected urine from

possums given choices between two diets containing different PSMs, each at the limiting concentration (Table 1). The choices (8 of 15 possible pairs) were between cineole and cymene, cineole and benzoate, cymene and benzoate, orcinol and salicin, orcinol and benzoate, orcinol and cineole, salicin and cineole, and salicin and benzoate. We also offered a diet containing orcinol and rutin (at their limiting and high concentrations, respectively; Table 1). We predicted that possums might avoid a diet containing orcinol in favor of rutin, because rutin did not limit feeding at any concentration tested. Thus, to ensure that they ate both compounds, we mixed them in a single diet. Each pair of PSMs was offered to three possums. Possums received the basal diet on the first night of each experiment, followed by one of the PSM pairs for the next two nights. DMI was measured each night, and urine was collected for 24 h on the first and third nights. All possums received three of the nine PSM pairs, with at least two nights separating experimental periods.

Urinary metabolites were extracted before and after hydrolysis with β-glucuronidase using the method described by Boyle et al. (2000). Standards were prepared by adding known amounts of metabolites to urine collected from a possum feeding on the basal diet. To quantify metabolites, 30 μL of urine extract was combined with 30 μL *N*,O-bis(trimethylsilyl) trifluoroactetamide (BSTFA) and was placed in a heating block at 70°C for 30 min to form trimethylsilyl (TMS) derivatives. Injections of 1 μL were separated by gas chromatography–mass spectrometry (GC–MS) using an Agilent 6890 GC fitted with an autosampler and coupled to an Agilent 5973 quadruple MS (Agilent, Palo Alto, California, USA). The GC was fitted with a Hewlett Packard HP-5 capillary column (30 m × 0.25 mm × 0.2 μm; Agilent, Palo Alto, California, USA) and the operating conditions were split injector at 250°C; detector at 300°C; and oven at 80°C for 2 min and then 3°C/min to 150°C, 10°C/min to 220°C, 30°C/min to 290°C, where it was held for 5 min. The carrier gas, ultrapure He, flowed at a constant rate of 2 mL/min. Metabolites were identified by their retention times and by reference to mass spectral data (NIST 2002; NIST Mass Spectral Search Program, version 2.0). We did not have standards for all cineole and cymene metabolites, so we quantified them using standard curves of similar compounds.

Part C: Food intake in relation to the detoxification pathways of PSMs

Experiment 1: Can a variety of PSMs in the diet overcome feeding limitations?—We compared the DMI of possums offered a choice of diets containing different PSMs with that of possums on a diet containing a single PSM. To ensure that all PSMs restricted feeding to a similar degree, we used the limiting concentration of each (Table 1). Seven possums were offered the following seven treatments for one night each in a digram balanced Latin square: basal, cineole, cymene, sodium benzoate, or a choice between cineole and cymene, cineole and benzoate, or cymene and benzoate. In a second experiment, 10 treatments were offered to eight possums in an incomplete Latin square. The treatments were: basal, cineole, sodium benzoate, salicin, orcinol, or a choice between orcinol and salicin, orcinol and benzoate, orcinol and cineole, salicin and cineole, or salicin and benzoate. In a digram Latin square, treatments follow each other an equal number of times, which allowed us to measure carryover effects from the previous treatment. In both experiments, treatment nights were consecutive, because most metabolites were excreted within 24 hours. We gave possums ~25 g DM of the basal diet each morning to provide food for those that had fed little during the night. They usually ate this entire ration.

Experiment 2: Do possums maintain their PSM preference over several nights?—In all cases, individual possums behaved differently when offered the choices in experiment 1, with some preferring certain diets. We were interested in whether possums maintain their preferences, so we measured DMI over four consecutive nights for four possums given a choice between the limiting concentrations (Table 1) of cineole and cymene, and four given cineole and benzoate. We selected these combinations to give one pair whose PSMs competed for detoxification and another pair requiring independent pathways.

Experiment 3: Is it better to ingest two PSMs in separate diets or both in a single diet?—We questioned how strictly possums were regulating their intake of PSMs and hypothesized that they might eat more if offered a diversity of flavors and the choice to eat the PSMs in any order and amount that they wished. Thus two PSMs were either mixed together to create a single diet or were added separately to give two diets. We measured DMI for eight possums offered four dietary treatments, for one night each in a Latin square design. Two of the treatments were choices: one between cineole and cymene, and the other between cineole and benzoate (all at their limiting concentration; Table 1). The other two treatments were single diets formed by mixing the diets in each choice combination. In other words, one diet contained both cineole and cymene at a final concentration of 0.3 mmol/g DM each, and the other contained both cineole and benzoate, again at 0.3 mmol/g DM each.

Experiment 4: Does glucuronic acid supply limit feeding?—We anticipated that although rutin itself did not limit feeding, it might further decrease intake of orcinol by appropriating some of the GA, slowing the rate of orcinol detoxification. Thus, we measured the DMI for six brushtail possums offered four diets for one night each: basal, orcinol, rutin, and orcinol plus rutin, with each PSM added at the limiting or high concentration (Table 1). Rutin and orcinol were mixed in the same diet because we thought that possums might avoid the limiting orcinol dose in favor of the rutin.

TABLE 2. A comparison of predicted and measured feeding responses (change in dry matter intake, DMI) in brushtail possums fed diets containing plant secondary metabolites (PSMs).

Paired PSMs and pathways	Shared pathway†	DMI change	
		Predicted	Actual
Paired PSMs with major pathways competing			
1,8-cineole and *p*-cymene	oxidation	=	=
Orcinol and salicin	GA conjugation	=	=
Orcinol and rutin	GA conjugation	↓	=
Paired PSMs with minor pathways shared			
Salicin and 1,8-cineole	oxidation and GA conjugation	↑	=
Orcinol and 1,8-cineole	GA conjugation	↑	↑
Salicin and sodium benzoate	glycine conjugation	↑	↑
p-cymene and sodium benzoate	glycine conjugation	↑	↑
Orcinol and sodium benzoate	GA conjugation	↑	↑
Paired PSMs with independent pathways			
1,8-cineole and sodium benzoate	none	↑	↑

Notes: We defined major and minor pathways arbitrarily as those that metabolized either >25% or <25% of an ingested PSM. Depending on the degree of competition between detoxification pathways, possums offered a choice between diets containing different PSMs were expected to eat more food (↑), or the same amount of food (=), compared to the amount eaten of a diet containing a single PSM. Possums were expected to eat less (↓) when rutin was added to a diet that already contained orcinol. The DMI of possums fed each pair of PSMs was either the same (=, $P > 0.05$) or significantly more (↑, $P < 0.001$) than when possums were offered each PSM individually.

† GA is glucuronic acid.

Statistical analysis

Experiments 1, 3, and 4 were analyzed using the ANOVA function in GenStat 7.1 (Numerical Algorithms Group 2003, Oxford, UK). The response variate was DMI (the sum of intake on both diets in choices), the treatment structure was the diet, or combination of diets offered, and the block structure for Latin square designs was the individual possum and day that the treatment was offered. Body mass was included as a covariate but did not affect DMI ($P > 0.05$).

For Part A and Experiment 2, data were analyzed using the residual maximum likelihood (REML) algorithm in GenStat 7.1. The fixed effects in Part A were the concentrations of PSMs offered, whereas the random effects were the possums and days of treatments. We analyzed Experiment 2 in two ways. First, DMI (the total amount eaten from both diets) was included as the response variate. Then, DMI was replaced by the amount of the cineole diet eaten. In both cases, the fixed effect was the day of the experiment and random effects were the individual possums and the within-possum differences between days. When a fixed effect was significant ($P < 0.05$), means were compared with a least significant difference test ($LSD_{0.05}$). All values are presented as means ± 1 SE.

RESULTS

Part A: Establishing limiting doses of PSMs

Increasing concentrations of all PSMs, except rutin, caused a decrease in DMI (cineole, cymene, benzoate, and orcinol; all $P < 0.001$). As expected, the intake of each PSM increased to a plateau (Appendix A: Fig. A1a–d), which represents the maximal ingestion of a PSM and presumably indicates saturation of the detoxification pathway. Possums ate the same amount regardless of the concentration of rutin ($P = 0.24$), and rutin intake increased linearly (Appendix A: Fig. A1e). Concentrations of ~0.6 mmol/g DM cineole, cymene, and benzoate and 0.9 mmol/g DM orcinol all limited intake to between 40 and 50 g DM (i.e., a 50% reduction from the basal diet DMI) and were designated as the limiting dose.

Part B: Detoxification pathways of PSMs in possums

Brushtail possums detoxified the six PSMs using a variety of metabolic pathways, including hydrolysis, oxidation, conjugation with GA, and conjugation with glycine (Appendix B). They produced the same urinary metabolites whether they ingested a PSM alone or in combination with another PSM, and the percentage recovered of each PSM usually remained the same for single and paired diets (Appendix B). This allowed us to identify pairs of PSMs with competing and independent detoxification pathways (Table 2).

Part C: Food intake in relation to the detoxification pathways of PSMs

Experiment 1: Can a variety of PSMs in the diet overcome feeding limitations?—Possums usually ate more when offered a choice of diets containing PSMs detoxified via different pathways, or by pathways with only minor overlap, compared to their intake of a diet containing a single PSM (Table 2, Fig. 1). For instance, they ate between 30 and 40 g DM when offered two bowls of food containing the same PSM, but significantly more ($P < 0.001$ for both parts of the experiment) when given a choice between cineole and benzoate, cymene and benzoate, orcinol and benzoate, orcinol and cineole, or salicin and benzoate. In contrast, when

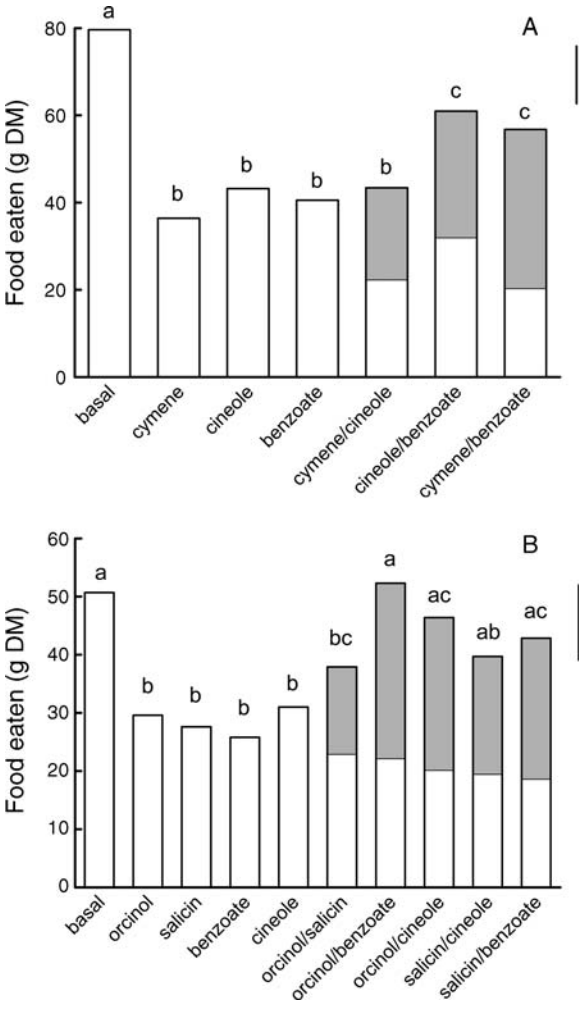

FIG. 1. The mean amount of food (on a dry matter [DM] basis) eaten by brushtail possums offered a diet containing a single PSM, or a choice of two diets with different PSMs. (A) Experiment in which seven possums were offered seven treatments for one night each in a digram balanced Latin square. (B) Experiment in which 10 treatments were offered to eight possums in an incomplete Latin square. Histogram bars indicate a choice of diets, with the white portion showing the amount eaten of the first diet listed on the *x*-axis and the gray portion the second. The two colors show that possums consumed some of each choice, but as we were interested only in the total amount eaten, we do not include separate error bars. The thin vertical bar at the right side shows the least significant difference ($P < 0.05$) between treatments for the total amount eaten. Treatments without a common lowercase letter are significantly different.

offered a choice between cineole and cymene, orcinol and salicin, or salicin and cineole, possums ate no more than when offered a diet containing one of the PSMs of the pair (Fig. 1).

Experiment 2: Do possums maintain their PSM preference over several nights?—Possums offered a choice between diets containing different PSMs maintained remarkably stable feeding patterns for four consecutive nights, suggesting that they did not alter their behavior with experience. Those offered diets containing cineole or cymene ate 39 ± 3 g DM per night over four consecutive nights ($P_n = 0.97$), of which most was the diet containing cineole (26 ± 3 g DM; $P_n = 0.75$). Likewise, possums offered a choice between cineole or benzoate ate, on average, 63 ± 6 g DM on four nights ($P_n = 0.89$), of which about half was the diet containing cineole (29 ± 7 g DM; $P_n = 0.65$).

Experiment 3: Is it better to ingest two PSMs in separate diets or both in a single diet?—Possums ate the same amount regardless of whether they were offered two PSMs in separate feeders, or the two PSMs mixed ($P = 0.80$; Fig. 2). They ate significantly more of the diet containing cineole and benzoate (or the choice) than they did of the diet containing cineole and cymene (or the choice; $P = 0.03$), reflecting the degree of competition between detoxification pathways.

Experiment 4: Does glucuronic acid supply limit feeding?—Adding orcinol to the diet reduced food intake ($P = 0.001$) compared to the amount eaten of the basal diet. Adding rutin (which, like orcinol, requires GA for detoxification) to either the basal diet or a diet that already contained orcinol did not affect food intake ($P = 0.97$; Fig. 3).

DISCUSSION

This is the first study to show that mammals can eat more if they select a mixed diet containing PSMs detoxified by different pathways. A poor understanding of the metabolic pathways of detoxification has hampered previous attempts to decipher the role of PSMs in diet mixing and in countering PSM-induced feeding

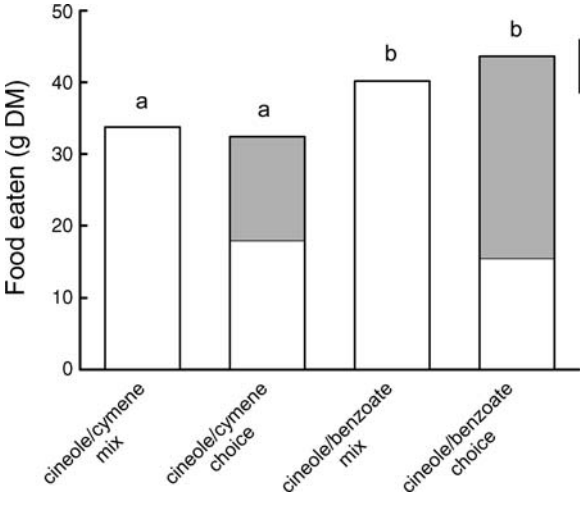

FIG. 2. The mean amount eaten by eight possums offered two PSMs in a single diet (unshaded), or as a choice (shaded, with the white referring to the PSM listed first on the *x*-axis). The two colors show that possums consumed some of each diet, but we were interested only in the total amount eaten so we do not include separate error bars. The thin vertical bar at the right side shows the least significant difference ($P < 0.05$) between treatments. Treatments without a common lowercase letter are significantly different.

depression. We overcame this problem by first analyzing the urinary metabolites produced by common brushtail possums fed six PSMs. Although possums excreted several metabolites for most of the PSMs, they tended to detoxify each PSM via one main pathway. Importantly, possums produced the same metabolites regardless of the concentration of a PSM, or whether they ingested one or two PSMs simultaneously. This implies that possums did not recruit alternative pathways to counter larger detoxification loads.

We did not investigate every combination of the six PSMs, because our focus was on understanding constraints imposed by PSMs that either compete or do not compete for detoxification pathways. In most cases, the feeding decisions of possums matched the expectations of the detoxification limitation hypothesis (Table 2). Compared to their intake of a diet containing one PSM, possums did not eat any more if their choice was between two diets containing PSMs that are both detoxified by oxidation (1,8-cineole and *p*-cymene) or by conjugation with GA (salicin and orcinol). In contrast, possums ate more when the choice was between compounds detoxified by different pathways, or those that competed only for minor pathways.

Two results did not fit our predictions. First, possums given a choice between cineole and salicin did not eat more. Salicin is predominantly detoxified by conjugation with GA, whereas cineole is mainly oxidized to cineolic and hydroxycineolic acids. At first glance, this suggests mutually exclusive pathways that would not compete. However, detoxification is a complex process and the detoxification of salicin and cineole are no exception. Some salicyl alcohol is oxidized to salicylic acid, presumably requiring the same alcohol dehydrogenase enzymes needed for oxidation of cineole alcohol metabolites. In addition, about half of the detected cineole metabolites are conjugated with GA. The feeding experiments suggest that this degree of competition, especially in oxidation, may be more important than first thought. As we will discuss, results from the orcinol/rutin experiment suggest that GA is not limiting, so there should not be competition for it. Another explanation for the apparent competition between the detoxification of cineole and salicin is that salicin inhibits cytochrome P450 enzymes (CYPs), which are critical in detoxifying cineole (Pass et al. 2001). Many compounds inhibit CYPs (Tanaka 1998, Lin and Lu 2001) and this can inhibit feeding due to lowered detoxification rates. For instance, tobacco hornworms treated with piperonyl butoxide, which inhibits CYPs, eat less of a diet containing nicotine than do untreated individuals (Snyder and Glendinning 1996).

The second unexpected result was the failure of rutin to depress feeding by possums given a diet that also contained orcinol. Rutin itself is unlikely to saturate detoxification pathways, because inclusion of up to 26.7% DM in the diet did not restrict feeding. However, possums conjugated most ingested rutin with GA and we envisaged that this would limit the GA available for the conjugation of orcinol. The failure of rutin to further depress feeding suggests that something other than GA availability limits feeding on orcinol. This, of course, is contingent on the metabolism of orcinol and rutin remaining the same, regardless of whether the compounds are fed simultaneously or separately, so that more GA is excreted when both compounds are eaten. Possums offered single diets containing orcinol excreted 15–20 mmol GA in 24 hours, whereas those offered single diets of rutin excreted 5–10 mmol GA in 24 hours. Unfortunately, wide variation in feeding between the three possums fed diets containing both orcinol and rutin during urine collections led to one possum excreting 11 mmol of GA in 24 hours, one 40 mmol, and the other 29 mmol, so the error was too large to confirm increased GA excretion. However, because the same metabolites were excreted in the same percentages on both the single and paired diets, we suggest that possums can detoxify both compounds simultaneously via GA conjugation.

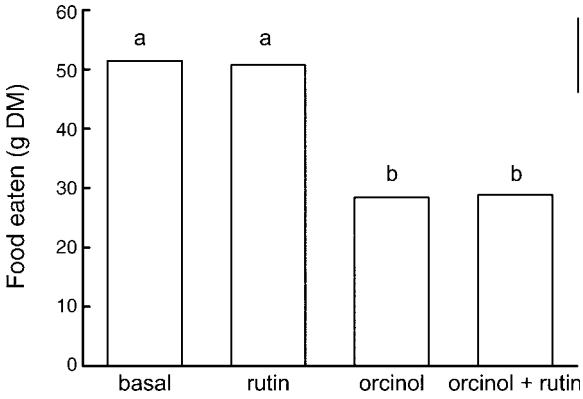

FIG. 3. The mean amount eaten by six brushtail possums offered a diet containing orcinol, rutin, or both compounds. The thin vertical bar at the right represents the least significant difference ($P < 0.05$) between treatments. Treatments without a common lowercase letter are significantly different.

Of course, if GA is in ample supply, how can we explain the failure of possums to eat more when offered salicin and orcinol simultaneously? It may be that there is adequate GA, but a deficiency of appropriate forms of UDP-glucuronosyltransferase (UGT) enzymes, to catalyze the conjugation. Although there is overlap in substrate specificity, different forms of UGT enzymes are available (Radominska-Pandaya et al. 1999). We hypothesize that the conjugation of orcinol and of salicin require the same UGT enzymes, whereas rutin uses a different form.

Our analyses of urinary metabolites did not account for all of a PSM ingested. Clearly, any metabolite that we fail to account for results in an underestimation of the contribution of a particular detoxification pathway. A worse outcome is that we may fail to identify a pathway for a focal PSM. However, we believe that our analyses provide an adequate overview of detoxification.

Our lowest recoveries were for the metabolites of cineole (33%) and cymene (51%). This is partly because we did not attempt to quantify all of the known metabolites. Even so, other researchers who tried to measure all metabolites still accounted for little more than we did (40–55% for cineole and ~60% for cymene; Boyle et al. 1999, 2000, Boyle and McLean 2004). Cineole can be detected in expired air (Boyle et al. 2002), but only traces of cineole, cymene, and their metabolites occur in feces (Boyle et al. 1999, 2000, Boyle and McLean 2004). Thus, we cannot account for the metabolites of a large amount of the cineole and cymene ingested by possums, but this is unlikely to affect our conclusions. We know that cineole and cymene compete for oxidation (Pass and McLean 2002), and we have demonstrated that this influences the possums' feeding behavior. Furthermore, our analysis accounts for all GA conjugates, so we are not underestimating the contribution of GA to cineole and cymene detoxification. The poor recovery of cineole and cymene metabolites suggests that other detoxification pathways may exist for these compounds, or that there are undiscovered metabolites from oxidation. In either case, this has little bearing on our study.

We could not account for ~10–30% of ingested orcinol, salicin, and rutin. Most metabolites of the PSMs that we studied are excreted within 24 hours. However, we found traces of orcinol glucuronide in the urine of a possum that had not ingested orcinol for more than 48 hours, which may explain some of the orcinol that we failed to detect. Most of the undetected salicin was probably excreted unchanged; a preliminary analysis revealed salicin in urine at up to 16% of the ingested dose, which is similar to previous findings (McLean et al. 2001). In contrast, there was little rutin or its aglycone, quercetin, in urine. In a separate group of possums, however, ~36% of the rutin dose appeared in the feces as quercetin (J. Sorensen, *unpublished data*). In humans, a high percentage of rutin is metabolized in the colon to hydroxyphenylacetic acids (Aura et al. 2002, Olthof et al. 2003). Differences in microbial flora in the hindguts of humans and brushtail possums might explain why the most abundant metabolite of rutin in possums was 4-methyl catechol.

There is also evidence that the speed of detoxification depends on the specific PSMs fed to possums. We recovered less benzoate and salicin when possums ate them in choice experiments than when they were offered singly, implying that a greater challenge slows the rate of detoxification. Pretreatment with compounds that deplete ATP is known to reduce the rate of conjugation of benzoate with glycine in rats (Gregus et al. 1996).

Detoxification is a complex process that involves many factors, including the detection of the PSM by the consumer, enzymes, cosubstrates, removal of end products, supply of ATP to fuel the process, and maintenance of homeostasis, such as acid–base balance. This suggests that there is really no such thing as truly independent detoxification pathways. Rather, there are degrees of separation. If so, it is difficult to ascertain the exact nature of detoxification and the causes of its limitations. This is particularly true for free-living herbivores, which may ingest, for example, soil, to negate the effects of a PSM that might otherwise be toxic (Gilardi et al. 1999). Determining the detoxification metabolites of PSMs enabled us to predict which PSMs should compete for detoxification and, consequently, limit feeding. However, it is clear from our results that when detoxification pathways compete, measuring the effects in terms of feeding becomes difficult if we cannot identify the limitation. This requires more specific studies, such as that by Pass and McLean (2002), which shows that cymene and cineole compete for cytochrome P450 enzymes, or that by Marsh et al. (2005) showing that the availability of glycine determines the detoxification rate of benzoate and, hence, feeding.

We purposely chose concentrations of PSMs that reduced feeding to 40–50 g DM, expecting possums to eat 80–100 g DM in a choice-feeding experiment if the detoxification pathways of the PSMs were independent. However, possums ate less than this. Feeding cineole and benzoate is a prime example. The pathways required for their detoxification appear to be independent, but possums ate about three-quarters of the expected amount when cineole and benzoate were offered as a choice. In addition, neither compound fed alone (0.3 mmol/g DM) in the limiting-dose studies inhibited feeding, so we did not expect an effect on feeding when they were combined at this concentration in a single diet. However, in Experiment 3, possums ate the same amount of cineole and benzoate combined as when offered a choice. Interestingly, these findings reflect those of other studies. For example, lambs offered a choice between diets containing amygdalin (a cyanogenic glycoside) and lithium chloride ate more than those lambs that were offered the compounds individually, but less than lambs fed the control diet (Burritt and Provenza 2000). This again suggests that detoxification pathways are separated by degrees rather than being truly independent.

Equally interesting was that possums offered a choice between cineole and cymene (PSMs with competing pathways) ate the same amount when they were combined in a single diet, even though choices may sometimes promote feeding because of different sensations or flavors (Rolls et al. 1981, Treit et al. 1983). This result has several implications. First, it suggests that possums base their feeding on cues from the PSMs they ingest, rather than from the number of different foods or tastes that confront them. Second, a possum feeding on a single diet, containing two PSMs, had no control over the percentage of each PSM it ate, or the order in which it ate them, even though it did when given the choice. A possum's choice could depend on a myriad of factors, such as which feeder it visits first or a preference for one diet or flavor over another, rather than on any innate ability to detoxify one compound better than another.

Finally, the result demonstrates how several PSMs, covarying in a natural diet, could lead us to relate food intake, rightly or wrongly, to any one of the compounds. The experiments with cineole illustrate this. It took 0.3 mmol/g DM of cineole to reduce intake below 40 g when 0.3 mmol/g DM cymene was also present (Experiment 3; Fig. 2), but 0.6 mmol/g DM when cineole was alone (Part A). When analyzing the diets of wild herbivores, we should not expect to correlate feeding with the concentration of one PSM if other PSMs, especially those that compete for detoxification, occur in the diet.

One interesting result remains. Possums that did not eat more when given a choice actually could have eaten as much by selecting just one of the diets. Instead, they ate a mixture of PSMs and maintained this choice for at least four days. This result implies that there is a benefit, other than possibly eating more, in sampling a variety of foods. Our study explored how limitations on detoxification influence feeding decisions, even though many other factors can dictate feeding behavior. For example, acquisition of nutrients, associative effects of PSMs, experience from sampling, transient food aversions due to satiety, and perhaps keeping detoxification systems primed, may all define foraging decisions. Variation in nutrient content cannot explain the present results, unless some PSMs reduce the availability of nutrients. In addition, there were no beneficial associative effects. However, we cannot discount the importance of sampling and transient or temporary food aversions. These aversions, often called sensory-specific satiety, probably contribute to animals eating a diverse diet. When animals or humans are presented with a variety of foods, they prefer an alternative to the one eaten most recently (Rolls et al. 1981, Treit et al. 1983, Provenza et al. 2003), because interactions among flavors, nutrients, and toxins cause transient aversions (Provenza et al. 2003). Thus, animals eat a variety of foods even though a single food may meet their nutritional needs and be free of toxins (Early and Provenza 1998). Sampling a variety of foods has another advantage: it allows animals to learn about the consequences of ingesting each food, or combination of foods, while enabling them to induce or maintain detoxification enzymes.

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APPENDIX A

The amount of 1,8-cineole, *p*-cymene, sodium benzoate, orcinol, and rutin ingested by brushtail possums at different dietary concentrations (*Ecological Archives* E087-130-A1).

APPENDIX B

A summary of the detoxification of six PSMs by brushtail possums (*Ecological Archives* E087-130-A2).